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Identification and characterization of Specific Spoilage Organisms (SSOs) in different food matrices

Scientific field AGR/16

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ABSTRACT:

This PhD thesis aims to improve the actual systems of management and quality control of food, expanding the knowledge about the microorganisms responsible of food spoilage (Specific Spoilage Organisms) and their degradative activities.
The analysed foods were of vegetable and animal origin: in particular they were ready-to-eat vegetables (carrots and green salads packaged in air and MAP), milk and dairy products (raw, pasteurized, UHT and micro-filtered milk and mozzarella cheese) and beef and hamburger packaged traditionally or in master bag.
Firstly the microbial quality of each food was monitored from the production, during the declared shelf life and even after the expiration date. The isolates were phenotypically and genotipically characterized and identified; for each food SSOs were recognized.
Some microorganisms appeared typical of each product, sometimes also depending on the packaging and storage conditions. *Leuconostoc* spp. was indicated as typical carrots spoiler; Enterobacteriaceae family was involved in spoilage of salads packaged under modified atmosphere; lactic acid bacteria were typical of food packaged in low oxygen concentrations (salads in MAP and meat in master bag) and *Brochothrix thermosphacta* was specific of beef.
Pseudomonas spp. appeared common to all the analysed products and dominant among the bacterial spoilers. For this genus more detailed studies were conducted: the classification was made up to the biotype and biovar level; the characterization focused on different enzymatic activities and in particular the proteolysis was qualitatively and quantitatively evaluated; a phylogenetic study, based on the gene codifying for the most common *Pseudomonas* protease, was made.
Subsequently each food was analysed with a different approach. For vegetables the influence of temperature on microbiota was verified, resulting that low temperature slowed down microbial growth and partially modified the composition of the microbiota. In milk proteolytic activity of *Pseudomonas* spp. was evaluated and the formation of Pseudo-GMPs, deriving from the cutting of K-casein (103-104 position), were recognized. In mozzarella the blue pigment formation was studied and a rapid method for the detection and the quantification of alive, dead and Viable But Non Colturable (VBNC) cells was set up. For meat, a primer specific for *Brochothrix* genus was built and the packaging conditions were studied to verify the evolution of microbiota and the possible effects on the growth of *Listeria monocytogenes* (challenge test).

RIASSUNTO:

Identificatine e caratterizzazione di Specific Spoilage Organisms (SSOs) in differenti matrici alimentari.

Lo scopo di questa tesi di dottorato è contribuire a implementare gli attuali sistemi di gestione e controllo della qualità di prodotti alimentari, ampliando la conoscenza dei microrganismi responsabili dell'alterazione degli alimenti (Specific Spoilage Organisms) e delle loro attività degradative.
Sono state considerate matrici alimentari sia di origine vegetale sia animale, in particolare sono stati analizzati vegetali di IV gamma (carote julienne e diverse insalate verdi a foglia confezionate in aria e in atmosfera protettiva), latte e latticini (latte crudo, sottoposto a trattamenti di sanificazione termici e di filtrazione, e mozzarella) e prodotti carnei (fette di manzo e hamburger, entrambi confezionati tradizionalmente e in master bag).
Inizialmente la qualità microbiologica di ogni alimento è stata monitorata dalla produzione, per tutta la durata della shelf life dichiarata dal produttore, fino a oltre la data di scadenza. I microrganismi isolati sono stati classificati e identificati e sono quindi stati riconosciuti i microrganismi specifici per ogni alimento (SSOs) responsabili delle alterazioni.
Alcuni microrganismi sono apparsi tipici e specifici del singolo alimento, a volte in dipendenza anche dalle condizioni di confezionamento e stoccaggio. Il genere *Leuconostoc* è stato individuato come tipico alterante delle carote; la famiglia delle Enterobacteriaceae è coinvolta nell'alterazione di insalate confezionate in atmosfera modificata; batteri lattici sono tipici di prodotti confezionati con ridotte concentrazioni d'ossigeno (insalate in MAP e prodotti carnei soprattutto confezionati in master bag) e *Brochothrix thermosphacta* è specifico di prodotti carnei.
Il genere *Pseudomonas* contrariamente a tutti i batteri precedenti è apparso comune a tutte le matrici analizzate e sempre dominante tra gli alteranti. Per questo genere sono quindi stati effettuati studi più approfonditi: la classificazione è stata fatta fino a livello di biotipo e biovar, la caratterizzazione ha previsto lo studio di diverse attività enzimatiche, in particolare la proteolisi è stata valutata con metodi qualitativi e quantitativi ed è stato condotto uno studio approfondito sul gene codificante per la più comune proteasi di *Pseudomonas* spp.
Successivamente ogni alimento è stato analizzato con una diversa finalità. Per i vegetali è stata verificata l'influenza della temperatura di conservazione sul microbiota, ritrovando che la bassa temperatura rallenta lo sviluppo microbico e modifica parzialmente la composizione del microbiota. Nel latte è stata valutata l'attività proteolitica del genere *Pseudomonas* ed è stata osservata la formazione di Pseudo-GMPs derivanti dal taglio della K-caseina in posizione 103-104 per opera di proteasi termoresistenti di *Pseudomonas* spp. In mozzarella è stata studiata la produzione di pigmenti blu ed è stato messo a punto un metodo rapido di determinazione e quantificazione di cellule vive, morte e vitali ma non coltivabili di *Pseudomonas* spp. Per i prodotti carnei è stato costruito un primer per l'identificazione specifica di *Brochothrix* spp. ed è stato studiato come il confezionamento influenzi l'andamento delle diverse popolazioni microbiche presenti, compreso lo sviluppo di patogeni come *Listeria monocytogenes* (challenge test).

1. INTRODUCTION

1.1 Food microbiology

Food spoilage is defined as any change that makes a product unacceptable for human consumption. It can result from different causes involving physical-chemical and biochemical changes and also microbial growth and activity (Huis in't Veld, 1996). In table 1.1 are shown the most common changes responsible of food spoilage.
In particular, microbial growth and activity are the most important causes of reduction of quality and shelf life of foods. The presence of a microorganism, at any production step, represents index of contamination; however not all microorganisms negatively operate on food.

In accordance to their activity, they can be divided into three groups:

- Pro-technological microorganisms: all the microorganisms whose presence is required and necessary for production or maturation of food; usually they are present in high number, but during shelf life they drastically decrease. This group includes lactic acid bacteria, but also yeasts, acetic acid bacteria and some moulds;
- Spoilage microorganisms (quality and process indices): they are specific for each food, according to its features and the storage conditions; they affect the shelf life of the product, therefore they should be present in low concentration since the beginning and should not increase too much during the conservation. All the utilized conservation methods aim to reduce the number of those microorganisms, preventing spoilage phenomena;
- Pathogen microorganisms (safety index): all microorganisms that represent a very serious danger for the safety of the consumer, for their presence or for their toxins production. They must be absent in 1 or 25 or 100g of food according to their hazard (Galli, 2005; Jay et al., 2005).

1.2 Specific Spoilage Organisms

At the end of the working process, each food has its own microbial population whose growth and activity depend on different interacting factors (Galli, 2005; Jay et al., 2005).
The intensity of the spoilage is not always linked to the total microbial concentration: in fact usually only a fraction of the population induces the spoilage and it is know as Specific Spoilage Organism (SSO). At the beginning, the SSOs usually are a minority, then during the shelf life they grow faster than the other microorganisms and produce the metabolites and the enzymes responsible of the spoilage (figure 1.1).
The SSOs are different microbial species depending on the food, in term of physical, chemical and structural features, on the type of packaging and the storage conditions, such as temperature, humidity and atmosphere composition. Another implicit parameter that affects the SSO population is the mutual influence among the organisms. Many microorganisms in fact can produce and release in the environment chemical molecules, called auto-inducers. Those substances are recognized from the microbiota and induce modifications in its gene expression. This event leads to changes in the composition of the microbiota and can generate loss of dominance and gradual replacement of certain species in the environment. This phenomenon is known as Quorum Sensing.
The SSOs can perform different activities depending on the physical-chemical features of food and on the ability of each microorganism to degrade nutrients. Example of SSOs activity is the fermentation of simple sugars that can lower the pH and produce gas, generating sour taste and

swelling of the package respectively. Complex carbohydrates instead are rarely attacked and only in the presence of pectinase, pectinesterase, cellulase, amylase and collagenase, which are enzymes typical of microorganisms responsible of rot on vegetables (*Pseudomonas* spp., *Erwinia carotovora* and some moulds).

Other activities of SSO can involve proteins and create free amino acids especially in milk, meat and fish. If this phenomenon happens in absence of oxygen it become putrefaction with a further degradation of amino acids in smelly compounds (putrescine, cadaverine, H_2S, NH_3).

The microbiological lipolytic activity can generate negative events inducing rancidity in the products, or can have positive and necessary effects for the production of the expected aroma of food (e.g. Taleggio cheese) (Feligini et al., 2012). The microbial spoilage can also affect the colour of foods (Franzetti & Scarpellini, 2007), which is not a dangerous phenomenon for human but it makes the product unpleasant for the consumer.

Fig.1.1 Growth and enzymes production of TBC and SSO during time (Huis in't Veld, 1996).

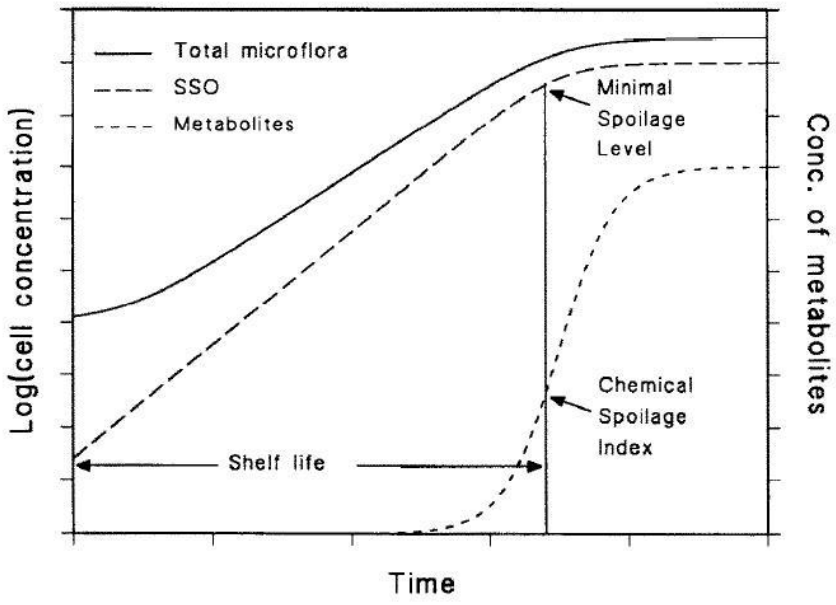


Table 1.1. The most common changes responsible of food spoilage.

Changes	Spoilage event	Effects
Physical	Water content variation	Dehydration / hydration Aggregation phenomena
Chemical	Oxidation	Rancidity
	Maillard reaction	Off-colours - Browning
Enzymatic	Polyphenol oxidase activity	Enzymatic browning
	Lipoxygenase e lipase activity	Rancidity
	Protease activity	Gelation - Structural changes
	Amylase activity	Structural changes
Microbial	Growth of spoilage microorganisms	Lost of sensorial features and shelf life reduction
	Growth of pathogen microorganisms	Health consumer risks
	Growth of pro-technological microorganisms	Positive changes of structure and flavours

1.3 Microbial food spoilage

Microorganisms difficultly attack starchy foods, such as cereals and flours, because their low a_w content prevents microbial growth: only some spore-forming bacteria (e.g. *Bacillus* spp.) and moulds can survive at values around 0.6 of a_w (Sorokulova et al., 2003; Jay et al., 2005) (figure 1.2).
Vegetables and fruit spoilage is principally due to the formation of slime, browning and rots (Barth et al., 2009) (figure 1.3). Ready-to-use vegetables are more perishable than the corresponding fresh products because of the stress to which they are subjected during the production (Riva et al., 2001; D'Egidio et al., 2009).
Usually in the spoilage of milk and dairy products, psychrotrophic bacteria are involved. *Pseudomonas* spp. for example produces heat-stable lipases and proteases (Deeth et al., 2002; Gunaskera et al., 2003) that can affect raw, pasteurized and sterilized milk and generate bitterness, off-flavours, coagulation and pigments of different colours (Datta & Deeth, 2001; Dogan & Boor, 2003; Giaccone, 2010). Typical events of spoilage in cheese are early and late swelling due to coliforms and *Clostridium* spp. respectively (Bassi et al., 2013) (figure 1.4).
In meat and meat products the most common microbial activity is the off-flavour production due to the putrescine and cadaverine, caused especially by *Pseudomonas* spp. and *Brochothrix termosphacta* (Ercolini et al., 2010; Limbo et al., 2010). The activity of *Brochotrix* is observed especially on meat packaged under modified atmosphere; in these conditions also some Clostridia can grow in the deepest part of the muscle (Gram et al., 2002).
Fishes are principally affected by Gram negative, aerobic, psychrothrofic bacteria such as *Pseudomonas* spp., *Acinetobacter* spp. *Shewanella putrefaciens* and different species belonging to the Vibrionaceae family, which produce off-flavours and fluorescence (Franzetti et al., 2001; Gram & Dalgaard, 2002; Franzetti et al., 2003) (figure 1.5).
The many microorganisms present on the surface of eggs can cross the eggshell and grow inside. In fact, although the lysozyme of the albumen has antimicrobial activity against Gram positives bacteria, the nutrients of the yolk represent optimal substrates for microbial growth (Hidalgo et al., 2008).
In alcohol-free drinks the spoilage can be present in terms of off-flavours, strange colours, turbidity, gas and films, caused by Lactic Acid Bacteria (principally *Leuconostoc* spp. and *Lactobacillus* spp.), Acetic Acid Bacteria, yeast and moulds (*Aspergillus* spp., *Penicillum* spp. and *Mucor* spp.).
Among the alcoholic drinks, beer is one of the most critics. Stringing can be due to *Acinetobacter* spp., *Lactobacillus* spp. or *Pediococcus cerevisiae*; honey smell can be caused by *Pediococcus cerevisiae*; souring is consequence of *Acinetobacter* spp. activity, and turbidity appeared for the presence of *Zymomonas anaerobia* and many yeasts among which *Saccharomyces cerevisiae*, *Pichia membranifaciens* and *Debaryomyces* spp. (Jay et al., 2005).

1.4 The most frequent SSO: *Pseudomonas* spp.

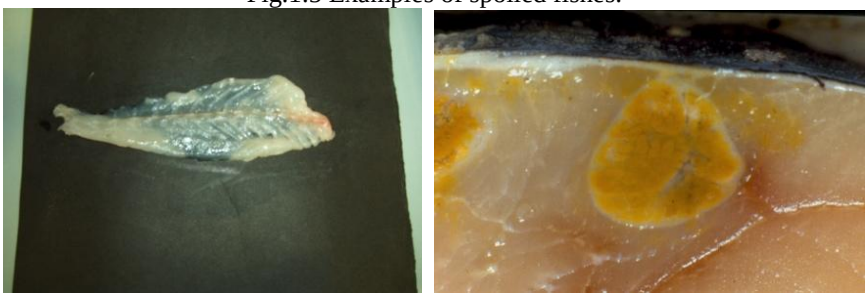
Pseudomonas spp. is one of the most common and dominant SSO. This genus is composed by Gram negative, aerobic, mesophilic and psychrotrophic rods belonged to Psedudomonadaceae family. They are oxidase and catalase positives and often pigment and fluorescence producers; they are considered ubiquitous because, thanks to their complex enzymatic systems, they can grow on different and even extreme substrates (e.g. soil, artic ice, hypersaline lakes, vegetables, animal tissues) (Giupta et al., 2008; Lee et al., 2012; Lopena et al., 2012; Phillips et al., 2012; Wilhelm et al., 2012).

Fig.1.2 Examples of microbial growth on bread.

Fig.1.3 Example of microbial growth on vegetables.

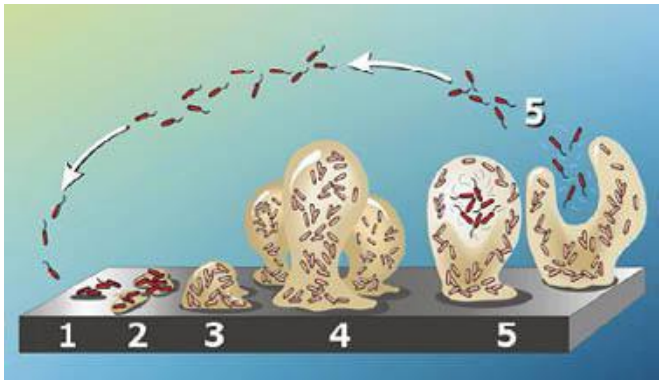
Fig.1.4 Example of swelling in cheese.

Fig.1.5 Examples of spoiled fishes.



The cells of *Pseudomonas* are very sensitive to high temperature and can be destroyed by heat treatment (pasteurization and sterilization); their extracellular enzymes instead are very thermo-stable and can spoilage food even after the cells death, a typical example is the UHT milk coagulation due to microbial proteases (Datta & Deeth, 2001).
The only human pathogen specie of this genus is *Pseudomonas aeruginosa*, which is opportunistic and causes many nosocomial infections (Stover et al., 2000). It produces endotoxins able to infect all the parts of human body, in host with compromised immune system. It can induce infections in urinary tract, respiratory tract, flat tissues, junctions, bones and gastrointestinal tract in patients affected by cancer, cystic fibrosis, burns and AIDS (Marquart et al., 2005; Reszka et al., 2010).
Some species are plant pathogen, for example *Pseudomonas syringae*, which has different phyto-variants able to affect more than 80 plants inducing necrosis (Hirano & Upper, 2000). In particular it enters inside the leaves using the stomata, growing in the interstitial spaces and generating the typical necrotic lesions (Buell et al., 2003). *Pseudomoans viridiflava* instead shows its activity against plats of *Arabidopsis thaliana*, creating translucent full of water spots that after two days degenerated to chlorotic lesions and then necrosis (Jakob et al., 2002).
A peculiar feature of *Pseudomonas* genus is the biofilm production. Many species in fact, especially *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*, can grow in two different ways: the traditional planktonic form and the aggregated form. In the fist way the cells are independent in a liquid environment; in the second instead the cells are closely linked to each other on a solid surface. This behaviour is induced by the Quorum Sensing chemical communication mechanism (Williams, 2007).
In figure 1.6 is shown the biofilm formation. At the beginning the biofilm starts with the adhesion of fluctuant microorganisms on a surface with weak and reversible Van der Waals interactions (phase 1), then if they are not immediately removed from the surface, they can bind firmly to the surface (phase 2). At this point the first colonizers produce exopolysaccharides that become a bridge among the cells and the surface facilitating the link of other cells (phase 3). In this way, the biofilm quickly grow on one hand thanks to the division of the cells and on the other hand by the link of external microorganisms, even of other microbial species (Drenkard & Ausubel 2002; Marino, 2008). Biofilms in fact are usually not composed by unique specie, but many species are present and unevenly distributed as micro-colonies dispersed and protected by the matrix (phase 4). In some biofilms water channels were recognized; those allow the distribution of nutrients and signal molecules (auto-inducers) inside the biofilm and also the removal of wastes outside the matrix (Sauer et al., 2002; Karatan & Watnick, 2009). After a while (phase 5), the cells present inside the biofilm are able to detach and constitute new independent agglomerate. This phenomenon depends from the sliding forces of the fluid, the presence of certain compound and the features of the bacterial species.
Biofilm formation represents a huge problem for food factories because microbial adhesion to surfaces in contact to foods or the foods itself, creates hygiene issue and economical looses (Marino, 2008).

Fig.1.6 Biofilm formation.



1.5 Conclusion and aim of the thesis

In conclusion, the presence in foods of SSOs is a big problem not in terms of consumers' dangers but because of their influence on shelf life of foods. For this reason it is very important to improve the knowledge about those microorganisms and their activities, to find rapid methods of detection and technological solutions (storage and packaging conditions) to slow down their growth and their metabolic activities. In this context, this PhD thesis is involved. The aim of this work is to study the main microorganisms responsible of foods spoilage.
The research involved foods of both vegetable and animal origin; the microbial quality of each food was verified and the SSOs were isolated. The isolates were identified and characterized. Their activities in foods were evaluated and the relationship between them and food spoilage were assessed. Rapid methods for the detection and quantification of *Pseudomonas* spp. (the SSO common to each product) were set up using Real Time-PCR (qPCR) and Retro Transcription-PCR (RT-PCR).

1.6 References

Barth M, Hankinson TR, Zhuang H, Breidt F. 2009. Microbiological spoilage of fruits and vegetables. In Compendium of the microbiological spoilage of foods and beverages, Food Microbiology and Food Safety, Sperber W.H and Doyle M.P. (eds), pp. 135–183, Springer, New York.

Bassi D, Fontana C, Gazzola S, Pietta E, Puglisi E, Cappa F, Cocconcelli PS. 2013. Draft genome sequence of Clostridium tyrobutyricum strain UC7086, isolated from Grana Padano cheese with late-blowing defect. Genome Announcement 1 (4): e00614-13.

Buell CR et al. 2003. The complete genome sequence of the Arabidopsis and tomato pathogen *Pseudomonas syringae* pv. tomato DC3000. PNAS 100 (18): 10181 - 10186.

D'Egidio V, Sinelli N, Limbo S, Torri L, Franzetti L, Casiraghi E. 2009. Evaluation of shelf-life of fresh-cut pineapple by using FIT-NIR and FT-IR spectroscopy. Postharvest biology and technology, 54: 87-92.

Datta N, Deeth HC. 2001. Age gelation of UHT-milk – a review. Food and Bioproducts Processing, 79: 197–210.

Deeth HC, Khusniati T, Datta N, Wallace RB. 2002. Spoilage patterns of skim and whole milks. J. Dairy Res., 69:227–241.

Dogan B, Boor KJ. 2003. Genetic diversity and spoilage potentials among *Pseudomonas* from fluid milk products and dairy processing plants. Applied and Environmental Microbiology, 69: 130–138.

Drenkard E, Ausubel FM. 2002. *Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation. Nature, 416: 740 - 743.

Ercolini D, Ferrocino I, La Storia A, Mauriello G, Gigli S, Masi P, Villani F. 2010. Development of spoilage microbiota in beef stored in nisin activated packaging. Food Microbiology, 27 (1): 137 - 143.

Feligini M, Panelli S, Buffoni JN, Bonacina C, Andrighetto C, Lombardi A. 2012. Identification of microbiota present on the surface of taleggio cheese using PCR-DGGE and RAPD-PCR. Journal of Food Science, 77 (11): 609-615.

Franzetti L, Martinoli S, Piergiovanni L, Galli A. 2001. Influence of Active Packaging on the Shelf-life of Minimally Processed Fish Products in a Modified Atmosphere. Packaging Technology and Science, 14: 267-274.

Franzetti L, Scarpellini M, Mora D, Galli A. 2003, *Carnobacterium* spp. in seafood packaged in modified atmosphere Ann. Microbiol., 53 189-198.

Franzetti L, Scarpellini M. 2007. Characterization of *Pseudomonas* spp. isolated from Foods, Annals of Microbiology, 57 (1): 39-47.

Galli A. 2005. Microbiologia degli alimenti. Casa Editrice Ambrosiana Milano.

Giaccone V. 2010. *Pseudomonas* e prodotti lattiero-caseari. Medicina Veterinaria Preventiva, Settembre, 32.

Giupta SK, Kumari R, Prakash O, Lal R. 2008. *Pseudomonas panipatensis* sp. nov., isolated from an oil-contaminated site. International Journal of Systematic and Evolutionary Microbiology, 58, 1339–1345.

Gram L, Dalgaard P. 2002. Fish spoilage bacteria – problems and solutions. Current Opinion in Biotechnology, 13:262–266.

Gram L, Ravn L, Rasch M, Bruhn JB, Christensen AB, Givskov M. 2002. Food spoilage-interactions between food spoilage bacteria. International Journal of Food Microbiology, 78: 79–97.

Gunasekera TS, Dorsch MR, Slade MB, Veal DA. 2003. Specific detection of *Pseudomonas* spp. in milk by fluorescence in situ hybridization using ribosomal RNA directed probes. Journal of Applied Microbiology, 94: 936–945.

Hidalgo A, Rossi M, Pompei C, Franzetti L. 2008. Chemical Markers for the Evaluation of Raw

Material Hygienic Quality in Egg Products, Journal Agricultural Food Chemistry, 56: 1289-1297.

Hirano SS, Upper CD. 2000. Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae* - a pathogen, ice nucleus, and epiphyte. Microbiology and Molecular Biology Reviews, 64: 624 – 653.

Huis in't Veld JHJ. 1996. Microbial and biochemical spoilage of foods: an overview. International Journal of Food Microbiology, 33: 1–18.

Jakob K, Goss EM, Araki H, Van T, Kreitman M, Bergelson J. 2002. *Pseudomonas viridiflava* and *P. syringae* - Natural Pathogens of Arabidopsis thaliana. Molecular Plant Microbe Interaction, 15 (12): 1195 – 1203.

Jay JM, Lossner MJ, Golden DA. 2005. Modern Food Microbiology. Springer Verlag New York 7th edition.

Karatan E, Watnick P. 2009. Signals, regulatory networks, and materials that build and break bacterial biofilms. Microbiology and Molecular Biology Reviews, 73 (2): 310 - 47.

Lee J, Teitzel GM, Greenber JT. 2012. SGT1b is required for HopZ3-mediated suppression of the epiphytic growth of *Pseudomonas syringae* on N. benthamiana. Plant Signaling & Behavior, 7 (9): 1 - 3.

Limbo S, Torri L, Sinelli N, Franzetti L, Casiraghi E. 2010. Evaluation and predictive modeling of shelf life of minced beef stored in high-oxygen modified atmosphere packaging at different temperatures. Meat Science, 84: 129–136.

Lopena L, Soria V, Varela H, Lupo S, Bergalli A, Guigou M,Pellegrino A, Bernardo A, Calvino A, Rivas F, Batista S. 2012, Extracellular enzymes produced by microorganisms isolated from maritime Antarctica. World J. Microb. Biotec. 28: 2249–2256.

Marino M. 2008. La formazione di biofilm nell'industria alimentare. Igiene alimenti – Disinfezione & Igiene ambientale, 25 (1): 3 – 6.

Marquart ME, Caballero AR, Chomnawang M, Thibodeaux BA, Twining SS, O'Callaghan RJ. 2005. Identification of a Novel Secreted Protease from *Pseudomonas aeruginosa* that Causes Corneal Erosions. Investigative Ophthalmology & Visual Science, 46 (10): 3761 – 3768.

Phillips K, Zaidan F 3rd, Elizondo OR, Lowe KL. 2012. Phenotypic characterization and 16S rDNA identification of culturable non-obligate halophilic bacterial communities from a hypersaline lake, La Sal del Rey, in extreme South Texas (USA). Aquatic Biosystems, 8 (1): 5-16.

Reszka KJ, Bilski PJ, Britigan BE. 2010. Quenching of Singlet Oxygen by Pyocyanin and Related Phenazines. Photochemistry and Photobiology, 86: 742 – 746.

Riva M, Franzetti L, Galli A. 2001. Effect of storage temperature on microbiological quality and shelf-life of ready to use salads. Annals of Microbiology, 51 (1): 39-52.

Sauer K, Camper AK, Ehrlich GD, Costerton JW, Davies DG. 2002. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. Journal of Bacteriology, 184 (4): 1140 – 1154.

Sorokulova IB, Reva ON, Smirnov VV, Pinchuk IV, Lapa SV, Urdaci MC. 2003. Genetic diversity and involvement in bread spoilage of *Bacillus* strains isolated from flour and ropy bread. Letters in Applied Microbiology, 37 (2): 169-173.

Stover CK et al. 2000. Complete genome sequence of *Pseudomonas aerugi*nosa PAO1, an opportunistic pathogen. Nature, 406: 959 – 964.

Wilhelm RC, Radtke KJ, Mykytczuk NC, Greer CW, Whyte LG. 2012. Life at the wedge: the activity and diversity of arctic ice wedge microbial communities. Astrobiology, 12: 347–360.

Williams P. 2007. Quorum sensing, communication and cross-Kingdom signaling in the bacterial world. Microbiology, 153: 3923-3938.

2. EFFECT OF STORAGE TEMPERATURE ON THE MICROBIAL COMPOSITION OF READY-TO-USE VEGETABLES

2.1 INTRODUCTION

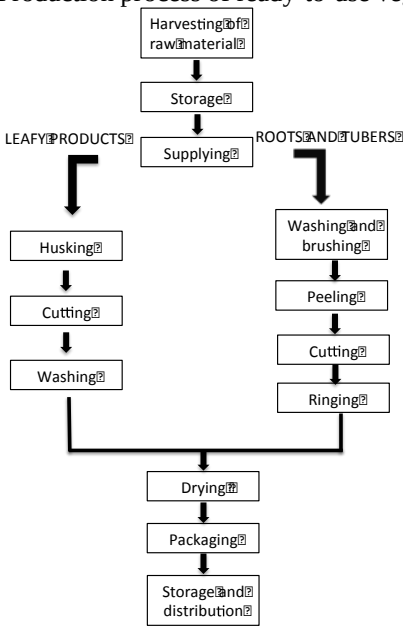
Ready-to-use vegetables are fresh products subjected to minimal processing to preserve their freshness. They belong to the convenience foods category, as they offer many added features like freshness, commodity of use and good retention of nutritional qualities. The products are very low-processed: they are picked, washed, dried, trimmed, cored, cut and packed, then marketed ready to eat without further handling (Baur et al., 2005; Della Rosa et al., 2007; Caponigro et al., 2010) (fig. 2.1). The above operations can increase the respiratory activity of vegetables, causing negative effects on their texture and colour, and rendering them more perishable than corresponding fresh vegetables. The stability of these products depends on the quality of raw materials, the handling procedures, the packaging, and the storage conditions (Gim'enez et al., 2003; Legnani et al., 2004; Morgante et al., 2008).

Microbial growth is a key factor in these products deterioration as the processing excludes the operations of sanitation and stabilization, and the raw material, characterized by a high nutrient and enzyme content and high water activity, is an excellent substrate for microorganisms (Ragaert et al., 2007; Riva et al., 2001).

In general, resident microorganisms in ready-to-use-vegetables are not pathogenic to humans, though there have been some reports in literature (Fröder et al., 2007; Little et al., 2007; Sant'Ana et al., 2012). The most important components of the microbiota of ready-to-use-vegetables are Gram negative microorganisms, belonging to the Pseudomonadaceae and Enterobacteriaceae families. However, the microbiota can vary, depending on the environment and the characteristics of the product (Tournas, 2005; Abadias et al., 2008; Caponigro et al., 2010; De Giusti et al., 2010; Franzetti et al., 1999; Oliveira et al., 2011).

The European Regulation 1441/2007 fixes a limit for *Salmonella* and *Escherichia coli* and the French regulations (Ministère de l'Economie, Article du 22-3-1993) fixes the maximum acceptable contamination values for the end of production and for retail sites. The respect of the cold chain (4°C) is determinant to control both enzymatic activity and microbial growth. More recently the modification of the atmosphere within the packaging showed to slow down the deterioration process (Sandhya, 2010).

Fig.2.1 Production process of ready-to-use vegetable.



2.1.1 Aim of the study

The aims of this work were *i)* to assess the microbiological quality and microbiota development of ready-to-use vegetables during shelf life, *ii)* to verify the influence of storage temperature and packaging method on microbiota composition and growth, *iii)* to characterize the most frequently found microbial strains responsible for deterioration phenomena.

2.2 MATERIALS AND METHODS

2.2.1 Samples

Five different ready-to-use-vegetables were analysed. In particular, they were julienne carrots packaged in air and four different green salads: "Songino" (*Valerianella olitoria*) and "Lattughino" (*Lactuca sub. secalina*) both packaged in bags of PP antifog in air, and "Lattuga foglia quercia" (*Lactuca sub. crispa*) and "Lattuga cappuccio" (*Lactuca sub. capitata*) packaged under Modified Atmosphere (the atmosphere composition was not declared).
All the samples were received into the laboratory, from the factory, the production day, and stored at 4°C and 10°C. The analyses were performed immediately at the reception (t_0) and daily until two days after the expiry date at both temperatures.

2.2.2 Microbiological analysis and isolation of the strains

Ten grams of sample were drawn and homogenized with 90ml of sterile 0,85% trypton salt solution in a sterile Stomacher bag, by the use of a Colworth 400 Stomacher for 2min. Decimal progressive dilutions were prepared and the following bacteriological determinations were carried out: Total Bacterial Count (TBC) (ISO 4833/2003), *Escherichia coli* (ISO 16649-2/2001), *Staphylococcus aureus* (UNI/EN/ISO 6888/2004), Lactic Acid Bacteria (De Man et al., 1960), yeasts and moulds (ISO 21527/2008); as safety indicators, *Salmonella* spp. (EN/ISO 6579/2007) and *Listeria monocytogenes* (ISO 11290-1/2004) were determined in 25g of product.
All microbiological analyses were carried out in triplicate, and the results were expressed as the mean log cfu g^{-1}. All colonies grown on the last dilution of PCA were collected, purified and stored in 20% (vol/vol) glycerol at -20°C.

2.2.3 Phenotypic characterization

The isolates were tested for morphology, motility, Gram staining by optical microscopy (1200X), catalase and oxidase tests.
Fluorescent and phenazine pigment production (King et al., 1954), levans production (Lelliot et al., 1966), and oxidative/fermentative metabolism (OF test, Hugh *et al.*, 1953) were used for a preliminary biochemical characterisation of Gram negative and catalase positive isolates. The enzymatic activities tested were: pectinolytic activity (Sands et al., 1972) and starch hydrolysis (Stanier et al., 1966).
Gram positive catalase negative no spore-forming bacteria were tested for gas production from glucose, NH_3 production from arginine, growth at 45 and 10°C, and (cocci only) esculin hydrolysis.

2.2.4 DNA extraction and PCR protocols

Genomic DNA from isolates was extracted from 300µl of an overnight culture diluted with 400µl of 1X TE buffer (10mM Tris-HCl and 1mM Na_2EDTA, pH 8.0) as described by Mora et al., 2000.
All PCR reactions were performed in a volume containing approximately 50-100ng of bacterial genomic DNA solution, 5µl of 10X PCR reaction buffer, 200µM of each dNTP, 2mM of

$MgCl_2$, 0.5µM of each primer and 0.5U of Taq Polymerase (Amersham- Pharmacia).
The primers and the amplification conditions used are shown in table 2.1, except for 16S rDA amplification that was performed using the following thermal profile: 2min at 94 °C; 5 cycles consisting of 94 °C for 45s, 55 °C for 1min, 72 °C for 2min; 35 cycles consisting of 92 °C for 45s, 60 °C for 45s, 72 °C for 2min; final extension of 72 °C for 2min; and final cooling at 4 °C.
All the amplifications were performed in a DNA thermal cycle (Biometra T gradient, Germany). Following the amplification, 7µl of each amplificate was analysed by electrophoresis at 100V (1% agarose gel, 0.2µg of ethidium bromide ml^{-1}) in TAE buffer.

2.2.5 Restriction analysis of the 16S rDNA gene and spacer gene (ITS1) - ARDRA analysis

Restriction digestion of 16S rRNA gene was carried out for 16 hours at 30°C in 25µl reaction mixture containing 15µl of amplified 16S rRNA template, 2.5µl of 10X PCR restriction buffer, 18.75U of one restriction enzyme, either H*aeIII* or V*spI* (Amersham Pharmacia Biotech). Restriction digestion of each amplified ITS1 was carried out for 16 hours at 65 °C in 25µl reaction mixture containing 15µl of ITS1template, 2.5µl of 10X PCR restriction buffer, 18.75U of T*aqI* (Amersham Pharmacia Biotech). The restriction digestions were then analysed by agarose gel electrophoresis (3% w/v) (Guasp et al., 2000).
The restriction results were interpreted using Quntity One 4.6 software package (Bio-Rad Laboratories, Milan, Italy).

2.2.6 Partial sequencing of the 16S rDNA gene

After amplification of the 16S rDNA gene from extracted DNA, the PCR product was purified according to the instructions of a commercial kit (Qiaquick, Qiagen), and the amplicons were sequenced with the 16S forward primer in a model 310 automatic DNA sequencer (Applied Biosystem, Foster City, CA). The obtained sequences were elaborated by using the software Chromas 2.13 (Technelysium Pty Ltd. Helensvale, Queensland, Australia). Sequence data of type strains were retrieved from NCBI sequence database and pairwise comparison was conduced using BLAST program.

2.2.7 Statistical analysis

The counts obtained at both temperatures for each product and at each time, were subjected to one-way analysis of variance (ANOVA). In case of statistically significant differences the Tukey multiple comparison test was performed ($P < 0.05$ and $P < 0.01$).

Table 2.1 PCR primers and conditions used.

Gene	Primers pair (5'-3')		Annealing temperature
Intergenic spacer region (ITS) (Mora et al., 2003)	ITSF: ITSR:	GTCGTAACAAGGTAGCCGTA CAAGGCATCCACCGT	54°C
Intergenic spacer region 1 (ITS1: 16S-ITS-23S) (Guasp et al., 2000)	16F945: 23R458:	GGGCCCGCACAAGCGGTGG CTTTCCCTCACGGTAC	55°C
16S rDNA region (Lane et al., 1985)	16SF: 16SR:	AGAGTTTGATCCTGGCTCAG CTACGGCTACCTTGTTACGA	See in the text
Pseudomonas fluorescens 16S rDNA partial region (Scarpellini et al., 2004)	16SPSEfluF: 16SPSEfluR:	TGCATTCAAAACTGACTG AATCACACCGTGGTAACCG	55.6°C

2.3 RESULTS AND DISCUSSION

The investigated products appeared of good hygienic quality: *Salmonella* sp. and *Listeria monocytogenes* were always absents in 25g, while *E. coli* and *Staphylococcus aureus* were found with values less than 1log cfu g^{-1}, the accepted level according to the European Regulation (1441/2007). Yeasts and molds were found only occasionally.
About the green salads, the TBC growth trend was similar in all products at both temperatures, although the initial values were higher in air packaged ones. In "Songino" and "Lattughino" initial TBC were 6.1log cfu g^{-1}, about 2log higher than "Lattuga foglia quercia" and "Lattuga cappuccio", both packaged under Modified Atmosphere. During storage this microbial parameter increased and at expiry (sixth day for air packaged products and eighth day for Modified Atmosphere packaged vegetables) all the values were similar falling between 7.1 and 8.2log cfu g^{-1}. For each product, the daily average at 4°C was compared with the corresponding average at 10°C (Tables 2.2 and 2.3).
However no statistically significant differences (at 95%) were found between the air-packed products stored at 4 and 10°C. The products packaged under Modified Atmosphere, instead, showed statistically significant differences. After varying times, depending on the type of product, the values are significant at 0.05 level, but on shifting this level to 0.01 the values were not statistically significant different. The differences found at $p<0.05$ are due to the overcoming of the limit imposed by the European regulation (1441/2007), that occurred for "Lattuga quercia" after 8 days at 10°C and for "Lattuga cappuccino" after 3 days at 10°C.
Carrots packaged in air seemed less contaminated than the green salads packaged under the same condition: the TBC initial value was 4.3log cfu g^{-1}, but the composition of this vegetable represents a favorable habitat for different microorganisms and during the shelf life at both temperatures TBC values reached values higher than 7log cfu g^{-1} (table 2.4). At the end of the analysis, after the expiration date of the product, those values appeared statistically significant different at $p<0.05$ and shifting this level to 0.01 the differences were not statistically significant.
Lactic Acid Bacteria were present in all investigated products, but they reached important values only in carrots, whereas in salads their presence was 3 or 4log cfu g^{-1} less than the TBC value (results not shown). In carrots instead (table 2.4), practically absent at the beginning, they grown faster and reached values similar to the TBC especially when stored at 10°C. The higher temperature in fact favored their growth allowing them to grow faster at the beginning (statistically significant different at $p<0.05$ values after 2 and 3 days of storage).

A total of 147 isolates were collected from PCA; biochemical investigations showed that over 80% of isolates were Gram negative aerobic oxidase positive rods, whereas the rest were Gram negative, facultative anaerobic, oxidase negative rods. About 70% of aerobic isolates were devoid of amylase activity, whereas more than 50% showed high pectinolytic activity, responsible for the browning phenomena of ready-to-use vegetables.
From MRS 84 strains were isolated and the 80% belonged to carrots. Over 85% of isolates, were hetero-fermentative cocci and the rest part was represented by omo-fermentative cocci.
Microbial diversity investigation of all isolates was performed using the ITS-PCR assay with the universal primers. ITS profiles were used to create different groupings, and no less than two representatives from each group were identified by partial 16S rDNA sequencing (Table 2.5).

TABLE 2.2 Evolution of the Total Bacterial Count of air-packaged ready-to-use salads during storage at 4°C and 10°C

	Songino			Lattughino		
Time (days)	TBC 4°C	TBC 10°C	Significative differences	TBC 4°C	TBC 10°C	Significative differences
0	6.1±0.1	6,1±0.1	n.s.d.	6.1±0.1	6.1±0.1	n.s.d.
1	6.4±0.2	6.5±0.3	n.s.d.	6.9±0.1	6.8±0.2	n.s.d.
2	6.5±0.1	6.6±0.2	n.s.d.	7.1±0.3	7.5±0.2	n.s.d.
3	7.4±0.2	7.6±0,2	n.s.d.	7.7±0.1	7.7±0.1	n.s.d.
6	7,6±0.2	7.8±0,1	n.s.d.	7.8±0.2	7.9±0.2	n.s.d.
8	7.7±0.3	8.5±0.4	n.s.d.	11.2±0.1	11.3±0.1	n.s.d.
n.s.d.: no significant difference, $p<0.05$						

TABLE 2.3 Evolution of the Total Bacterial Count of modified atmosphere ready-to-use salads during storage at 4°C and 10°C

	Lattuga foglia quercia			Lattuga cappuccio		
Time (days)	TBC 4°C	TBC 10°C	Significative differences	TBC 4°C	TBC 10°C	Significative differences
0	4,2±0,3	4,2±0,3	n.s.d.	4,1±0,1	4,1±0,1	n.s.d.
1	5,7±0,4	5,3±0,2	n.s.d.	5,5±0,3	5,1±0,2	n.s.d.
2	6,3±0,4	6,3±0,2	n.s.d.	6,0±0,1	6,5±0,2	n.s.d.
3	6,0±0,2	6,1±0,2	n.s.d.	6,4±0,2	7,1±0,1	*
6	6,3±0,2	6,5±0,1	n.s.d.	7,1±0,4	7,5±0,3	n.s.d.
8	7,1±0,1	7,7±0,2	*	7,4±0,4	8,2±0,1	n.s.d.
10	7,7±0,3	8,2±0,3	n.s.d.	7,5±0,3	8,3±0,2	n.s.d.
n.s.d.: no significant difference, $p<0.05$; *: significant difference, $p<0.05$						

TABLE 2.4 Evolution of the Total Bacterial Count and Lactic Acid Bacteria of carrots during storage at 4°C and 10°C

	Carrots					
Time (days)	TBC 4°C	TBC 10°C	Significative differences	LAB 4°C	LAB 10°C	Significative differences
0	4.3±0.1	4.3±0.1	n.s.d.	0.0±0.0	0.0±0.0	n.s.d.
1	5.7±0.2	5.7±0.2	n.s.d.	3.6±0.2	4.1±0.3	n.s.d.
2	5.9±0.2	6.2±0.2	n.s.d.	4.5±0.3	5.5±0.2	*
3	6.5±0.3	7.3±0.2	n.s.d.	4.9±0.2	6.2±0.3	*
7	7.0±0.2	7.3±0.2	n.s.d.	5.2±0.2	5.7±0.1	n.s.d.
9	7.1±0.1	7.7±0.1	*	6.5±0.3	6.5±0.4	n.s.d.
n.s.d.: no significant difference, $p<0.05$; *: significant difference, $p<0.05$						

TABLE 2.5 16S rDNA partial sequencing of isolates.

Group	16S region partial sequencing	Accession number
A	*Leuconostoc mesenteroides*	HM218785.1
B	*Leuconostoc mesenteroides*	GQ351324.1
C	*Leuconostoc mesenteroides*	HM058900.1
D	*Leuconostoc mesenteroides*	HM218070.1
E	*Leuconostoc mesenteroides*	HM058934.1
F	*Leuconostoc mesenteroides*	EU099617.1
G	*Leuconostoc mesenteroides*	AB593362.1
H	*Leuconostoc pseudomesenteroides*	EU177643.1
I	*Leuconostoc gasicomitatum*	GU470976.1
L	*Leuconostoc palmae*	AM940225.1
M	*Leuconostoc inhae*	AY675244.1
N	*Leuconostoc citreum*	AB572028.1
O	*Enterococcus mundtii*	AB576587.1
P	*Enterococcus mundtii*	GU372708.1
Q	*Carnobacterium maltaromaticum*	AY543035.1
α	*Pantoea agglomerans*	FJ756348.1
β	*Enterobacter cloacae*	EU733519.1
γ	*Citrobacter freundii*	EU365679.1
δ	*Rahnella aquatilis*	DQ440548.1
ε	*Erwinia rhapontici*	U80206.1|ERU80206
η	*Serratia fonticola*	NR_025339.1
χ	*Enterobacter ludwigii*	JX666242
1	*Pseudomonas fluorescens*	GU198125.1
2	*Pseudomonas fluorescens*	HM439956.1
3	*Pseudomonas fluorescens*	EU169164.1
4	*P. marginalis pv. marginalis/ P. fluorescens*	HM190225.1 / GU198116.1
5	*P. fluorescens / P. poae*	HM439956.1 / AB495132.1
6	*P. fluorescens / P. poae*	GU198111.1 / FJ179369.1
7	*P. fluorescens/ P. extremaustralis/ P. veronii*	GU198116.1/AJ583501.3/AB334768.1
8	*P. poae / P. fluorescens*	AB495132.1 / GU198126.1
9	*P. grimontii / P. fluorescens*	NR_025102.1 / GU198125.1
10	*P. fluorescens / P. jessenii*	EU169164.1 / AM933510.1
11	*P. fluorescens / P. kilonensis*	FN675867.1 / DQ377772.1
12	*Pseudomonas koreensis*	HM367598.1
13	*Pseudomonas viridiflava*	AM182934.1
14	*Pseudomonas argentinensis*	AY691188.2
15	*Pseudomonas putida*	AY450555.1
16	*Pseudomonas putida*	EU118779.1
17	*Pseudomonas putida*	AB016428.1
18	*Pseudomonas koreensis*	FM202488.1
19	*Pseudomonas veronii*	AF539745.1
20	*Pseudomonas veronii*	AY512620.1
21	*Pseudomonas fragi*	GU549487.1
22	*Pseudomonas fragi*	AM062695.1
23	*Pseudomonas cf. synxantha V4*	AJ244725.1
24	*Pseudomonas frederiksbergensis*	AY785733.1

Among Lactic Acid Bacteria, *Leuconostoc mesenteroides* was the predominant specie showing high bio-variability. The other species of *Leuconostoc* found in low concentration (*L. gasicomitatum, L. palmae, L. inhae, L. citreum* and *L. pseudomesenteroides*) were associated with gaseous spoilage of modified-atmosphere-packaged products. Occasionally we found homofermentative cocci, *Enterococcus mundtii* and rods like *Carnobacterium maltaromaticum.*
About 80% of Enterobacteriaceae came from Modified Atmosphere packaged products, and the most frequent specie found was *Citrobacter freundii*, followed by *Rahnella aquatilis* and *Pantoea agglomerans*. A major variety of species (24 groups) were found among *Pseudomonas*, but *Pseudomonas fluorescens* was dominant. For some groups it was not possible to make unequivocal identification. In order to unequivocally ascribe the species, the isolates were tested with species-specific primers for *P. fluorescens* (Scarpellini et al., 2004) (results not shown). The most part showed the typical profile of this species (850bp), whereas only two groups did not show the amplification, and were classified as *Pseudomonas jessenii* and *Pseudomonas kilonensis* respectively (groups 10 and 11).
For all the *P. fluorescens* strains (9 groups), the differentiation of biovars was performed according to Scarpellini et al., 2004 (figure2.2). Over 60% of *P. fluorescens* belonged to biovar G, 5% belonged to biovar 3, and the rests part was equally divided between biovars B and C. Table 2.6 summaries ARDRA analysis results.

The species distribution (Figures 2.3) showed that in the air-packaged salads, *Pseudomonas* was, at the beginning of the shelf life, the dominant genus, particularly *P. fluorescens,* was the most frequently found specie. At the end of the shelf life it remained the most represented, however the presence of Enterobacteriaceae, less psychrotrophic, increased especially in the product stored at 10°C. Instead, packaging under modified atmosphere is a more favourable environment to Enterobacteriaceae. Their number increased during the storage and at the end of shelf life they became the prevalent microbial population.
For carrots, the distribution of the microbial composition is reported in figure 2.4. At t_0 the microbiota was composed by *Pseudomonas koreensis* and two species belonging to Enterobacteriaceae family. The storage at the two different temperatures didn't affect the founded species: *Pseudomonas* became the dominant genus at both temperature and in addition to *P. koreensis*, *P. fluorescens*, *P. veronii*, *P. fragi* and *P. putida* were found. During the shelf life appeared the LAB, absent at the beginning. At the two temperatures, we found the same species and in the same percentage (figure 2.5).

Fig. 2.2 Electrophoretic profiles of the digestion with a) T*aqI*, b) H*aeIII* and c) V*spI;* Marker Gene Ruler 100 bp (MBI Fermentas).

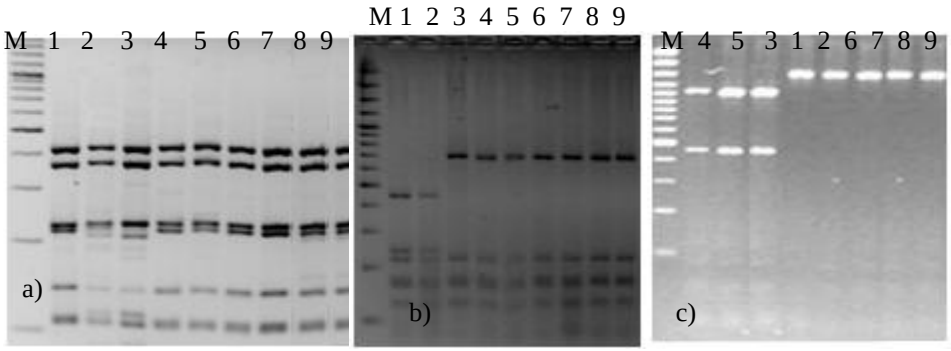


TABLE 2.6 Differences and distribution of *Pseudomonas fluorescens'* biovars.

Biovar	T*aq I* on 16S-ITS-23S rDNA	H*ae III* on 16S rDNA	V*sp I* on 16S rDNA	Levans production	Recognized groups
A	420, 380, 280, 230, 180, 150, 110	700, 220, 180, 150	1600	+	-
B	420, 380, 230, 220, 150, 110	450, 250, 220, 180, 150	1600	+	1, 2
C	420, 380, 230, 220, 150, 110	700, 220, 180, 150	1150, 450	+	3, 4
3	420, 380, 230, 210, 150, 110	700, 220, 180, 150	1150, 450	-	5
G	420, 380, 230, 210, 150, 110	700, 220, 180, 150	1600	+	6, 7, 8, 9

Fig. 2.3 TBC percentage composition in a) air packaged product; b) Modified Atmosphere packaged product during the shelf life at 4 and 10°C.

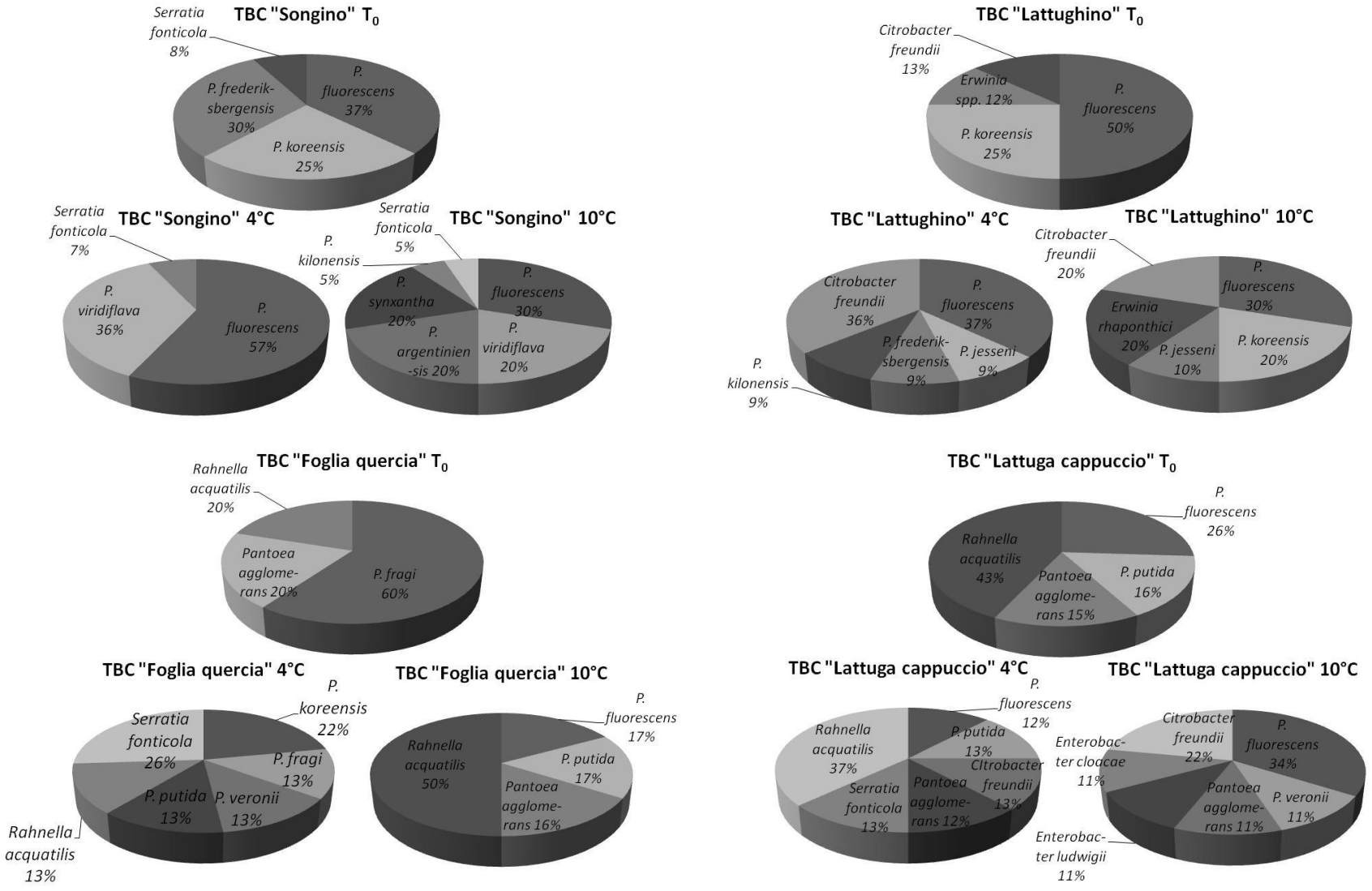


Fig. 2.4 TBC percentage composition in carrots during the shelf life at 4 and 10°C.

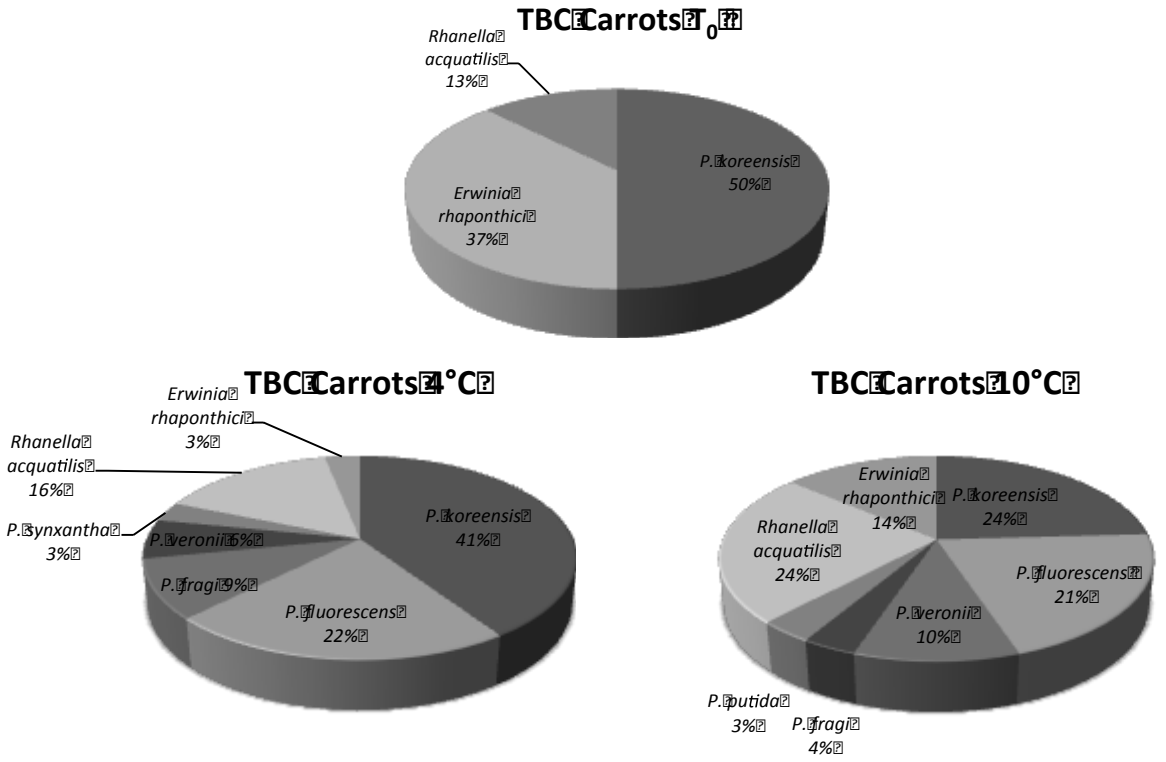
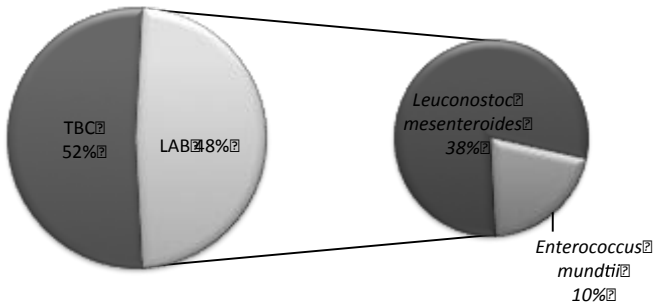


Fig. 2.5 TBC and LAB percentage and composition in carrots during shelf life.



2.3 CONCLUSIONS

In conclusion the investigated products' quality was good. Total Bacterial Count and Lactic acid bacteria were the most important indices to define the quality of the final product. According other authors (Fröder et al., 2007; Caponigro et al., 2010; Sandhya, 2010; Sant'Ana et al., 2012) the microbial quality of the raw vegetables, the processing, the packaging technique and the storage temperature are important factors influencing the qualitative and quantitative microbial composition in the final product.

The predominant genus of TBC (80%) was *Pseudomonas* and in particular we found specie *fluorescens*. Over 60% of *P. fluorescens* belonged to biovar G, 5% belonged to biovar 3, and the rests part was equally divided between biovars B and C. These isolates were characterized by an important pectinolytic activity, responsible of browning phenomena of ready to use vegetables. Only 20% of the TBC belonged to species of Enterobacteriaceae family, and about 80% of these came from Modified Atmosphere packaged products. These species are typical of vegetables environment (soul and water) and are characterized by a pectinolytic activity and starch hidrolisys.

The LAB were a minority except in carrots (figure 2.5), *Leuconostoc mesenteroides* was the predominant specie (75%). In air packaged salads it was the only specie found, whereas more variability was observed in packaged under Modified Atmosphere lettuces. *Leuconostoc* spp. produces viscous and slimy material that allows adhering on the surface of vegetables, from which they are difficultly removed. Nevertheless LAB presence is important because with their metabolic activity (acidification) they influence the development of different microorganisms.

The temperature seemed not to change the distribution of microbial population found in each product, the only detected difference is the appearance of certain microbial species at 10°C not found at 4° C (figures 2.3 and 2.4). The recovered species are typical of vegetables environment (soil and water) and are characterized by pectinolytic activity and starch hydrolysis. These results highlight how each vegetable has its own ecosystem and the composition (microbiota) of this system is influenced by the quality of raw materials, the processing and the packaging conditions and the storage temperature.

2.4 REFERENCES

Abadias M, Usall J, Anguera M, Solsona C, Viñas I. 2008. Microbiological quality of fresh, minimally-processed fruit and vegetables, and sprouts from retail establishments. International Journal of Food Microbiology, 123: 121-129.

Baur S, Klaiber R, Hua Wei H, Hammes WP, Carle R. 2005. Effect of temperature and chlorination of pre-washing water on shelf-life and physiological properties of ready-to-use iceberg lettuce Innovative Food Science and Emerging Technologies, 6: 171-182.

Caponigro V, Ventura M, Chiancone I, Amato L, Parente E, Piro F. 2010. Variation of microbial load and visual quality of ready-to-eat salads by vegetable type, season, processor and retailer. Food Microbiology, 27: 1071 - 1077.

De Giusti M, Aurigemma C, Marinelli L, Tufi D, De Medici D, Di Pasquale S, De Vito C, Boccia A. 2010. The evaluation of microbiological safety of fresh ready-to-eat vegetables produced by different technologies in Italy. Journal of Applied Microbiology, 109: 996 - 1006.

De Man JC, Rogosa M, Sharpe ME. 1960. A medium for the cultivation of Lactobacilli. Journal of Applied Microbiology, 23: 130 - 135.

Della Rosa M, Rocculi P. 2007. Prodotti di IV gamma, aspetti qualitativi e tecnologici. Agricoltura, 34: 33-34.

Franzetti L, Galli A. 1999. Microbial quality indicators of minimally processed stick carrots. Annals of Microbiology and Enzimology, 49: 137 - 144.

Fröder H, Martins CG, De Souza KL, Landgraf M, Franco BD, Destro MT. 2007. Minimally processed vegetable salads: microbial quality evaluation. Journal of Food Protection, 70: 1277 - 1280.

Gim´enez M, Olarte C, Sanz S, Lomas C, Ech´avarri JF, Ayala F. 2003. Relation between spoilage and microbiological quality in minimally processed artichoke packaged with different films. Food Microbiology, 20: 231 - 242.

Guasp C, Moore ERB, Lalucat J, Bennasar A. 2000. Utility of internally transcribed 16S-23S DNA spacer regions for the definition of *Pseudomonas stutzeri* genomovars and other *Pseudomonas* species. International Journal of Systematic and Evolutionary Microbiology, 50: 1629 - 1639.

Hugh R, Leifson E. 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram negative bacteria. Journal of Bacteriology, 66: 24-26.

King EO, Ward WK, Raney DE. 1954. Two simple media for the determination of pyocyanine and fluorescine. Journal of Laboratory and Clinical Medicine, 44: 301 - 307.

Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML, Pace NR. 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc. Natl. Acad. Sci USA. 82: 6955-6959.

Legnani PP, Leoni E. 2004. Effect of processing and storage conditions on the microbiological quality of minimally processed vegetables. International Journal of Food Science and Technology, 39: 1061 - 1068.

Lelliot RA, Billing E, Hayaward AC. 1966. A determinative for the fluorescent plant pathogenic *Pseudomonas*. Journal of Applied Bacteriology, 29: 470 - 489.

Little CL, Taylor FC, Sagoo SK, Gillespie IA, Grant K, McLauchlin J. 2007. Prevalence and level of *Listeria monocytogenes* and other *Listeria* species in retail pre-packaged mixed vegetable salads in the UK. Food Microbiology, 24: 711 - 717.

Ministère de l'Economie, des Finances et du budget. 1993. Article du 22-3-1993 relatif aux re`gles d'hygiène applicable aux végeétaux et préparations de végétaux crus prets a l'emploi

a la consumation humaine. Journal Official De la République Francaise 1627:5586–5588.
Mora D, Fortina MG, Parini C, Daffonchio D, Manachini PL. 2000. Genomic subpopulations within the species *Pediococcus acidilactici* detected by multilocus typing analysis: relationship between pediocin AcH/PA-1 producing and non- producing strains. Microbiology, 146: 2027 - 2038.
Mora D, Ricci G, Guglielmetti S, Daniele Daffonchio D, Fortina MG. 2003. 16S–23S rRNA intergenic spacer region sequence variation in *Streptococcus thermophilus* and related dairy streptococci and development of a multiplex ITS-SSCP analysis for their identification. Microbiology, 149: 807–813.
Morgante RA, Mencaroni G, Scuota S, Cenci T. 2008. Survey on Minimally processed vegetables. Indagine conoscitiva sui vegetali di IV gamma. Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche, Webzine Sanità Pubblica Veterinaria, 50: 1581 - 1592.
Oliveira MA, Maciel de Souza V, Morato Bergamini AM, Pereira de Martinis EC. 2011. Microbial quality of ready-to-eat minimally processed vegetables consumed in Brazil. Food Control, 22 (8): 1400-14003.
Ragaert P, Devlieghere F, Debevere J. 2007. Role of microbiological and physiological spoilage mechanisms during storage of minimally processed vegetables. Postharvest Biology and Technology, 44: 185-194.
Riva M, Franzetti L, Galli A. 2001. Microbiological quality and shelf life modeling of ready-to-eat Cicorino. Journal of Food Protection, 64: 228 - 234.
Sandhya E. 2010. Modified atmosphere packaging of fresh produce: Current status and future needs. LWT - Food Science and Technology, 43: 381 - 392.
Sands DC, Hankin L, Zucker M. 1972. A selective medium for pectolytic fluorescent *Pseudomonas*. Phytopatology, 62: 998 - 1000.
Sant'Ana AS, Barbosa MS, Destro MT, Landgraf M, Franco BDGM. 2012. Growth potential of *Salmonella* spp. and *Listeria monocytogenes* in nine types of ready-to-eat vegetables stored at variable temperature conditions during shelf-life. International Journal of Food Microbiology, 157: 52-58.
Sant'Ana A, Igarashi MC, Landgraf M, Destro MT, Franco BDGM. 2012. Prevalence, population and pheno- and genotypic characteristics of *Listeria monocytogenes* isolated from ready-to-eat vegetables marketed in Sao Paulo, Brazil. International Journal of Food Microbiology, 155: 1-9.
Scarpellini M, Franzetti L, Galli A. 2004. Development of PCR assay to identify *Pseudomonas fluorescens* and its biotype. FEMS Microbiology Letters, 236: 257 - 260.
Stanier RY, Palleroni NJ, Doudoroff M. 1966. The aerobic pseudomonads: a taxonomic study. Journal of General Microbiology, 43: 159 - 271.
Tournas VH. 2005. Moulds and yeasts in fresh and minimally processed vegetables and sprouts. International Journal of Food Microbiology, 99: 71 - 77.

3 EVALUATION OF MILK DESTABILIZATION BY PROTEOLYTIC ACTIVITY OF *Pseudomonas* spp.

3.1 INTRODUCTION

Different technological tools can be adopted to reduce microbial presence in raw milk, which represents a suitable environment for microbial growth; in particular, the refrigeration of raw milk is imposed by EU Hygiene Regulations to keep microbial growth under control. However, low temperature creates selective conditions for psychrotrophic bacteria, especially those belonging to *Pseudomonas* spp. (Cousin, 1982; Datta et al., 2003; Lafarge et al., 2004; Hantsis-Zacharov et al., 2007). This genus, and particularly *Pseudomonas fluorescens*, has significant spoilage potential due to its ability in producing heat-stable enzymes (proteases and lipases), which are not inactivated by pasteurisation and UHT treatments. These enzymes may cause reduction in cheese yield, gelation of UHT milk and off-flavours in many dairy products (Sorhaug et al., 1997; Woods et al., 2001; Dogan et al., 2003; Marchand et al., 2009a).
While milk destabilisation can be a positive event in cheese production and maturation (Coker et al., 2005), it represents a very negative phenomenon in milk intended for direct consumption where it can lead to product waste with high economic losses. During storage at room temperature, UHT milk can undergo gelation and two different mechanisms have been proposed to explain this phenomenon. One relates to the heat-induced formation of a complex between the β-lactoglobulin and k-casein, which is then progressively released from the micelles into the milk serum and, when it reaches a certain concentration, it forms a three-dimensional network (McMahon, 1996). More recently, another mechanism has been proposed that seems to be more relevant. It relates to a slow proteolysis of k-casein that leads to the formation of a gel similar to the gel obtained by rennet coagulation (Garcìa-Risco et al., 1999; Datta et al., 2001; Dupont et al., 2007). Thus destabilisation of UHT milk has been linked to residual or reactivated proteolytic enzymes (Nicodème et al., 2005; Liu et al., 2007; Dufour et al., 2008; Baglinière et al., 2013). These proteases are both native, as plasmin, and produced by psychrotrophic bacteria. These latter predominantly attack k-casein, and therefore seem to be more directly involved in gel formation (Baglinière et al., 2013), and can also affect whey proteins (Datta et al., 2001). In parallel, residual plasmin activity may contribute to further casein degradation, markedly αs2-casein (Chavan et al., 2011).
AprX protease is the most common alkaline metallo-protease produced by *Pseudomonas* spp. and is the only one produced by *P. fluorescens* (Woods et al., 2001). It is a heat-stable protease belonging to the serralysin family and requiring Zn and Ca^{2+} for its activity; it has an optimal pH of activity at 8.5 and an optimal temperature at 45 °C (Dufour et al., 2008).
The *aprX* gene codifies for this protease and is located in an operone with other genes encoding for lipase (*lipA*), protease inhibitor (*inh*), protease secretion apparatus (*aprDEF*) and autotransporter proteins (*prtA* and *prtB*). The organization of such operon varies from one strain to another (Liao and McCallus, 1998; Johnson et al., 1992; Ahn et al., 1999Kawai et al., 1999; Woods et al., 2001). The regulation of the expression of this gene is very complex and not completely understood; usually the expression occurred in the exponential or stationary phase of microbial growth (Griffiths, 1989), and it is affected by different parameters, such as temperature (Nicodème et al., 2005), iron content (Woods et al., 2001), Quorum Sensing (Juhas et al., 2005; Liu et al., 2007) and phase variation (van den Broeck et al., 2005).

3.1.1 Aim of the study

Chemical and biochemical approaches have been used since long time for the study of UHT milk coagulation and destabilization phenomena (Mottar et al., 1985; Lopez Fandino et al., 1993; Picard et al., 1996; Recio et al., 1996, Deeth et al., 2000). In this study we aim to improve the knowledge about milk destabilization and to examine a possible correlation with the proteolytic activity of *Pseudomonas* spp. In particular, the correlation between the molecular detection of *aprX* gene and the coagulation of milk was investigated.

3.2 MATERIALS AND METHODS

3.2.1 Samples

Commercial samples pasteurized, micro-filtered and UHT milk were analysed. The samples were collected during three years and analysed after different times of storage at 4°C or room temperature, in some cases after the expiration date. In addition, two samples of raw milk were provided by a milk manufacturer. Some samples were subjected to a previous inoculation with overnight culture of *Pseudomonas fluorescens* DSM50106^{T} before the analysis; the total count of the inoculum was determined by microscopy quantification (Burker chamber), and the concentration of the alive cells of the inoculum was quantified by culture dilutions plating on CFC selective *Pseudomonas* Agar Base (PAB) (VWR International, Italy).

3.2.2 Microbial analysis and identification of the strains

Decimal progressive dilutions in sterile trypton salt solution (0.85% w/v) were prepared and the following bacteriological determinations were carried out: Total Bacterial Count (TBC) (ISO 4833/2003), *Escherichia coli* (ISO 16649-2/2001), *Staphylococcus aureus* (UNI/EN/ISO 6888/2004), Lactic Acid Bacteria (De Man et al., 1960), *Pseudomonas* spp. (ISO, 1372:2010), incubation at 35 °C for 5 days (Blazevic et al., 1973), yeasts and moulds (ISO 21527/2008).
All microbiological analyses were carried out in triplicate, and the results were expressed as the mean log cfu ml^{-1}. All colonies grown on the last dilution of PCA and CFC selective PAB were collected, purified and stored in 20% (vol/vol) glycerol at -20°C. The identification of the isolates was conduced following the methods previously reported (chapter 2, paragraphs from 2.2.3 to 2.2.6).

3.2.3 Molecular microbiology analysis of milk

3.2.3.1 DNA and RNA extractions and amplifications

For each sample, 0.2 ml of milk was used for the DNA and/or RNA extraction.
DNA was extracted using QIAamp® DNA Stool kit (Qiagen, Milano, Italy) and DNA Isolation System (Alfa Wassermann Diagnostic, West Caldwell, New Jersey), following instructions of the manufacturers, quantified with a Smart Spec™ Plus Spectrophotometer (Bio-Rad Laboratories, Milan, Italy), and diluted to obtain a solution of 1ng/µl used for amplification.
RNA was extracted using RNeasy® Plus Universal kit (Qiagen, Milano, Italy), followed by a treatment with DNase1 amplification Grade (Sigma-Aldrich, Milano, Italy) to purify RNA and remove the eventual DNA residues. After quantification with Eppendorf Biophotometer, the reverse transcription was conduced on 1µg of RNA, using iScript™ cDNA Synthesis kit (Bio-Rad Laboratories, Milan, Italy).
The obtained DNA and cDNA were subjected to Real Time-PCR amplification specific for the genus *Pseudomonas* built on the 16S rRNA region and/or specific for *aprX* gene, which codify for the most common metallo-protease of *Pseudomonas* spp. The primers used are reported in table 3.1. PCR reaction was carried out in a final volume of 15µl containing 7.5µl SSO Fast™ Eva Green® Supermix (Bio-Rad Laboratories, Milan, Italy), 0.3µM of each primer and 5ng of extracted DNA. The amplifications were performed in CFX96 Real-Time PCR System (Bio-Rad Laboratories, Milan, Italy), using the programs reported in table 3.2. A mixture of all PCR reagents without any DNA was used as a negative control; a response was considered positive when the amplification curve of the two replicates exceeded the fluorescence threshold line, which was positioned by a background-based algorithm calculated by the software.

3.2.3.2 Calibration curves and quantification

Calibration curves generated by plotting *CT* versus log10 of starting genomic quantities were used to determine the limit of the detection (LOD) and the limit of quantification (LOQ) of the assay. Different calibration curves were constructed for DNA and RNA method by using known quantities of genomic DNA and RNA of *P. fluorescens* DSM50106^{T}, extracted from a ten fold dilution series in a concentration range of about 0.96 to 6.96log cfu ml^{-1}. The number of cfu for each dilution was obtained by the standard plate count method on CFC selective PAB.
The concentrations of the extracted nucleotides were measured with Smart Spec^{TM} Plus Spectrophotometer (Bio-Rad Laboratories, Milan, Italy) and with an Eppendorf Biophotometer for the DNA and RNA respectively. High importance was given also to the ratio between the OD obtained at 260 and 280nm, which indicates the nucleic acid purity and it was always between 1.6 and 2.

3.2.3.3 ANOVA analysis

The repeatability of the methods was verified. Analysis of variance was conducted to determine whether there were statistically significant differences among mean quantities of *Pseudomonas* for each sample. Amplification efficiencies and detection sensitivities among different experiments were investigated: slopes of the calibration curves were calculated by performing a linear regression analysis. Amplification efficiency (*E*) was estimated by using the slope of the calibration curve and the formula $E = (10^{-1/slope}) - 1$. A reaction with 100% efficiency will generate a slope of -3.32.

3.2.4 Evaluation of proteolysis in milk

3.2.4.1 Soluble peptides and proteins (HPLC)

A 25ml aliquot of milk at 25°C was acidified at pH 4.6 using 2N HCl; after centrifugation (5000g, 20min at 10°C) and dilution with phosphate buffer (pH 6.7, 0.1M) the sample was filtered through a 0.45µm membrane filter (Millipore) and analysed under the conditions described by De Noni et al. (2007). The chromatographic separation was performed with an Alliance system (Waters, Milford, MA, USA) equipped with a PLRP-S column (300 A pore size, 5µm particle size, Polymer Laboratories Ltd, UK) and a 996 diode array detector (DAD) (Waters, Milford, MA, USA) operating at 280nm. Chromatogram acquisition was made using Millennium^{R} software (Waters).

3.2.4.2 Casein fractions (CZE)

A 250µl aliquot of milk was dissolved in 1ml of solubilisation buffer and kept for 4h at 25°C with agitation every 30min; after that, 250µl of the sample were dissolved in 1ml of extraction buffer and filtered through a 0.22µm membrane filter (Millipore) and analysed according to the conditions described by Recio et al. (1996). Electrophoretic separations were performed on a capillary electrophoresis system (P/ACE MDQ, Beckman Coulter, Fullerton, CA) equipped with a DAD. A bare fused-silica capillary (Agilent, Milan, Italy) of 50µm of diameter and 60cm total length (50cm to the detector window) was utilized. Prior to each run, the capillary was flushed with the running buffer for 3min with a pressure of 138kPa. Samples were introduced into the capillary column by hydrodynamic injection at 3.5kPa for 12.5s. Analyses were performed at 25°C using a separation voltage of 50kV/m and with UV detection at 214nm.

Table 3.1 PCR primers used.

Gene	Primers pair (5'-3')
16S *Pseudomonas* spp. specific (Calisti, 2008)	P94F: CGGACGGGTGAGTAATGCCTAG P649R: CAGGAAATTCCACCACCCTCTACC
aprX gene (Marchand *et al*., 2009b)	SM2F: AAATCGATAGCTTCAGCCAT SM3R: TTGAGGTTGATCTTCTGGTT

Table 3.2 PCR conditions used.

	***Pseudomonas* spp.**	***aprX* gene**
Denaturation step	95°C 3 min	94°C 3 min
Amplification step	(95°C 10s, 62.4°C 30s, 72°C 30s, with a single fluorescence measurement) x40 times	(95°C 30s, 60°C 30s, 72°C 60s, with a single fluorescence measurement) x40 times
Melting curve program	65-95°C hold 5s, fluorescence measurement every 0.5°C	65-95°C hold 5s, fluorescence measurement every 0.5°C

3.3 RESULTS AND DISCUSSION

3.3.1 Evaluation of the microbial population and identification of the isolates

As expected, different microbial concentrations were found in the milk samples, depending on the production process. No microbial growth was recognized for both micro-filtered and UHT milk samples, indicating that their manufacturing processes were suitable to remove and/or destroy all alive cells. In pasteurized milk samples instead Total Bacterial Count of about 1log cfu ml^{-1} was found. Not correctly stored raw milk was analysed and showed extremely high TBC value of 6.78log cfu ml^{-1}.
Microbial isolates, all belonging to raw and pasteurized milk, were subjected to characterization. None of the isolates had amylolytic activity, furthermore, 36% of the isolates had lipolytic activity, 60% had lecithinasic activity and around 50% had pectinolytic activity. Proteolytic activity was widespread and generally very high; for some isolates it was favourite at low temperature. All isolates produced diffusible pale pigments (white and yellow), some produced also intense red colour, and only a few were florescent.
The identification of raw milk isolates showed that *Pseudomonas* spp. were the dominant contaminants (60% of raw milk isolates) followed by Enterobacteriaceae (30%) such as *Serratia marcescens*, *Hafnia alvei*, and *Citrobacter freundii*. Quantitatively less important (10% of the isolates), Gram-positive isolates were found, belonging to the genera *Staphylococcus* and *Lactococcus*. For pasteurized milk, the same microbial groups were found, but in different concentrations: more important was the number of Lactic Acid Bacteria (50%), followed by Pseudomonadaceae (35%) and Enterobacteriaceae (15%).

3.3.2 Artificial inoculation of micro-filtered and UHT milk: visual approach

Samples artificially inoculated with *P. fluorescens* DSM 50106^T were studied and non-inoculated samples were used as control. For these preliminary analyses the coagulation was only visually evaluated. Tables 3.3 and 3.4 report the results of plate count on CFC selective PAB for micro-filtered and UHT milk respectively.
No viable growth of *Pseudomonas* spp. and no visual coagulation were detected for each control in both type of milk at both temperatures. Instead the growth of the strains was monitored in the inoculated samples and it is shown in figure 3.1. The trends of growth appeared similar in the two types of milk that showed comparable values at each time. About the storage temperature, this experiment confirmed that the growth rate of the strain is dramatically temperature-dependent. According to literature (Baglinière et al. 2013), a visual destabilisation appeared progressively over time and it was distinguished as the presence of two distinct phases: one solid and hyaline and the other liquid and whitish. In our samples the appearance was recognized at different concentration levels: it was very recognizable after two days at 25°C in both types of milk, whereas after 21 days at 4°C it was just at the beginning (Fig. 3.2).

3.3.3 Proteolysis evaluation in naturally coagulated UHT milk

Evaluation of proteolysis in naturally coagulated UHT milk was conducted as well. Figure 3.3 reports the HPLC chromatograms of four samples, two coagulated UHT milk (1 and 2 samples) and two non-coagulated UHT milk (3 and 4 samples). In the figure it is possible to recognize the peaks of β-LG, α-LA and BSA; proteose peptones, deriving from the action of plasmin on β-CN, are clearly visible as well. The main difference was the presence in the two coagulated samples of many small peptides, among which two highest picks appeared interesting because they remained the glycomacropeptides (GMPs) originated form the rennet activity on the K-

casein but showing a small delay in the elution time, and in accordance to the literature (Recio et al. 1996, Recio et al., 2000, Dupont et al., 2007) were defined Pseduo-GMPs. According Nieuwenhuijse (1995), the coagulation of UHT milk could be related to proteolytic activity of *P. fluorescens*.
The identification of the two peaks of Pseudo-GMPs was carried out with Electrospray ionization mass spectrometry (HPLC/ESI-MS) (Rovaris, 2011). The detected mass showed that the k-CN hydrolysis occurred on the 103-104 bond. The two peaks showed a difference in their mass due to their different amino acid composition: Pseudo-GMP A has a Rt of 8min, a mass of 6933Da and the AA Thr_{136} and Asp_{148}; Pseudo-GMP B has a Rt of 10min, a mass of 6901.6Da and the AA Ile_{136} and Ala_{148}.

3.3.4 Chemical analysis of artificially inoculated micro-filtered milk

A micro-filtered pasteurized milk was inoculated with a proteolytic strain of *P. fluorescens* (DSM 50106^T) and analysed after different time (from zero to 62h) of incubation at 35°C. The control was the same milk non-inoculated and incubated for 32h at 35°C.
The samples were analysed by HPLC (Fig. 3.4) and CZE (Fig. 3.5) to evaluate proteolysis extent. Plate count (Fig. 3.6) was determined as well on CFC selective PAB (VWR International, Italy).
The HPLC chromatograms of the control and the samples after 24, 32, 48 and 62h of incubation are shown in figure 3.4. The control showed a chromatogram comparable to the time zero, as if the incubation didn't affect the proteins. In the inoculated samples instead, the formation of Pseudo-GMPs started very quickly: Pseudo-GMP A is forming after 24h and Pseudo-GMP B after 32h of incubation. After 48h and even more after 62h, the proteolysis was so abundant that it was not possible do distinguish the Pseudo-GMPs among the many small peptides formed in milk.
The CZE electrophoresis (Fig. 3.5) allowed to recognize the different fractions of casein (CN). In particular, in accordance to the HPLC results, the k-casein (K-CN) gradually decreased during incubation, until a complete disappearance at 62h of incubation; as a consequence, a Pseudo-para-K-casein was forming during incubation. The decreasing of the fractions of casein seemed to be due to the activity of *P. fluorescens* because after the removal of somatic cells, due to the micro-filtration, the plasmin activity should be reduced. Figure 3.6 showed the growth of *P. fluorescens* in blue, and the degradation of K-CN in red, expressed as the peak area ratio between K-CN and α-LA. The control after 32h, indicated with dotted circles, showed the same value of K-CN (around 1.6) of the T_0 always in absence of the microorganism, confirming that the coagulation was due to the activity of strain and not to the temperature. It was assumed that the incubation at 35°C didn't induce the coagulation. Nevertheless it is important to remind that the destabilization of milk by *Pseudomonas* spp. is strain-dependent (Baglinière et al., 2012).

Table 3.3 The growth of *Pseudomonas* spp. in micro-filtered pasteurized milk stored at 4 and 25°C. Values expressed as means of the triplicate in log cfu ml^{-1} ± Standard Deviation; ND: non determined; /: visually non coagulated; *: visually coagulated.

Time (days)	**Storage temperature: 4°C**		**Coagulation**	**Storage temperature: 25°C**		**Coagulation**
	Control	Inoculated		Control	Inoculated	
0	<1	3.00±0.10	/	<1	3.00±0.10	/
1	<1	3.60±0.20	/	<1	7.79±0.10	/
2	<1	5.32±0.30	/	<1	7.90±0.20	*
3	<1	5.23±0.30	/	<1	7.95±0.10	*
7	<1	7.70±0.20	/	ND	ND	ND
10	<1	8.36±0.10	/	ND	ND	ND
13	<1	8.40±0.20	/	ND	ND	ND
21	<1	9.67±0.20	*	ND	ND	ND

Table 3.4 The growth of *Pseudomonas* spp. in UHT milk stored at 4 and 25°C. Values expressed as means of the triplicate in log cfu ml^{-1} ± Standard Deviation; ND: non determined; /: visually non coagulated; *: visually coagulated.

Time (days)	**Storage temperature: 4°C**		**Coagulation**	**Storage temperature: 25°C**		**Coagulation**
	Control	Inoculated		Control	Inoculated	
0	<1	3.00±0.10	/	<1	3.00±0.10	/
1	<1	3.54±0.10	/	<1	7.70±0.20	/
2	<1	4.48±0.20	/	<1	7.85±0.10	/
3	<1	5.85±0.20	/	<1	8.60±0.20	/
7	<1	7.95±0.30	/	<1	8.48±0.30	*
10	<1	8.70±0.20	/	ND	ND	ND
13	<1	9.00±0.10	/	ND	ND	ND
21	<1	9.85±0.20	*	ND	ND	ND

Fig. 3.1 Growth of *Pseudomonas* spp. in micro-filtered and UHT milk at 4 and 25°C

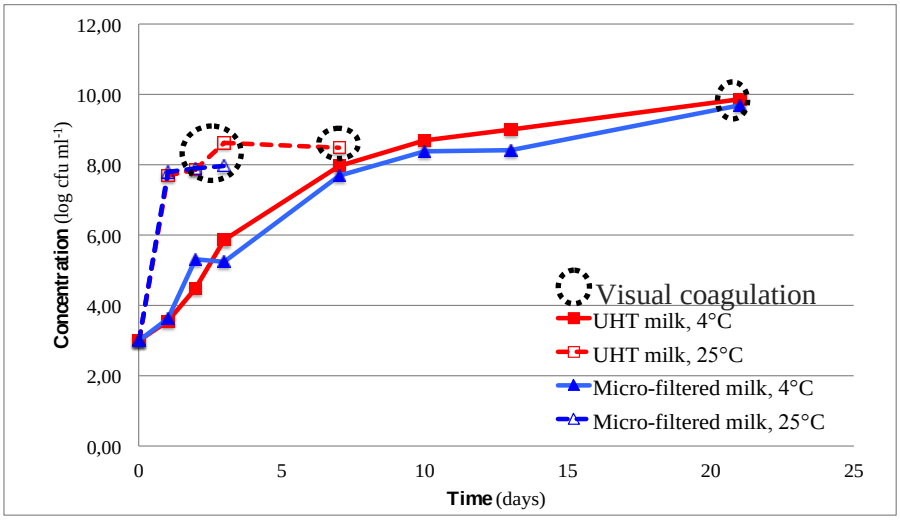
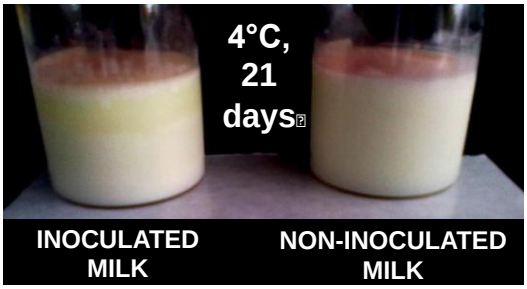


Fig. 3.2 Visual coagulation of inoculated and non-inoculated micro-filtered milk, stored at 4 and 25°C.



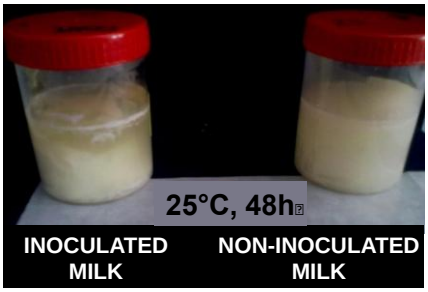


Fig. 3.3 HPLC chromatograms of different UHT milk.
Samples 1 and 2: naturally coagulated milk; samples 3 and 4: non-coagulated milk

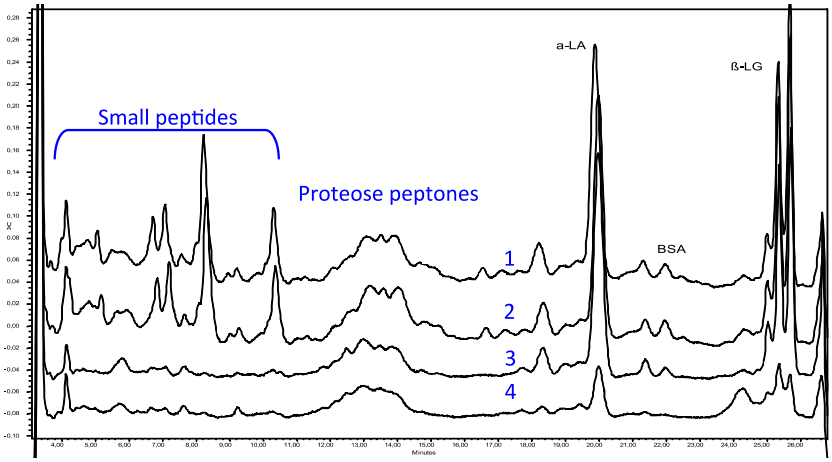


Fig. 3.4 HPLC chromatograms of micro-filtered milk at different time of incubation at 35°C after inoculation with *P. fluorescens* DSM50106^{T}.

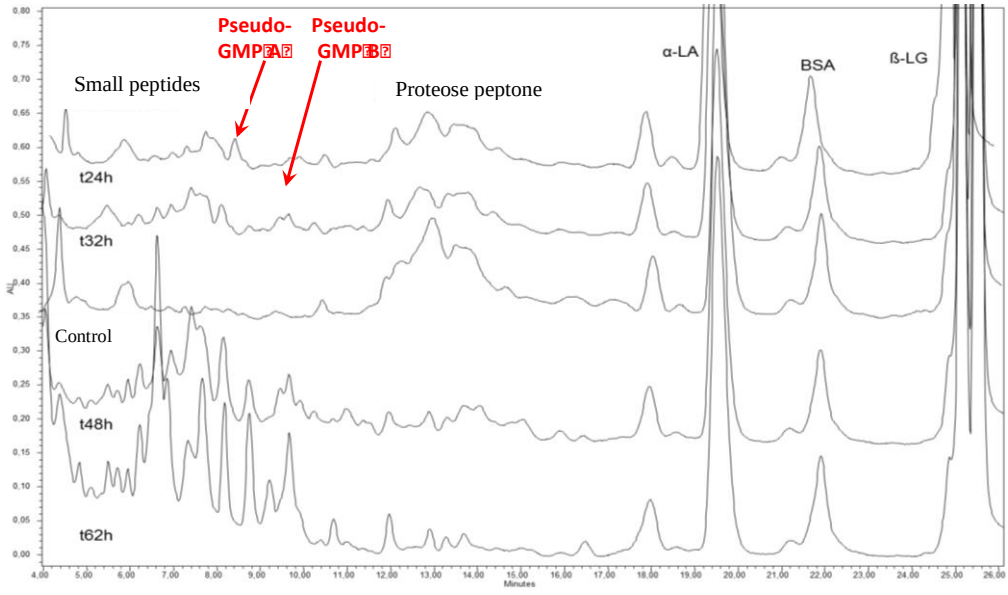


Fig. 3.5 CZE of micro-filtered milk at different time of incubation after inoculation with *P. fluorescens* DSM50106^T.

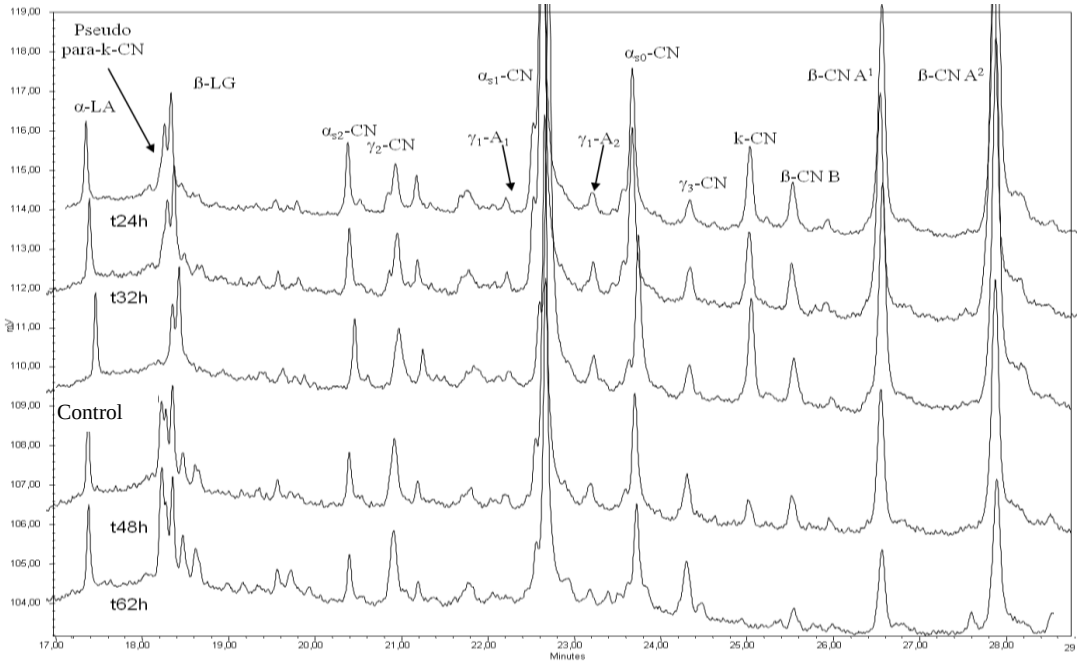
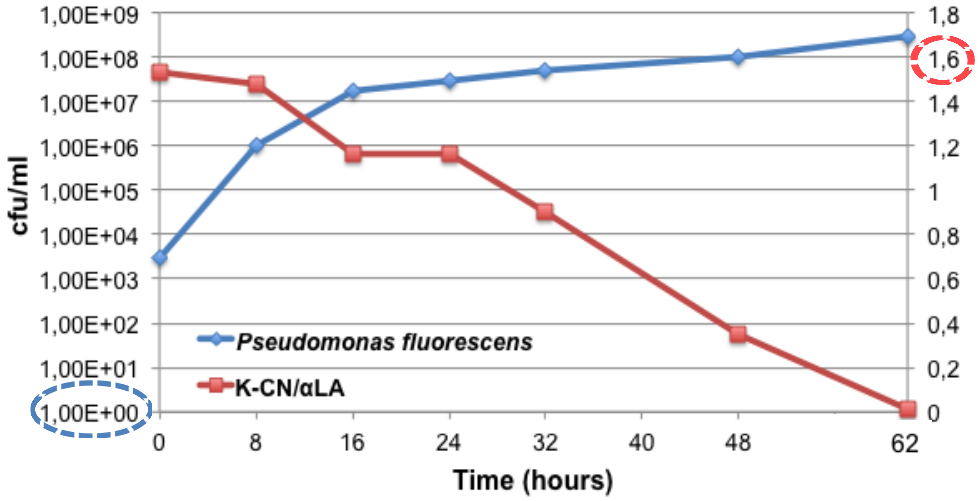


Fig. 3.6 Growth of *P. fluorescens* DSM50106^T and decrease of K-CN in inoculated micro-filtered milk during incubation at 35°C.



3.3.5 Evaluation of commercial kits for DNA extraction

To choose the DNA extraction method, two commercial kits were compared extracting the DNA of different samples of milk (0.2ml) both as such and inoculated with known concentrations of overnight colture of *P. fluorescens* DSM50106^T. For each extraction kit a calibration curve was created and the results of the amplification of the 16S specific for *Pseudomonas* genus were compared. The average values of the replicates expressed as log cfu g^{-1} are shown in table 3.5.
The ANOVA analysis was made for two different purposes: to verify the eventual differences between the two extraction kits and between each single kit and the plate count method. Both the tested kits evidenced presence of *Pseudomonas* spp. in the inoculated UHT milk, with a slightly significant ($p<0.05$) difference with respect to plate count, and not in the control UHT milk. However, only the QIAamp® DNA Stool kit was able to detect the target bacteria in the pure culture and the level was not significantly different from that of the plate count. For this reason, QIAamp® DNA Stool kit was adopted for further experiments.

3.3.6 Calibration curves and repeatability of DNA extraction

In order to quantify the cells of *Pseudomonas* spp. (16S gene based quantification) in milk samples, several calibration curves (three independent experiments) were created starting from serially diluted cells in trypton salt solution (0.85% w/v); DNAs were extracted and subjected to qPCR. The linearity range was found from 1 to 7log cfu ml^{-1}, the efficiency was 85.327% and the determination coefficient (R^2) was 0.986 (Fig. 3.7). In this case, the limit of detection (LOD) and the limit of quantification (LOQ) coincided and their value was 1log cfu.
The repeatability of the quantification of DNA of *Pseudomonas* spp. was evaluated on two samples (A and B) of milk from the same production batch. From each sample, ten independent DNA extractions and quantifications were conducted. In table 3.6, the average values and the standard deviations of the analyses are shown. The good repeatability of the method was confirmed; no statistically significant differences were found among the samples within the same analysis (intra-analysis) and even between the two different analyses (inter-analysis).
Also for the *aprX* gene based quantification, several calibration curves (three independent experiments) were created. The linearity range was found from 6.48 to 1.48log cfu ml^{-1}, the efficiency was 92.726% and the determination coefficient (R^2) was 0.965 (Fig. 3.8). LOD and LOQ coincided and their value was 1.48log cfu.
The repeatability of the DNA quantification of *aprX* gene was evaluated on pasteurized milk. The good repeatability of the method was confirmed; no statistically significant differences were found among the samples (C and D) within the same analysis (intra-analysis) and even between the two different analyses (inter-analysis) (table 3.7).

3.3.7 Calibration curve and repeatability of RNA extraction

As for DNA analysis, for *Pseudomonas* spp. quantification calibration curves (two independent experiments) were created starting from serially diluted cells in trypton salt solution (0.85% w/v). The linearity range was from 6.70 to 1.70log cfu ml^{-1}, the efficiency was 82.855% and the determination coefficient (R^2) was 0.982 (Fig. 3.9). LOD and LOQ showed the same value of 1.70cfu.
The repeatability of the RNA method was evaluated on two samples (E and F) of milk from the same production batch. From each sample, ten independent RNA extractions, reverse trascription and quantifications were conducted. This analysis confirmed a good repeatability of the method, no statistically significant differences were found (table 3.8).

Table 3.5 Comparison of QIAamp® DNA Stool and DNA Isolation System.
Values expressed as means of the triplicate in log cfu ml^{-1} ± Standard Deviation; N/A: non-detected.

Sample	**Extraction kit**	**Plate count** (log cfu ml^{-1})	**Real time-PCR quantification** (log cfu ml^{-1})	**ANOVA analysis** (kit-plate count)	**ANOVA analysis** (kit-kit)
Pure overnight colture ($DSM50106^T$)	QIAamp® DNA Stool kit	6.00±0.10	6.47±0.10	n.s.d	**
	DNA Isolation System		N/A	**	
UHT milk	QIAamp® DNA Stool kit	<1.00±0.00	N/A	n.s.d	n.s.d.
	DNA Isolation System		N/A	n.s.d	
UHT milk inoculated	QIAamp® DNA Stool kit	5.85±0.20	6.73±0.20	*	n.s.d.
	DNA Isolation System		6.58±0.20	*	
n.s.d.: no significant difference at $p<0.05$; *: significant difference at $p<0.05$; **:significant difference at $p<0.01$					

Fig. 3.7 Standard curve for *Pseudomonas* spp. DNA quantification, showing the linear relationship between the Threshold Cycle (CT) values and Log cfu for serially diluted DNA obtained from an overnight *P. fluorescens* culture.

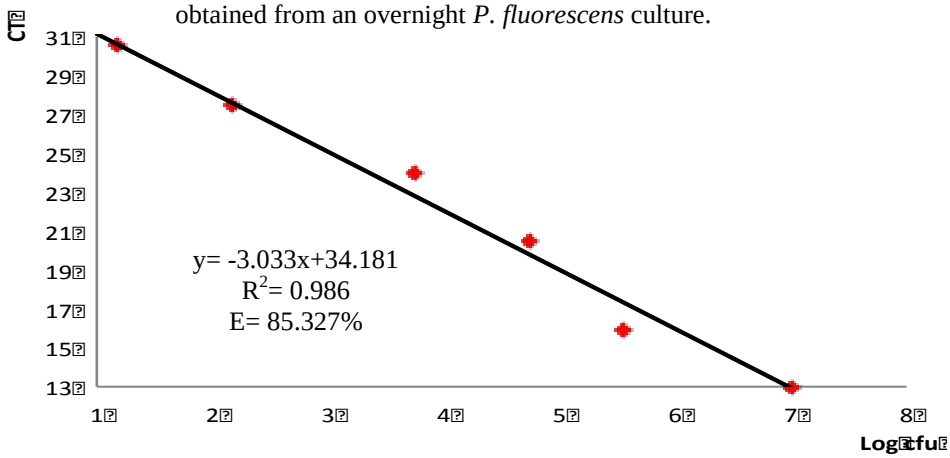


Fig. 3.8 Standard curve for *aprX* gene DNA quantification, showing the linear relationship between the Threshold Cycle (CT) values and Log cfu for serially diluted DNA obtained from an overnight *P. fluorescens* culture

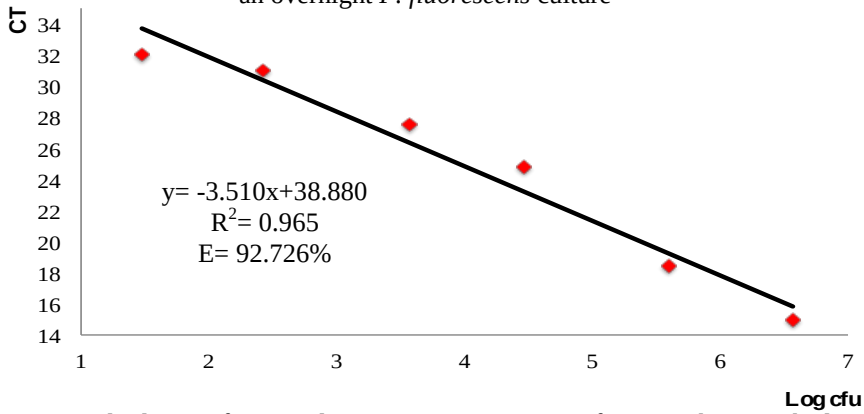


Fig. 3.9 Standard curve for *Pseudomonas* spp. RNA quantification, showing the linear relationship between the Threshold Cycle (CT) values and Log cfu for serially diluted DNA obtained from an overnight *P. fluorescens* culture

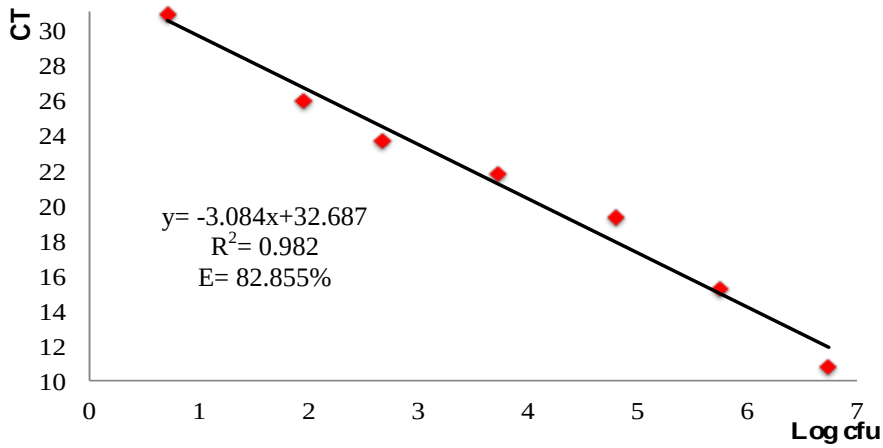


TABLE 3.6 Repeatability of the quantification of DNA of *Pseudomonas* spp. (16S gene) in UHT milk.

	Values (log cfu g^{-1})										**Mean**	**SD**	**ANOVA intra-analysis**	**ANOVA inter-analyses**
Sample A	3.38	3.40	3.93	3.09	3.38	3.96	3.35	3.77	3.70	3.31	3.53	0.29	n.s.d.	n.s.d.
Sample B	3.35	3.03	3.80	3.86	3.77	3.43	3.64	3.69	3.37	3.47	3.41	0.26	n.s.d.	
n.s.d.: no significant difference at $p<0.05$														

TABLE 3.7 Repeatability of the quantification of *aprX* gene (DNA analysis) in pasteurized milk.

	Values (log cfu g^{-1})										**Mean**	**SD**	**ANOVA intra-analysis**	**ANOVA inter-analyses**
Sample C	4.44	4.66	4.33	4.25	4.28	4.86	4.58	4.79	4.51	4.60	4.53	0.21	n.s.d.	n.s.d.
Sample D	4.38	4.51	4.65	4.37	4.97	4.52	4.39	4.47	4.73	4.49	4.55	0.19	n.s.d.	
n.s.d.: no significant difference at $p<0.05$														

TABLE 3.8 Repeatability of the quantification of RNA of *Pseudomonas* spp. (16S gene).

	Values (log cfu g^{-1})										**Mean**	**SD**	**ANOVA intra-analysis**	**ANOVA inter-analyses**
Sample E	3.41	3.42	3.40	3.53	3.38	3.27	3.55	3.48	3.37	3.35	3.42	0.09	n.s.d.	n.s.d.
Sample F	3.58	3.23	3.38	3.49	3.37	3.46	3.67	3.29	3.35	3.66	3.45	0.15	n.s.d.	
n.s.d.: no significant difference at $p<0.05$														

3.3.8 Molecular analysis of raw, pasteurized and UHT milk

Different raw, pasteurized and UHT milk were analysed from the molecular point of view (tab. 3.9). Results for two raw milk samples analysed at time zero are reported; *Pseudomonas* spp. appeared to be present with very high values, alive cells were detected by traditional plate count on selective medium and by RNA quantification with values higher than 6.60log cfu ml^{-1}. No significant differences were found for the results obtained by DNA and RNA *Pseudomonas* spp. quantification, indicating that almost all of the cells were alive. The quantification of *aprX* gene suggested that many cells (more than 4.20log cfu ml^{-1}) potentially could produce proteases. For pasteurized milk (two samples at time zero), no viable cells were detected by plate count, while around 3.40log cfu ml^{-1} were recognized as Viable But Non-Culturable (VBNC) cells (RNA quantification); the quantified DNA value represents the DNA of both VBNC and dead cells. For pasteurized samples, the most part of detected *Pseudomonas* spp. showed the presence of *aprX* gene in their genome.
All the UHT milk reported in table 3.9 were analysed between three and twelve months after their expiration dates. The two naturally coagulated and semi-skimmed milk samples showed levels of 3.17 and 4.41log cfu ml^{-1} of *Pseudomonas* spp. and levels of 4.32 and 3.47log cfu ml^{-1} of *aprX* gene DNA respectively. The three normal samples, independently from the quantity of fat present inside the milk, showed lower values for both the indices. For all the UHT samples, the presence of DNA values in absence of plate count growth suggested the presence of dead cells. For all those products, the *aprX* gene quantification was between 3.45 and 4.66log cfu ml^{-1} and the correlation between this value and the coagulation of milk wasn't observed: high value didn't correspond to the effective coagulation of milk.

3.3.9 Combination of molecular and chemical approaches in UHT milk analysis

In table 3.10 and figure 3.10 the molecular results and the HPLC and CZE patterns of two semi-skimmed UHT milk samples are shown respectively; one sample was normal milk (in light blue) and the other one was coagulated (in blue). These preliminary results suggested a correlation between the molecular detection of *aprX* gene and the chemical degradation of casein. In fact the coagulated sample showed high values of *Pseudomonas* spp. and *aprX* DNA, high levels of Pseudo-GMPs and Pseudo-para-K-CN and the absence of intact K-CN; on the contrary the non-coagulated sample showed low or undetectable values of *Pseudomonas* spp. and *aprX* DNA, normal level of K-CN in absence of Pseudo-GMPs and Pseudo-para-K-CN.
A full-cream and a semi-skimmed UHT milk samples, both naturally coagulated, were analysed after 90 and 130 days from the production (expiration date at 120 days from the production) (table 3.11 and figures 3.11 and 3.12). In absence of viable *Pseudomonas* spp. (selective plate count value), the full-cream milk showed almost constant value of *Pseudomonas* spp. DNA (3.3-3.6log cfu ml^{-1}), no detected value for *aprX* gene and slightly decreasing values of proteose peptones, Pseudo-GMPs and small peptides. Those trends suggest that the total DNA detected value derived only from dead cells, and that the initial proteolytic activity recognized in the sample, only partially characteristic of *Pseudomonas*, has been inactivated by the sterilization process. For the semi-skimmed milk, the absence of alive *Pseudomonas* spp. was confirmed. High values for *Pseudomonas* spp. and *aprX* gene were detected in the sample at 90 days from production, both decreasing during the subsequent storage time, probably due to a partial degradation of DNA. Besides, in the semi-skimmed sample, both the Pseudo-GMPs and proteose peptone fractions slightly decreased whereas the small peptides increased by 40% approximately. The absence of K-CN, in favour of high value of Pseudo-para-K-CN (fig.3.12) detected after 90 days from the production, explained these trends: the increasing of small peptides could be due to non-specific protease activities occurred on other casein fractions during the considered storage.

TABLE 3.9 Raw, pasteurized and UHT milk samples analysis. Values expressed as means of the triplicate in log cfu ml^{-1} ± Standard Deviation; ND: non-determined; N/A: non-detected.

Sample	**Selective plate count**	***Pseudomonas* spp. quantification**		***aprX* gene quantification**
		DNA	**RNA**	**DNA**
Raw milk	6.81±0.10	6.62±0.10	6.97±0.20	4.48±0.10
Raw milk	6.60±0.05	6.14±0.20	6.77±0.15	4.27±0.20
Pasteurized milk	<1	4.22±0.20	3.40±0.10	4.45±0.30
Pasteurized milk	<1	4.64±0.10	3.42±0.25	4.66±0.20
Naturally coagulated, semi-skimmed UHT milk	<1	3.17±0.10	ND	4.32±0.10
Naturally coagulated, semi-skimmed UHT milk	<1	4.41±0.20	ND	3.47±0.30
Normal, semi-skimmed UHT milk	<1	3.34±0.10	ND	4.01±0.20
Normal, semi-skimmed UHT milk	<1	2.96±0.15	ND	N/A
Normal, full-cream UHT milk	<1	2.96±0.20	ND	3.45±0.20

TABLE 3.10 Semi-skimmed UHT milk samples analysis. Values expressed as means of the triplicate in log cfu ml^{-1} ± Standard Deviation; N/A: non-detected.

Sample	**Selective plate count**	***Pseudomonas* spp. DNA quantification**	***aprX* gene DNA quantification**
Naturally coagulated milk	<1	5.39±0.20	5.53±0.10
Non-coagulated milk	<1	3.57±0.30	N/A

TABLE 3.11 Naturally coagulated UHT milk samples analysis. Values expressed as means of the triplicate in log cfu ml^{-1} ± Standard Deviation; N/A: non-detected; Before: analysed before the expiration date (90 days after production); After: analysed after the expiration date (130 days after production).

Sample	**Selective plate count**		***Pseudomonas* spp. DNA quantification**		***aprX* gene DNA quantification**	
	Before	After	Before	After	Before	After
Semi-skimmed milk	<1	<1	5.39±0.20	4.13±0.10	5.53±0.10	N/A
Full-cream milk	<1	<1	3.36±0.20	3.61±0.10	N/A	N/A

Fig. 3.10 HPLC chromatograms and CZE electropherograms of two samples of semi-skimmed UHT milk: naturally coagulated sample (in blue), and non-coagulated sample (in light blue).

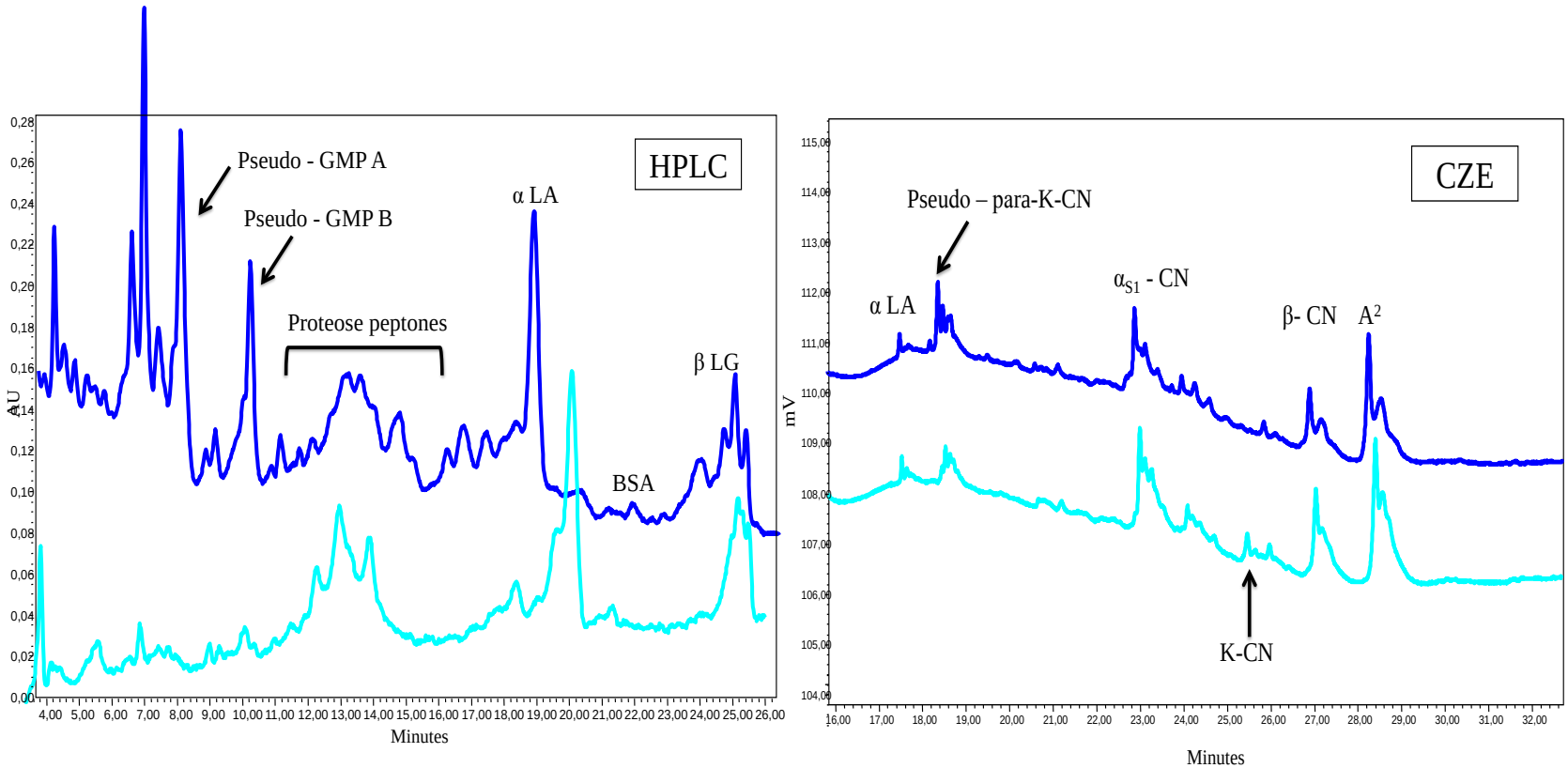


Fig. 3.11 Quantification of small peptides, proteose peptones and Pseudo-GMPs before and after expiration date in two naturally coagulated milk: semi-skimmed UHT milk (in blue) and in full-cream UHT milk (in red).

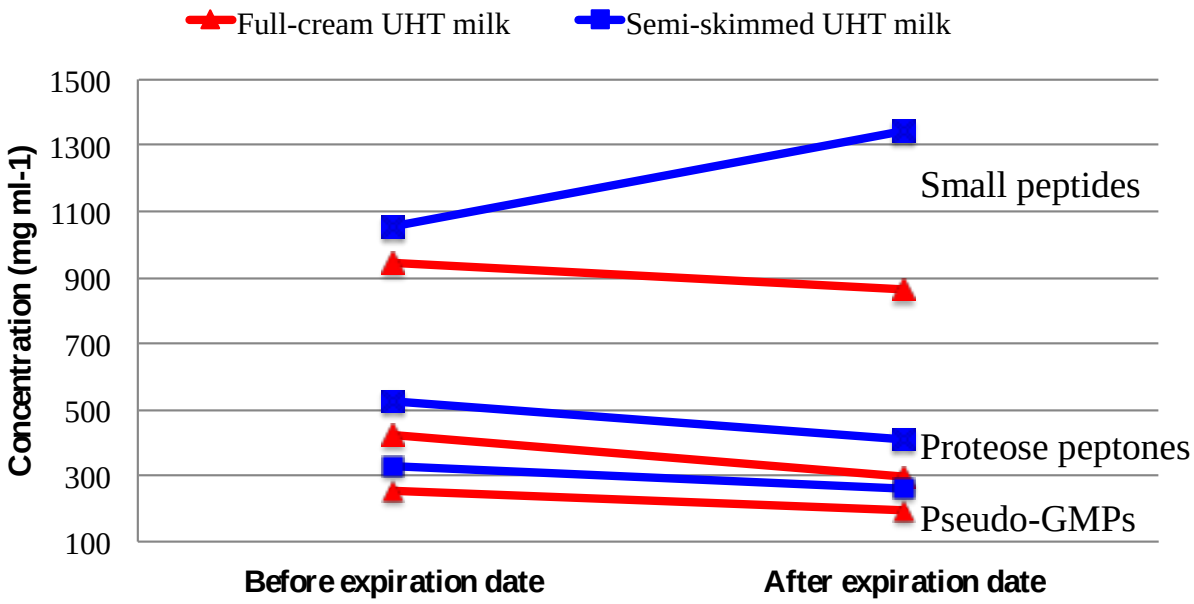
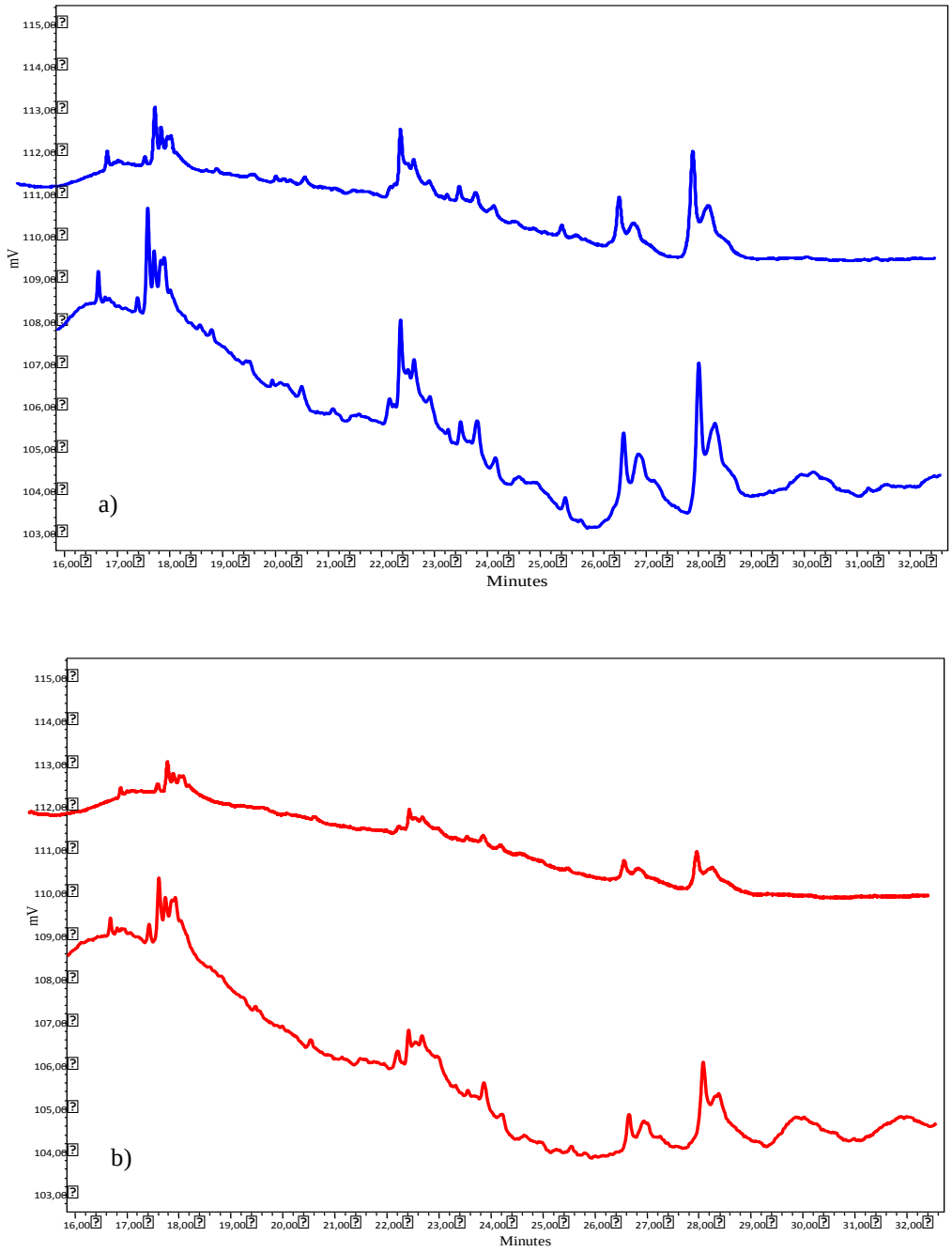


Fig. 3.12 CZE electropherograms before and after expiration date in a) naturally coagulated semi-skimmed UHT milk b) naturally coagulated full-cream UHT milk.



3.4 CONCLUSIONS

In conclusion the psycrhotrofic bacteria appeared the most important contaminants in raw and pasteurized milk. In particular *Pseudomonas* spp., especially the specie *fluorescens*, showed high spoilage potential, thanks to their heat-stable enzymes. Our results suggested that the proteolytic activity of *Pseudomonas* spp. is very important in destabilization and coagulation of milk, being responsible for the cutting of K-CN, producing Pseudo-GMPs and Pseduo-para-K-CN. The amino acid composition of the two fractions of Pseudo-GMPs was studied and a difference between the Pseudo-GMPs and the GMPs obtained with the rennet activity was found: the hydrolysis due to the proteases of *Pseudomonas* spp. in fact occurred on the 103-104 bond of k-casein, instead the 105-106 bond cut by rennet.
The only *aprX* gene detection appeared a not enough specific parameter for the prediction of milk coagulation, in fact the detection of this gene didn't always correspond to the effective coagulation of the sample, indeed according to literature (Martins et al., 2005; Dufour et al., 2008), the presence of the gene doesn't necessary imply its expression. Besides our quantification referred to the gene of dead cells and it could be active only if the production of the proteases occurred before the destruction of the cells by UHT treatment. Nevertheless the quantifications of *aprX* gene and *Pseudomonas* spp. DNA combined with the chemical approach can give an indication about the possible destabilization and coagulation of milk; HPLC and CZE appeared suitable for the detection of the coagulation and the modification of the fractions of casein in milk.

3.5 REFERENCES

Ahn JH, Pan JG, Rhee JS. 1999. Identification of the *tliDEF* ABC transporter specific for lipase in *Pseudomonas fluorescens* SIK W1. Journal of Bacteriology, 181: 1847–1852.

Baglinière F, Tanguy G, Jardin J, Matéos A, Briard V, Rousseau F, Robert B, Beaucher E, Humbert G, Dary A, Gaillard JL, Amiel C, Gaucheron F. 2012. Quantitative and qualitative variability of the caseinolytic potential of different strains of *Pseudomonas fluorescens*: Implications for the stability of casein micelles of UHT milks during their storage. Food Chemistry 135: 2593–2603.

Baglinière F, Matéos A, Tanguy G, Jardin J, Briard-Bion V, Rousseau F, Robert B, Beaucher E, Gaillard JL, Amiel C, Humbert G, Dary A, Gaucheron F. 2013. Proteolysis of ultra high temperature-treated casein micelles by AprX enzyme from *Pseudomonas fluorescens* F induces their destabilisation. International Dairy Journal 31: 55-61.

Blazevic DJ, Koepcke MH, Matsen JM. 1973, Incidence and identification of *Pseudomonas fluorescens* and *Pseudomonas putida* in the clinical laboratory. Appl. Microbial. 25: 107-110.

Calisti C. (2008). Sviluppo di metodiche molecolari per il rilevamento rapido di *Pseudomonas* in matrici alimentari e ambientali. PhD thesis in Foods Biotechnology, Università degli studi della Tuscia, Viterbo.

Chavan RS, Chavan SR, Khedkar CD, Jana AH. 2011. UHT milk processing and effect of plasmin activity on shelf life: a review. Comprehensive Reviews in Food Science and Food Safety, 10: 251-268.

Coker CJ, Crawford RA, Johnston KA, Singh H, Creamer LK. 2005. Towards the classification of cheese variety and maturity on the basis of statistical analysis of proteolysis data - a review. International Dairy Journal, 15: 631-643.

Cousin MA. 1982. Presence and activity of psychrotrophic microorganisms in milk and dairy products: a review. Journal of Food Protection, 45: 172–207.

Datta N, Deeth HC. 2001. Age gelation of UHT milk – a review. IChem, 79: 197-210.

Datta N, Deeth HC. 2003. Diagnosing the cause of proteolysis in UHT milk. Lebensm Wiss Technol, 36: 173-182.

Deeth HC, Datta N. 2000. Age gelation of UHT milk. Quality Quarterly, 9 (4): 6-8.

De Noni I, Pellegrino L, Cattaneo S, Resmini P. 2007. HPLC of proteose peptones for evaluating ageing of packaged pasteurized milk. International Dairy Journal, 17: 12-19.

Dogan B, Boor KJ. 2003. Genetic diversity and spoilage potentials among *Pseudomonas* spp. isolated from fluid milk products and dairy processing plants. Applied and Environmental Microbiology, 69: 130-138.

Dufour D, Nicodème M, Periin C, Driou A, Brusseaux E, Humbert G, Gaillard JL, Dary A. 2008. Molecular typing of industrial strains of *Pseudomonas* spp. isolated from raw milk and genetical and biochemical characterization of an extracellular protease by one of them. International Journal of Food Microbiology, 125: 188-196.

Dupont D, Lugand D, Rolet-Repecaud O, Degelaen J. 2007. ELISA to detect proteolysis of ultrahigh-temperature milk upon storage. J. Agric. Food Chem., 55: 6857-6882.

Garcìa-Risco MR, Ramos M, Lòpez-Fandino R. 1999. Proteolysis, protein distribution and stability of UHT milk during storage at room temperature. Journal of the Science of Food and Agriculture 79:1171-1178

Griffiths MW. 1989. Effect of temperature and milk fat on extracellular enzyme synthesis by

psychrotrofic bacteria during growth in milk. Milchwissenschaft, 44: 537-543.
Hantsis-Zacharov E, Harpern M. 2007. Culturable psychrotrophic bacterial communities in raw milk and their proteolytic and lipolytic traits. Appl Environ Microbiol, 73: 7162-7168.
Johnson LA, Beacham IR, MacRae IC, Free ML. 1992. Degradation of triglycerides by a pseudomonad isolated from milk: molecular analysis of a lipase-encoding gene and its expression in *Escherichia coli*. Applied and Environmental Microbiology, 58: 1776–1779.
Juhas M, Eberl L, Tummer B. 2005. Quorum sensing: the power of cooperation in the world of *Pseudomonas*. Envirnmental Microbiology, 7 (4): 459-471.
Kawai E, Idei A, Kumura H, Shimazaki K, Akatsuka H, Omori K. 1999. The ABC- exporter genes involved in the lipase secretion are clustered with the genes for lipase, alkaline protease, and serine protease homologues in *Pseudomonas fluorescens* no. 33. Biochimica et Biophysica Acta, 1446: 377–382.
Lafarge V, Ogier JC, Girard V, Maladen V, Leveau JY, Gruss A, Delacroix-Buchet A. 2004. Raw cow milk bacterial population shifts attributable to refrigeration. Appl Environ Microbiol, 70: 5644-5650.
Liao CH, McCallus DE. 1998. Biochemical and genetic characterization of an extracellular protease from *Pseudomonas fluorescens* CY091. Applied and Environmental Microbiology, 64: 914–921.
Lopez Fandino R, Olano A, San Jose C, Ramos M. 1993. Application of reverse-phase HPLC to the study of proteolysis in UHT milk. Journal of Dairy Research, 60 (1): 111-116.
Liu M, Wang H, Griffiths MW. 2007. Regulation of alkaline metalloprotease promotor by N-acyl homoserine lactone quorum sensing in *Pseudomonas fluorescens*. Journal of Applied Microbiology, 103: 2174-2184.
Marchand S, Heylen K, Messens W, Coudijzer K, De Vos P, Dewettinck K, Herman L, , De Block J, Heyndrickx M. 2009a. Seasonal influence on heat-resistant proteolytic capacity of Pseduomonas lundensis and *Pseudomonas* fragi, predominant milk spoilers isolated from Belgian raw samples. Environmental Microbiology, 11(2): 467-482.
Marchand S, Vandriesche G, Coorevits A, Coudijzer K, De Jonghe V, Dewettinck K, De Vos P, Devreese B, Heyndrickx M, De Block J. 2009b. Heterogeneity of heat-resistant proteases from milk *Pseudomonas* species. Int. J. of Food Microb. 133: 68-77.
Martins ML, de Araùjo EF, Mantovani HC, Moraes CA, Vanetti MCD. 2005. Detection of *apr* gene in psychrotrophic bacteria isolated from refrigerated raw milk. Internationa Journal of Food Microbiology, 102: 203-211.
McMahon DJ 1996. Age-gelation of UHT milk: changes that occur during storage, their effect on shelf life and the mechanism by which age-gelation occurs. In heat treatments & alternative methods. International Dairy Federation, 315-325.
Mottar J, van Renterghem RV, de Vilder J. 1985. Evaluation of the raw material for UHT milk by determing the degree of protein breakdown through HPLC. Milchwissenschaft, 40(2): 717-721.
Nicodème JP, Grill G, Gaillard JL. 2005. Extracellular protease activity of different *Pseudomonas* strains: dependence of proteolytic activity on culture conditions. Journal of Applied Microbiology, 99: 641-648.
Nieuwenhuijse JA 1995. Changes in heat-treatment milk products during storage. In Heat-induced changes in milk. Special issue 9501. International Dairy Federation, 236-251.
Picard C, Plard I, Collin JC. 1996. Application of the inhibition ELISA method to study of proteolysis caused by heat-resistant *Pseudomonas* proteases specific towards *k-casein*. Michwissenschaft, 51 (8): 438-442.
Recio I, Lopez-Fandino R, Olano A, Oleiman C, Ramos M. 1996. Study of the formation of caseinomacropeptides in stored ultra-high-temperature-treated milk by capillary electrophoresis. J. Agric. Food Chem., 44: 3845-3848.

Recio I, Garcia-Risco MR, Ramos M Lopez-Fandino R. 2000. Characterization of peptides produced by the action of psychrotrophic proteinases on k-casein. J Dairy Res., 67: 625-630.

Rovaris M. 2011. Valutazione dell'attività di protease termoresistenti di Pseduomonas fluorescens nel lette UHT. Thesis in Foods Science and Technology, Università degli studi di Milano, Milano.

Sorhaug T, Stepaniak L, 1997. Psychrotrophs and their enzymes in milk and dairy products: quality aspects. Trends in Food Science and Technology 8: 35–40.

van den Broeck D, Bloemberg VG, Lugtenberg B. 2005. The role of phenotypic variation in rhizosphere *Pseudomonas* bacteria. Environmental Microbiology, 7 (11): 1686-1997.

Woods RG, Burger M, Beven CA, Beacham IR. 2001. The *aprX-lipA* operon of *Pseudomonas fluorescens* B52: a molecular analysis of metalloprotease and lipase production. Microbiology, 147:345-354.

4. Distinguishing among dead, alive and VBNC cells of *Pseudomonas* spp. in mozzarella cheeses

4.1 INTRODUCTION

Pseudomonas is the most common genus responsible for the spoilage of different kind of food. All the species of this genus, in fact, have simple nutritional requests and are characterized by a strongly developed adaptability, producing many enzymes (e.g. proteases, lipases, pectinases) that can allow them growing on different matrices.

Although the cells are easily destroyed with high temperatures, many of their enzymes are heat-stables and can spoil food even after the death of cells. It has been showed that *Pseudomonas* spp.'s proteases degrade casein, inducing bitterness in milk, gelation of UHT-milk and decreasing yields of soft cheese; lipases hydrolysing the triglycerides produce alterations of flavour and lecithinases are able to disrupt milk fat globule membranes, increasing the susceptibility of milk fat to the action of lipases (Datta & Deeth, 2001;Herrera, 2001; Deeth *et al.*, 2002; Gunasekera *et al.*, 2003; Ray, 2004).

Mozzarella is a typical Italian cheese, whose producing process is characterized by the stretching of the curd, a treatment necessary to render the curd elastic. In particular the processing requires the addition of lactic acid bacteria (*Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus* or *Lactobacillus helveticus*) to the pasteurized milk, coagulating at 34-37°C, breaking and acidification of the curd (pH 5.1 – 5.2), stretching of the curd with hot water (90°C), formation, cooling and packaging in a governing liquid, mainly composed of tap water, brine and whey.

The contamination of mozzarella mainly represented by *Pseudomonas* spp. and other psycrotrophic bacteria (Baruzzi *et al.*, 2012) can occur at two steps, generating different problems. The first possibility is a contamination of the product before the heat-treatment; in this case the eventual heat-stable enzymes will survive and can induce instability into the final product, for example influencing the hardness, meltability and stretchability of Mozzarella (Oommen *et al.*, 2002). However the most common contamination occurs after the heat process: dipping into the governing liquid can increase microbial loads and turbidity over the storage time and can cause alterations even under refrigeration conditions (Baruzzi *et al.*, 2012).

The interest in the role of *Pseudomonas*, especially the *fluorescens* specie, in the spoiling of mozzarella increased in summer 2010 when the "blue mozzarella" event occurred in Italy and then spread in many countries of Europe (Nogarol et al., 2013; Andreani et al., 2014). Official laboratory analysis and health authorities in fact linked the anomalous blue coloration of the cheese to the presence of strains of *P. fluorescens* group.

For the quantification of nucleic acids Real Time-PCR has been widely used (Bustin 2002, Vanysacker et al., 2013; Wilson et al., 2013). This technique has many advantages: sensitiveness, speed, precision, high throughput and high degree of potential automation. Recently thanks to its significant advantages for the enumeration of bacteria directly from food samples, it found successful applications in food microbiology (Malorny *et al.*, 2004; Oliveira *et al.*, 2005; Alarcon *et al.*, 2006; Wang *et al.*, 2007; Rantsiou *et al.*, 2008; Pennacchia *et al.*, 2009).

Traditionally the cell viability was verified by growing on solid media, but nowadays is clear that the absence of colonies does not necessarily mean that the cells are dead. Instead it is possible that some Viable But Non-Culturable (VBNC) cells are present; those are damaged or dormant cells, not able to grow on culture media (Díaz *et al.*, 2010). The causes can be multiple stresses (chemical or heat shock, osmotic stress and dehydration); in this state the cells maintain their metabolic activities and remain alive, with the possibility of an influence on many processes.

The aim of this work is the set up of a rapid method for the total detection and the quantification of *Pseudomonas* genus in mozzarella cheese, discriminating among the dead, the alive and the VBNC cells.

4.2 MATERIALS AND METHODS

4.2.1 Samples

Mozzarella cheeses coming from GDO were analysed. The samples were collected during two years, from October 2011 to September 2013, analysed after different times of storage at 4°C. Some samples were subjected to a previous inoculation with overnight culture of *P. fluorescens* DSM50106^{T} before the analysis; the total count of the inoculum was determined by microscopy quantification, and the concentration of the alive cells of the inoculum was quantified by culture dilutions plating on CFC selective *Pseudomonas* Agar Base.

4.2.2 Microbiological analysis

The entire content of the package (mozzarella and governing liquid) was homogenized into a sterile Stomacher bag, by using a Colworth 400 Stomacher for 2 minutes. Decimal progressive dilutions in sterile trypton salt solution (0.85% w/v) were prepared and the concentration of *Pseudomonas* spp. was determined by spread technique on plates of CFC selective *Pseudomonas* Agar Base (VWR International, Italy). To allow the adaptation and growth of the eventual stressed *Pseudomonas* cells revivification step was set up: for each sample, 0.2g of mozzarella were incubated at 35°C for 4 hours, and the concentration of *Pseudomonas* spp. was compared to that in the samples without the incubation period. All the analyses were conducted in triplicate.

4.2.3 DNA and RNA extractions

For each product, 0.2 - 1g of mozzarella were used for the DNA and/or RNA extraction. For DNA extraction different commercial kits were tested following the manufacturer's instructions: QIAamp® DNA Stool kit (Qiagen, Milano, Italy), DNeasy® Mericon™ Food (Qiagen, Milano, Italy) and InstaGene™ Matrix (Bio-Rad Laboratories, Milan, Italy). For all the DNAs, after quantification with a Smart Spec™ Plus Spectrophotometer (Bio-Rad Laboratories, Milan, Italy), a solution of 1ng/µl was prepared and used for the amplification.
Two volumes of RNA Protector Bacteria Reagent (Qiagen, Milano, Italy) were used to prepare the samples for RNA extraction. RNeasy® Plus Universal kit (Qiagen, Milano, Italy) was used to extract RNA, followed by a treatment with DNase1 amplification Grade (Sigma-Aldrich, Milano, Italy) to purify RNA and remove the residual DNA. After that, the RNAs were quantified with Eppendorf Biophotometer and 1µg was utilized for reverse transcription using iScript™ cDNA Synthesis kit (Bio-Rad Laboratories, Milan, Italy) with random primers. The resultant cDNA was subjected to qRT-PCR; a control PCR of the same samples without reverse transcriptase was included to assure the absence of DNA contamination.
For PMA pre-treatment, 20µl of PMA 20mM in H_2O (Biotium Inc., Hayward, USA), diluted at 2.5mM in 20% DMSO, were added to 980µl of stomached mozzarella. Following an incubation period of 5min in the dark, samples were light-exposed for 15min using a 400-W halogen light source placed 20 cm from the sample tubes. During exposure, samples were placed on ice to avoid excessive heating. After photo-induced cross-linking, cells were pelleted at 6000g for 5min prior to DNA and RNA isolation.

4.2.4 Amplifications

All the obtained DNA and cDNA were subjected to Real Time-PCR amplification specific for the genus *Pseudomonas*. The primers used (P94F 5'-CGGACGGGTGAGTAATGCCTAG-3'; P649R 5'-CAGGAAATTCCACCACCCTCTACC-3') were built on the 16S rRNA region of *Pseudomonas* spp. (Calisti 2008) and the specificity was tested with melting curves during amplification and by 1% agarose gels.
PCR reaction was carried out in a final volume of 15µl containing 7.5µl SSO Fast™ Eva

Green® Supermix (Bio-Rad Laboratories, Milan, Italy), 0.3µM of each primer and 5ng of extracted DNA. The amplifications were performed in CFX96 Real-Time PCR System (Bio-Rad Laboratories, Milan, Italy), using the following program: denaturation step (95°C for 3 min), amplification and quantification steps repeated for 40 cycles (95°C for 10s, 62.4°Cfor 30s, 72°C for 30s with a single fluorescence measurement). A mixture of all PCR reagents without any DNA was used as a negative control; a response was considered positive if the amplification curve of the two replicates exceeded the fluorescence threshold line, which was positioned by a background-based algorithm calculated by the software.

4.2.5 Calibration curves and results quantifications

Calibration curves generated from plotting *CT* versus log10 of starting genomic quantities were used to determine the limit of the detection (LOD) and the limit of quantification (LOQ) of the assay. Different calibration curves were constructed for DNA and RNA method by using known quantities of genomic DNA and RNA of *P. fluorescens* DSM50106^{T}, extracted from a ten fold dilution series in a concentration range of 1to 7log cfu ml^{-1}. The number of cfu for each dilution was obtained by the standard plate count method on CFC selective *Pseudomonas* Agar Base.
The concentrations of the extracted nucleotides were measured for the DNA with Smart Spec™ Plus Spectrophotometer (Bio-Rad Laboratories, Milan, Italy) and for the RNA with an Eppendorf Biophotometer. High importance was given also to the ratio between the OD obtained at 260 and 280 nm, which indicates the nucleic acid purity and was always between 1.6 and 2.

4.2.6 ANOVA analysis

The repeatability of the methods was verified. Analysis of variance was conducted to determine whether there were statistically significant differences among mean quantities of *Pseudomonas* for each sample. Amplification efficiencies and detection sensitivities among different experiments were investigated: slopes of the calibration curves were calculated by performing a linear regression analysis. Amplification efficiency (*E*) was estimated by using the slope of the calibration curve and the formula $E = (10^{-1/\text{slope}}) - 1$. A reaction with 100% efficiency will generate a slope of -3.32.

4.3 RESULTS

4.3.1 Confronting DNA extraction kits

During period of the analysis we verified the presence of *Pseudomonas* genus in mozzarella by plating on selective medium and by amplification with Real-Time PCR. None of the samples showed a detectable concentration on the selective medium, but they all were positive to the amplification. Even after the expiration date, the concentration on plate appeared less than the minimum relievable (<1log cfu g^{-1}); nevertheless after the expiration date some samples appeared spoiled, in terms of visual degradation of mozzarella.
To choose the DNA extraction method, three commercial kits were compared extracting the DNA of different samples of mozzarella (0.2g) as such and inoculated with know concentrations of overnight colture of *P. fluorescens* DSM50106^{T}. For each extraction kit a calibration curve was created and the results were compared. The averages of the replicates expressed as log cfu g^{-1} are shown in tables 4.1 and 4.2.
The ANOVA analysis was made for two different evaluations: verify the eventual differences between the two extraction kits and the eventual differences between each single kit and the plate count method.
The ANOVA between each kit and the plate count method showed no statistically significant differences ($p<0.05$) in the inoculated samples; instead in absence of inoculation (samples as such) it showed statistically significant differences until $p<0.01$.
Seeing the absence of statistically significant differences between the two kits ($p<0.05$), QIAamp® DNA Stool kit was preferred due to the higher extraction yield.
In table 4.2, the averages of the replicates made for the comparison of Stool kit and Instagene Matrix kit are reported. The InstaGene^{TM} Matrix is a purifying kit based on the DNA retention on a resin. This analysis suggested that the resin interfered with the amplification; in fact to obtain an amplification signal a previous dilution of the samples was necessary, utilizing a solution of 0.01ng/µl. In addition, the resulting values appeared about three log cycles higher than the DNA amount actually present, showing high statistically significant differences with the DNA really present. For this reason, QIAamp® DNA Stool kit was adopted for further experiments.

TABLE 4.1 Comparison of DNeasy® mericonTM Food and QIAamp® DNA Stool DNA extraction kits.

Sample	**Extraction kit**	**Plate count** (log cfu g^{-1})	**Real-Time PCR quantification** (log cfu g^{-1})	**ANOVA analysis** (kit-plate count)	**ANOVA analysis** (kit-kit)
Inoculated mozzarella	DNeasy® mericon ™ Food	7.9±0.0	7.73±0.2	n.s.d.	n.s.d.
Inoculated mozzarella	QIAamp® DNA Stool		7.95±0.1	n.s.d.	
Mozzarella as such	DNeasy® mericon ™ Food	<1	6.50±0.1	**	n.s.d.
Mozzarella as such	QIAamp® DNA Stool		5.47±0.1	**	
n.s.d.: no significant difference at p<0.05; *: significant difference at p<0.05; **:significant difference at p<0.01					

TABLE 4.2 Comparison of QIAamp® DNA Stool and InstaGene™ Matrix DNA extraction kits.

Sample	**Extraction kit**	**Plate count** (log cfu g^{-1})	**Real-Time PCR quantification** (log cfu g^{-1})	**ANOVA analysis** (kit-plate count)	**ANOVA analysis** (kit-kit)
Mozzarella as such	QIAamp® DNA Stool	<1	3.71±0.1	*	*
Mozzarella as such	InstaGene™ Matrix		6.16±0.2	**	
Inoculated mozzarella	QIAamp® DNA Stool	4.04±0.1	5.00±0.0	n.s.d.	*
Inoculated mozzarella	InstaGene™ Matrix		7.19±0.1	*	
Inoculated mozzarella	QIAamp® DNA Stool	5.97±0.0	6.62±0.2	n.s.d.	*
Inoculated mozzarella	InstaGene™ Matrix		9.15±0.1	*	
n.s.d.: no significant difference at p<0.05; *: significant difference at p<0.05; **:significant difference at p<0.01					

4.3.2 Optimization and repeatability of DNA extraction

To optimize the DNA extraction, the same mozzarella was analysed starting from different quantities (0.2-1g) and using different stomaching times (2-5minutes). Those samples were also compared with the corresponding ones subjected to the revivification treatment at 35°C for 4 hours, in order to allow the adaptation and the growth of the stressed cells and to quantify them as difference between the values obtained for the two corresponding samples (with and without the treatment) (table 4.3).

From table 4.3 appeared that none of the considered variables influenced the analysis: there are no statistically significant differences at 95% among all the samples. Also the incubation treatment seemed not to modify the detected concentrations, suggesting that this is not a good way to quantify the stressed cells; for this reason this step was no longer utilized.

In order to quantify the cells in food samples, several calibration curves (three independent experiments) were created starting from serially diluted cells in trypton salt solution (0.85% w/v); DNAs were extracted and subjected to qPCR. The linearity range was found from 1 to 7log cfu ml^{-1}, the efficiency was 85.327% and the determination coefficient (R^2) was 0.986 (Fig.4.1).

The repeatability of the DNA method was evaluated conducing independent analyses of mozzarella cheeses from the same production line batch. From each sample, ten DNA extractions and quantifications were conduced. In table 4.4 are shown the average and the standard deviations of the two analyses.

The good repeatability of the method was confirmed; no statistically significant differences were found among the samples inside the same analysis (intra-analysis) and even between the two different analyses (inter-analysis).

Fig. 4.1 Calibration curve for DNA quantification, showing the linear relationship between the Threshold Cycle (CT) values and Log cfu for serially diluted DNA obtained from an overnight *P. fluorescens* culture.

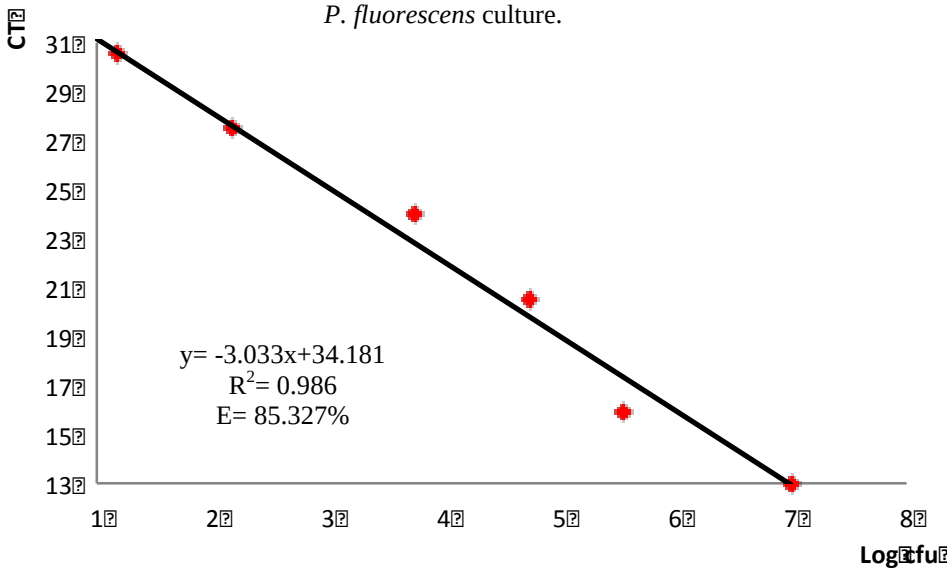


TABLE 4.3 QIAamp® DNA Stool DNA extraction kit optimization.

Sample (g)	**Revivification treatment**	**Stomacher Time**	**Plate count** (log cfu g^{-1})	**Real-Time PCR quantification** (log cfu g^{-1})	**ANOVA analysis**
0.2	/	2 min	<1	5.65±0.03	n.s.d.
0.2	•		<1	5.35±0.22	
1	/		<1	5.13±0.01	
1	•		<1	5.06±0.10	
0.2	/	5 min	<1	5.75±0.06	
0.2	•		<1	5.49±0.03	
1	/		<1	5.27±0.00	
1	•		<1	5.23±0.05	
/= Sample non-subjected to the treatment; •=sample subjected to treatment n.s.d.: no significant difference at $p<0.05$					

TABLE 4.4 Repeatability of the DNA analysis.

	Values (log cfu g^{-1})										**Mean**	**SD**	**ANOVA intra-analysis**	**ANOVA inter-analyses**
First analysis	5.65	5.35	5.43	5.13	5.06	5.75	5.49	5.29	5.27	5.23	5.36	0.19	n.s.d.	n.s.d.
Second analysis	5.35	5.03	5.80	5.86	5.77	5.43	5.64	5.69	5.37	5.47	5.54	0.26	n.s.d.	
n.s.d.: no significant difference at $p<0.05$														

4.3.3 Seasonality

Optimized the method, samples of mozzarella as such and inoculated with known concentrations of overnight colture of *P. fluorescens* were analysed in different moment of the year. Table 4.5 shows the means of the results obtained in the warmer period (spring and summer), which is the most critical period for milk quality.
In both seasons, the DNA concentrations in the non-inoculated samples were very high and showed statistically significant differences with the plate count, suggesting the presence of stressed or dead cells which represented the background noise of the matrix. These values were not always the same; in particular in summer mozzarella it was significantly higher than the spring-time sample. This kind of differences was explained by the different quality of the raw milk and the seasonality of the production (Barron et al., 2001; Caridi et al., 2003; Alonso et al., 2011).
Looking at the DNA quantification of the inoculated samples was affected by the background value. Depending on the noise concentration, the quantification of the inoculated samples were differently affected: statistically significant differences ($p<0.01$ and/ or $p<0.05$) were found in spring mozzarella for all the samples inoculated with concentrations lower than 3.67log cfu g^{-1} (sample D), whereas in summer mozzarella for almost all the samples inoculated with concentrations lower than 5.60log cfu g^{-1} (sample F).

TABLE 4.5 DNA analyses on mozzarella during the year.

Spring-time analysis						**Summer-time analysis**					
Sample; inoculum (log cfu g^{-1})	**Plate count** (log cfu g^{-1})		**Real-Time PCR quantification** (log cfu g^{-1})		**ANOVA analysis**	**Sample; inoculum** (log cfu g^{-1})	**Plate count** (log cfu g^{-1})		**Real-Time PCR quantification** (log cfu g^{-1})		**ANOVA analysis**
	Means	**SD**	**Means**	**SD**			**Means**	**SD**	**Means**	**SD**	
A (0)	<1	0.00	3.26	0.72	**	A (0)	<1	0.00	5.57	0.47	**
B (1)	1.59	0.41	3.68	0.18	**	B (1)	1.91	0.29	5.86	0.24	**
C (2)	2.66	0.33	4.10	0.24	**	C (2)	3.02	0.25	5.89	0.03	**
D (3)	3.67	0.44	4.13	0.24	*	D (3)	3.82	0.44	6.27	1.19	**
E (4)	4.44	0.29	4.97	0.58	n.d.s.	E (4)	4.81	0.30	5.96	0.03	**
F (5)	5.61	0.61	6.06	0.82	n.d.s.	F (5)	5.60	0.44	6.60	0.28	*
G (6)	6.29	0.04	7.07	0.58	n.d.s.	G (6)	6.70	0.43	7.40	0.25	n.d.s.
n.s.d.: no significant difference at $p<0.05$; *: significant difference at $p<0.05$; **:significant difference at $p<0.01$											

4.3.4 Set up of RNA extraction for evaluating alive cells

Seeing that it was not possible to establish a priori the background noise of the matrix and to quantify only the live cells the RNA was analysed.
As for DNA analysis, calibration curves (three independent experiments) were created starting from serially diluted cells in trypton salt solution (0.85% w/v). The linearity range was from 1.7 to 6.7log cfu ml^{-1}, the efficiency was 82.855% and the determination coefficient (R^2) was 0.982 (Fig. 4.2).
The repeatability of the RNA method was evaluated conducing three independent analyses of mozzarella cheeses from the same production line batch. From each sample, seven RNA extractions and quantifications were carried out (table 4.6). This analysis confirmed a good repeatability of the method: no statistically significant differences were found.
To quantify the background noise of the death and uncultivable (VBNC) cells, we compare the quantification of the total DNA and RNA of mozzarella samples as such and inoculated with increasing and known concentrations of overnight colture of *P. fluorescens* DSM50106^T (table 4.7). The values of plate count, DNA and RNA quantifications of each sample were used to quantify the dead and uncultivable cells. Again the background noise of the matrix (DNA of non-inoculated sample) affected the DNA quantification especially of the samples inoculated with low concentrations. The dead cells were quantified by subtraction of RNA concentration from DNA quantification; this value is around 4.5log cfu g^{-1} and it increased in the last three samples for the presence of inoculum dead cells. The Viable But Non-Culturable cells instead were quantified by subtraction of plate count results from RNA quantification; this value was around 2.5log cfu g^{-1} and became higher in the lasts samples due to the inoculum.

Fig. 4.2 Calibration curve for RNA quantification, showing the linear relationship between the Threshold Cycle (CT) values and Log cfu for serially diluted DNA obtained from an overnight *P. fluorescens* culture.

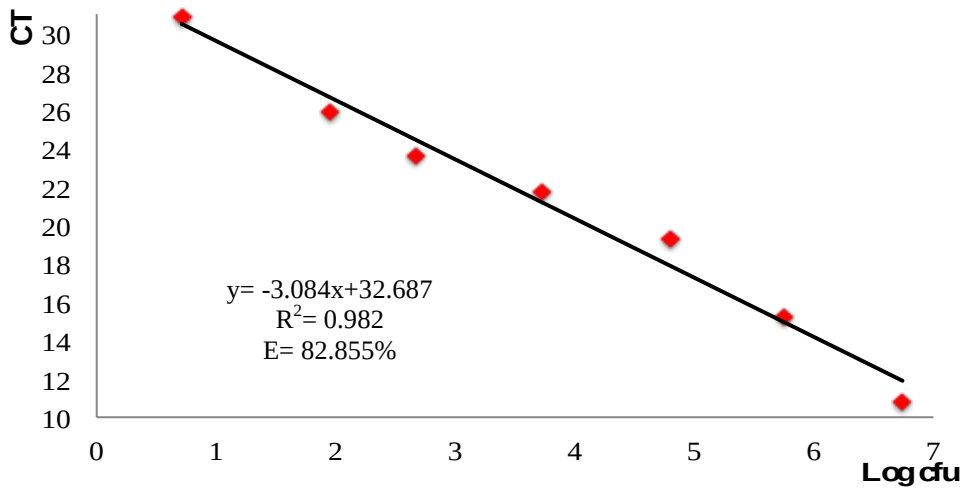


FIGURE 4.2

TABLE 4.6 Repeatability of the RNA analysis.

	Values (log cfu g^{-1})							**Mean**	**SD**	**ANOVA intra-analysis**	**ANOVA inter-analyses**
First analysis	3.49	3.83	3.42	3.92	3.72	3.82	3.92	3.73	0.20	n.s.d.	n.s.d.
Second analysis	2.69	2.84	3.08	3.20	2.83	3.02	2.38	2.86	0.28	n.s.d.	
Third analysis	3.90	4.04	4.08	3.64	3.01	3.65	3.14	3.64	0.42	n.s.d.	
n.s.d.: no significant difference at $p<0.05$											

Table 7 – DNA and RNA comparison.

Sample	**Plate count** (log cfu g^{-1})	**DNA RT-PCR quantification** (log cfu g^{-1})	**RNA RT-PCR quantification** (log cfu g^{-1})	**Dead cells** (log cfu g^{-1})	**VBNC cells** (log cfu g^{-1})
Mozzarella as such	<1	4.52±0.2	1.94±0.1	4.51±0.3	1.94±0.1
Inoculated mozzarella	1.81±0.2	4.40±0.2	2.32±0.1	4.40±0.3	2.16±0.4
Inoculated mozzarella	2.79±0.1	4.56±0.1	2.94±0.3	4.55±0.4	2.41±0.2
Inoculated mozzarella	4.11±0.1	4.81±0.3	4.13±0.2	4.71±0.4	2.70±0.4
Inoculated mozzarella	4.91±0.2	5.29±0.2	4.93±0.1	5.05±0.3	3.49±0.4
Inoculated mozzarella	5.91±0.3	6.23±0.0	5.91±0.2	5.95±0.2	3.95±0.3
Inoculated mozzarella	6.95±0.2	7.17±0.1	6.96±0.1	6.75±0.2	4.81±0.3

4.3.5 Method application

Thinking about the "blue mozzarella event" of 2010, we tried to reproduce the blue pigmentation. In particular different mozzarella cheeses form the same production line batch were inoculated with a note concentration (2.30log cfu g^{-1}) of blue pigment producer *P. fluorescens*, incubated at 4°C, and 12°C for 30 days, and at 25°C for 2 days. Non-inoculated samples from the same production line batch were incubated and analysed as controls. The plate count specific for *Pseudomonas* spp. was done after different time (figure 4.3) and 17 days of storage the molecular analysis was conduced; in the same analysis a sample of another production line batch, recalled from the market because naturally become blue, was also analysed (table 4.8).
In figure 4.3 the continuous lines represented the non-inoculated samples and show different trends of growth according to the temperature. The dashed lines instead represented the inoculated mozzarellas: the strain grew very quickly from the beginning producing the blue pigmentation at all temperatures when it reached around ten to the eight cfu g^{-1}.
In table 4.8 are reported the molecular analysis conduced after 17 days of storage. The samples of this analysis were the only non inoculated samples (A and C) positives to the plate count plating (around 3log cfu g^{-1}); their VBNC value instead was variable: at 12°C the revivification seemed to be favourite, showing lower VNBC value (2.58log cfu g^{-1}) than that at 4°C (3.86log cfu g^{-1}). For B and D samples (inoculated samples), the dead cells were higher for the inoculum (7log cfu g^{-1}) and the VBNC were approximately zero, indicating that the revivification was complete. For the E sample very high alive and VBNC cells values were found (8.75 and 8.49log cfu g^{-1} respectively). Interesting was that the blue pigmentation appeared when the *Pseudomonas* concentrations reached around 8.0log cfu g^{-1} of alive cells.

Fig. 4.3 Growth of blue pigment producer strain of *Pseudomonas* in mozzarella at different temperature of storage.

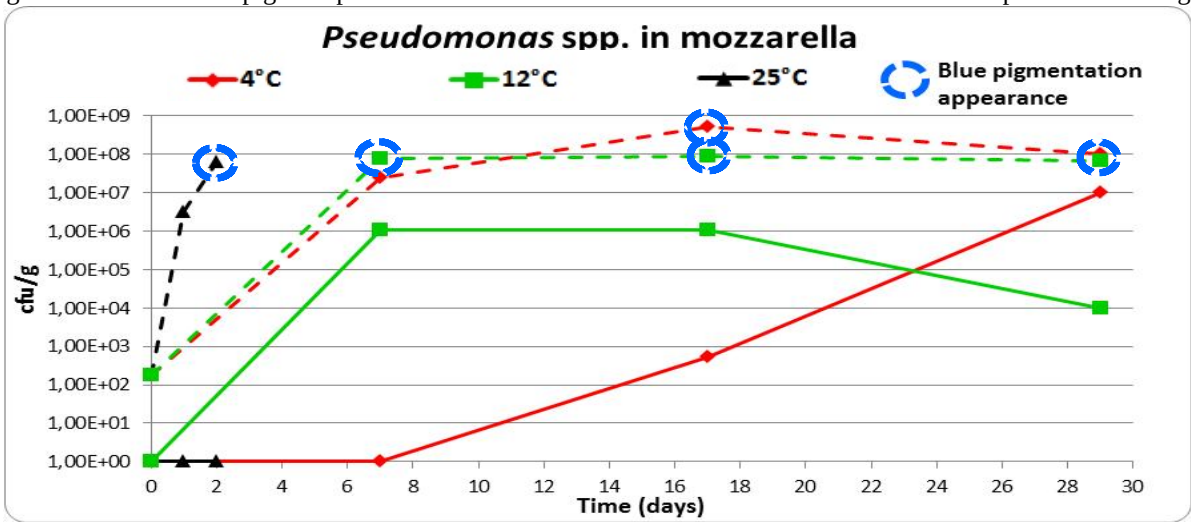


TABLE 4.8 DNA and RNA comparison of blue mozzarellas.

Sample		**Storage temperature**	**Sample colour**	**Plate count** (log cfu g^{-1})	**DNA RT-PCR quantification** (log cfu g^{-1})	**RNA RT-PCR quantification** (log cfu g^{-1})	**Dead cells** (log cfu g^{-1})	**VBNC cells** (log cfu g^{-1})
A	As such	4°C	Normal	2.74±0.1	3.95±0.2	3.89±0.1	3.06±0.3	3.86±0.2
B	Inoculated	4°C	Blue	8.52±0.1	8.79±0.1	8.76±0.2	7.01±0.3	0.24±0.3
C	As such	12°C	Normal	3.91±0.2	4.01±0.1	3.93±0.2	3.04±0.3	2.58±0.4
D	Inoculated	12°C	Blue	7.96±0.1	8.30±0.2	7.96±0.1	7.03±0.3	0.00±0.2
E	As such	Cold chain during sale	Blue	8.75±0.2	8.97±0.1	8.94±0.2	7.79±0.3	8.49±0.4

4.3.6 PMA pre-treatment: preliminary results

The identified method appeared rapid, effective and suitable for the detection of *Pseudomonas* spp., but economically expensive; trying to remedy this problem, a pre-treatment with Propidium MonoAzide was utilized. This is a dye able to enter inside cells with compromised membranes and, after photo-activation, to intercalate into the nucleic acids creating stable cross-links, which prevent from the amplification of the DNA of dead cells. The reducing of the costs will be due to the excluding of RNA quantification and the possible quantification of the only DNA after the pre-treatment with PMA.
We analysed mozzarellas as such and inoculated with different and known concentrations of *P. fluorescens* DSM 50106^{T}. DNA and RNA were extracted and quantified in absence of PMA and after pre-treatment.
In our analysis, RNA after PMA pre-treatment was used as control to check the selectivity of the dye: in fact RNA quantification allow to quantify alive cells, and for this reason the PMA treatment shouldn't have any effects on this quantification, they should show similar values. If the two values appear different it could means that the PMA penetrated also into the live cells, damaging and killing them. In addition RNA was also a control, to check the efficacy of the pre-treatment: DNA after pre-treatment in fact should show the same values obtained for RNA.
In table 4.9 are shown the preliminary results. For the non-inoculated mozzarella, the RNA values in presence and absence of PMA appeared the same (around 1.9log cfu g^{-}1) and close to the plate count value. The pre-treatment with PMA seemed to have a partial effect on DNA concentration that from 4.5log cfu g^{-1} became 2.38log cfu g^{-1}, but nevertheless remaining too high.
For mozzarella inoculated with low concentration of *P. fluorescens* (3.95log cfu g^{-1}), the PMA treatment worked very well: DNA quantification passed from 4.79log cfu g^{-1} to 3.97log cfu g^{-1} after pre-treatment reaching RNA (with and without PMA).
Different results instead were shown on mozzarella inoculated with higher *P. fluorescens* concentration (7.22log cfu g^{-1}). In this case in fact it seemed that PMA could penetrat into live cells showing about 2log cfu g^{-1} less then the quantity really present in the sample.
The results here presented are only a preliminary study and more analyses have to be done.

TABLE 4.9 PMA pre-treatment analysis.

Sample and inoculum (log cfu g^{-1})	**Plate count** (log cfu g^{-1})	**Real-Time PCR quantification** (log cfu g^{-1})			
		DNA		**RNA**	
		Before PMA	**After PMA**	**Before PMA**	**After PMA**
Mozzarella as such (0)	<1	4.50±0.2	2.38±0.1	1.90±0.2	1.92±0.1
Inoculated mozzarella (4)	3.95±0.2	4.79±0.1	3.97±0.2	3.92±0.2	3.90±0.2
Inoculated mozzarella (7)	7.22±0.2	7.35±0.1	5.38±0.2	7.21±0.3	5.29±0.1

4.4 DISCUSSION AND CONCLUSIONS

Pseudomonas genus is known to be an important spoilage organism of foods (Caldera & Franzetti, 2013); in particular it can deteriorate foods due to its life on the surface: growing and producing biofilms, anomalous colours, browning and enzymes or even after the death of the cells: many of its extracellular enzymes in fact are heat stable, survive the heat treatments and remain active in the derived products (Marchand *et al.*, 2009; Arslan *et al.*, 2011). For this reason, it is very important to rapidly detect and quantify the presence of live *Pseudomonas* but also dead and Viable But Non-Culturable cells to prevent deterioration of dairy products. In fact, as Gunasekera *et al.* (2003) suggested for milk, a rapid method for the detection and enumeration of *Pseudomonas* can facilitate the identification of specific contamination sources and the prediction of food shelf life.

Therefore, the goal of this study was the set up of a rapid method for the detection, the quantification and the distinction of total, live, dead and VBNC cells of *Pseudomonas* genus in mozzarella.

The method consists of the DNA and RNA extraction from mozzarella, reverse transcription of RNA into cDNA, amplification of DNA and cDNA in Real Time-PCR, quantification with the calibration lines and calculation of all the indices concentrations. The DNA values represents the totality of alive, VBNC and dead cells; RNA concentration is composed by alive and VBNC cells; the dead cells were quantified as difference between DNA and RNA and the VBNC cells were quantified as difference between RNA and plate count or inoculum.

Our analysis suggested that the quality of raw milk used for mozzarella production affects the amount of dead and non-culturable cells present in the final product. In fact during the four seasons of the year the value of DNA concentration was variable among 3log cfu g^{-1} and 6log cfu g^{-1} (non inoculated samples). These values affected the DNA quantification of *Pseudomonas* spp. in samples previously contaminated with known concentrations of *P. fluorescens* DSM 50106^{T}.

A *P. fluorescens* blue pigment producer was intentionally inoculated on mozzarella. On the inoculated samples and on the naturally blue product recalled from the market, the blue coloration appeared when the microbial concentration reached 8log cfu g^{-1} and, in accordance to Sechi *et al.* (2013), even when they were kept in refrigeration conditions.

The developed method requiring less than 4 hours is much faster than the 48 hours for presumptive identification using *Pseudomonas* selective media; it is also much specific and allows to distinguish among the dead, VBNC and live cells in mozzarella. It has also the possibility of be adapted for the detection of other organisms or single interesting *Pseudomonas* specie (such as *P. fluorescens*) by utilizing specific primers in the amplification. The possible costs reducing, by only quantify DNA after PMA treatment, is still under evaluation.

In conclusion, the development of rapid method for detection, enumeration and recognition of the state (live, dead, VBNC) of *Pseudomonas* cells in mozzarella are important for quantitative population analysis of bacteria in this product.

4.5 REFERENCES

Alarcon B, Vicedo B, Aznar R. 2006. PCR-based procedures for detection and quantification of *Staphylococcus aureus* and their application in food. Journal of Applied Microbiology, 100 (2), 352-364.

Alonso R, Picon A, Rodríguez B, Gaya P, Fernández-García E, Nuñez M. 2011. Microbiological, chemical, and sensory characteristics of Hispánico cheese manufactured using frozen high pressure treated curds made from raw ovine milk. Internationa Dairy Journal, 21 (7): 484-492.

Andreani NA, Martino ME, Fasolato L, Carraro L, Montemurro F, Mioni R, Bordin P, Cardazzo B. 2014. Tracking the blue: A MLST approach to characterise the *Pseudomonas fluorescens* group. Food Microbiology, 39: 116-126.

Arslan S, Eyi A, Özdemir F. 2011. Spoilage potentials and antimicrobial resistance of *Pseudomonas* spp. isolated from cheeses. Journal of Dairy Science, 94: 5851-5856.

Barron LJR, Fernàndez de Labastida E, Perea S, Chàvarrib F, de Vega C, Vicente MS, Torres MI, Nàjera AI, Virto M, Santisteban A, Pérez-Elortondo FJ, Albisu M, Salmeròn J, Mendìa C, Torre P, Ibànez FC, de Renobales M. 2001. Seasonal changes in the composition of bulk raw ewe's milk used for Idiazabal cheese manufacture. International Dairy Journal, 11: 771-778.

Baruzzi F, Lagonigro R, Quintieri L, Morea M, Caputo L. 2012. Occurrence of non-lactic acid bacteria populations involved in protein hydrolysis of cold-stored high moisture Mozzarella cheese. Food Microbiology, 30: 37-44.

Bustin SA. 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. Journal of Molecular Endocrinology, 29: 23-39.

Caldera L, Franzetti L. 2013. Specific spoilage bacteria in different type of packaged fresh foods, Microbial Spoilers in Food 2013, ISBN: 978-2-916248-45-5, p14.

Calisti C. 2008. Sviluppo di metodiche molecolari per il rilevamento rapido di *Pseudomonas* in matrici alimentari e ambientali. PhD thesis in Foods Biotechnology, Università degli studi della Tuscia, Viterbo.

Caridi A, Micari P, Foti F, Ramondino D, Sarullo V. 2003. Ripening and seasonal changes in microbiological and chemical parameters of the artisanal cheese Caprino d'Aspromonte produced from raw or thermized goat's milk. Food Microbiology, 20: 201-209.

Datta N, Deeth HC. 2001. Age gelation of UHT-milk – a review. Food and Bioproducts Processing, 79: 197–210.

Deeth HC, Khusniati T, Datta N, Wallace RB. 2002. Spoilage patterns of skim and whole milks. Journal of Dairy Research, 69: 227–241.

Díaz M, Herrero M, García LA, Quirós C. 2010. Applicationof flow cytometry to industrial microbial bioprocesses. Biochemical Engineering Journal, 48: 385-407.

Gunasekera TS, Dorsch MR, Slade MB, Veal DA. 2003. Specific detection of *Pseudomonas* spp. in milk by fluorescence in situ hybridization using ribosomal RNA directed probes. Journal of Applied Microbiology, 94: 936–945.

Herrera AG. 2001. Psychrotrophic microorganisms. J.F.T. Spencer and de A. L. R. Spencer, ed. Humana Press Inc., in Food Microbiology Protocols (pp. 3-11). Totowa, New Jersey, NJ.

Malorny B, Paccassoni E, Fach P, Bunge C, Martin A, Helmuth R. 2004. Diagnostic real-time PCR for detection of *Salmonella* in food. Applied and Environmental Microbiology, 70 (12): 7046-7052.

Marchand S, Vandriesche G, Coorevits A, Coudijzer K, De Jonghe V, Dewettinck K, De Vos P, Devreese B, Heyndrickx M, De Block J. 2009. Heterogeneity of heat-resistant proteases from milk *Pseudomonas* species. International Journal of Food Microbiology, 133: 68-77.

Nogarol C, Acutis PL, Bianchi DM,Maurella C, Peletto S,Gallina S, Adriano D, Zuccon F, Borrello S, Caramelli M, Decastelli L. 2013. Molecular Characterization of *Pseudomonas fluorescens* Isolates Involved in the Italian "Blue Mozzarella" Event. Journal of Food Protection, 76 (3): 376-551.

Oliveira TC, Barbut S, Griffiths MW. 2005. Detection of *Campylobacter jejuni* in naturally contaminated chicken skin by melting peak analysis of amplicons in real-time PCR. International Journal of Food Microbiology, 104 (1): 105-111.

Oommen BS, McMahon DJ, Oberg CJ, Broadbent JR, Strickland M. 2002. Proteolytic specificity of *Lactobacillus delbrueckii* subsp. *bulgaricus* influences functional properties of mozzarella cheese. Journal of Dairy Science, 85: 2750-2758.

Pennacchia C, Ercolini D, Villani F. 2009. Development of a Real-Time PCR assay for the specific detection of *Brochothrix thermosphacta* in fresh and spoiled raw meat. International Journal of Food Microbiology, 134 (3): 230-236.

Rantsiou K, Alessandria V, Urso R, Dolci P, Cocolin L. 2008. Detection, quantification and vitality of *Listeria monocytogenes* in food as determined by quantitative PCR. International Journal of Food Microbiology, 121 (1): 99-105.

Ray B. 2004. Fundamental Food Microbiology. CRC Press, Boca Ra- ton, FL.

Sechi P, Vizzani A, Scuota S, Zicavo A, Parmegiani S, Cenci Goga B. 2013. Anomalous blue colouring of mozzarella cheese intentionally contaminated with pigment producing strains of *Pseudomonas fluorescens*. Italian Journal of Food Safety, 1: 81-84.

Vanysacker L, Denis C, Roels J, Verhaeghe K, Vankelecom IFJ. 2013. Development and evaluation of a TaqMan duplex real-time PCR quantification method for reliable enumeration of *Candidatus Microthrix.* Journal of Microbiological Methods, http://dx.doi.org/10.1016/j.mimet.2013.11.016.

Wang L, Li Y, Mustaphai A. 2007. Rapid and simultaneous quantitation of *Escherichia coli* 0157:H7, *Salmonella*, and *Shigella* in ground beef by multiplex real-time PCR and immunomagnetic separation. Journal of Food Protection, 70 (6): 1366-1372.

Wilson B, Muirhead A, Bazanella M, Huete-Stauffer C, Vezzulli L, et al. 2013. An Improved Detection and Quantification Method for the Coral Pathogen *Vibrio coralliilyticus*. PLoS ONE 8(12): e81800. doi:10.1371/journal.pone.0081800.

5. INFLUENCE OF LOW OXYGEN TENSION PACKAGING SYSTEM ON MICROBIAL EVOLUTION IN BEEF AND HAMBURGERS

5.1 INTRODUCTION

Meat is a very perishable food. It represents a favourable substrate for microbial growth thanks to its chemical composition: it presents a very high a_w value (around 0.99) and a slightly acid pH (5.5-6.5) and contains all the nutrient substances (Marshall et al., 2001). Microorganisms firstly use glucose, and then they deteriorate amino acids and proteins realizing volatile compounds, such as putrescine and cadaverine, that induce off-flavours (Ercolini et al., 2010; Limbo et al., 2010). The spoilage of meat can occur as strong off-odours and off-flavours, discoloration, superficial slime, uncontrolled acidification, putrefaction and presence of fluorescence (Nychas et al., 2008).

Wide ranges of microorganisms from different sources can be transferred onto meat at different moments of the production: before the slaughter, during slaughter and *post mortem*. Before the slaughter, the contamination is usually due to intestinal microorganisms and it happens if the animal is very stressed or under ill health. During the slaughter it is caused by environmental and intestinal bacteria, conveyed by contaminated tools, operators and delayed evisceration. The *post mortem* contamination is due to environmental microorganisms and usually affects the surface of meat (Galli Volonterio, 2005).

High variety of microorganisms can contaminate meat surface. The most frequent bacteria are *Pseudomonas* spp., *Aeromonas* spp., *Acitenobacte*r spp., *Moraxella* spp., *Micrococcus* spp., *Streptococcus* spp., *Leuconostoc* spp., Enterobacteriaceae, *Clostridium* spp. *Flavobacterium* spp. *Brochothrix thermosphacta* and *Lactobacillus* spp.; and among yeast has been recognized *Saccharomyces* spp., *Candida* spp. and *Rhodotorula* spp. and the most common moulds are *Mucor* spp., *Alternaria* spp., *Penicillium* spp., *Aspergillus* spp. (Gram et al., 2002; Olsson et al., 2003; Koutsoumanis et al., 2008). In particular according to Nychas et al. (2008), aerobically stored meat is commonly spoiled by *Pseudomonas* (especially *fluorescens*, *fragi* and *lundensis* species), instead Enterobacteriaceae (e.g. *Hafnia alvei*, *Serratia liquefaciens*, *Enterobacter agglomerans*) have been considered as indicators of food safety. Both Lactic Acid Bacteria and *Brochothrix thermosphacta* are the main cause of spoilage in term of souring of meat packed under vacuum or modified atmospheres.

To avoid meat deterioration different technological methods were used, in particular the packaging conditions have been widely studied. Vacuum packaging combined with storage under chilled conditions (Venter et al., 2006; Pennacchia et al., 2011; Nowak et al., 2012b), antimicrobial packaging films (active packaging) (Skandamis et al., 2001; Mauriello et al., 2004; Ercolini et al., 2006; Ercolini et al., 2010) and modified-atmosphere packaging with different gases concentrations (Faber, 1991; Gill, 2003; Jeremiah, 2001, Nowak et al., 2012b) have been used and studied since long time.

The solution utilized in this work, optimized by Limbo et al. (2013), consists of enclosing the traditionally packaged meat (trays overlapped with a film characterized by high gas permeability) into a master bag, able to contain multiple packages in low oxygen tension. At the opening of the master bag, meat has to be bloomed to get back the desirable red colour. This solution allows to facilitate the retailer labor and distribution, reducing waste of products and prolonging meat storage at retail store.

5.1.1 Aim of the study

The aims of this work were *i)* to assess the microbiological quality and microbiota development of slices of beef and hamburgers during shelf life, *ii)* to verify the influence of maser bag storage and the following display-life on microbiota composition and growth, *iii)* to identify the most frequently found microbial strains responsible for deterioration phenomena, *iv)* to conduce a challenge test to assess the growth of *Listeria monocytogenes* in hamburgers.

5.2 MATERIALS AND METHODS

5.2.1 Samples

Slices of beef and hamburgers packaged into trays overwrapped with a PVC stretch film with high oxygen permeability, were introduced in master bag, made by a coextruded film and containing 30% of CO_2 and 70% of N_2 and two oxygen absorbers. The master bag were stored at 0-1°C; at the opening times the trays were stored for 4 hours at 3°C in the dark to promote the oxygenation of the myoglobin (blooming) and then stored at 5°C in the presence of light to simulate the conditions of supermarket distribution (Limbo et al., 2013).

5.2.2 Microbiological analysis

Microbiological analyses were carried out at time zero, after 7, 14 and 21 days of slices of beef storage inside master bags immediately after the blooming, at time zero, after 4, 8 and 13 days of hamburgers storage inside master bags, and after 24 and 48h of display life for both products. Slices of beef and hamburgers traditionally packaged into trays overwrapped with a PVC stretch film with high oxygen permeability were analysed during their shelf life (three days at 4°C) as control.
Each time sampling 10g of meat were drawn from two trays and homogenized with 90ml of sterile 0,85% trypton salt solution in a sterile Stomacher bag, by the use of a Colworth 400 Stomacher for 5 minutes. Decimal progressive dilutions were prepared and the following bacteriological determinations were carried out: Total bacterial count (TBC) was assessed by pour plates on Plate Count Agar (Merck Germany) (ISO, 2003), incubation at 30 °C for 72h. Enterobacteriaceae were assessed by double layer pour plates on Violet Red Bile Dextrose Agar (VRBD) (Merck Germany) (ISO, 2004), incubation at 37 °C for 24h. *Escherichia coli* was measured by pour plates on TBX (Merck Germany) (ISO, 2001), incubation at 37 °C for 24h. *Pseudomonas* spp. was assessed by spread technique on *Pseudomonas* Agar Base added with CFC Selective (Merck Germany) (ISO, 2010), incubation at 35 °C for 5 days. Lactic acid bacteria (LAB) were measured by pour plates on APT (Merck Germany), incubation at 30 °C for 48h under anaerobic condition (gas pack). Spores of *Clostridium perfringens* was counted in the presence of Sulphite Polimixin Sulfadiazine after pasteurization at 80 °C for 10min according to Angelotti et al. (1962). *Brochrotix thermosphacta* was assessed in STAA (Merck Germany) by spread plates, incubation at 30 °C for 48–72h (Dainty et al., 1980). *Salmonella* spp. (ISO, 2007) and *Listeria monocytogenes* (ISO, 2004) were determined in 25g of product.
All microbiological analyses were carried out in triplicate, and the results were expressed as the mean log cfu g^{-1}.

5.2.3 Isolation and phenotypic characterization

All colonies grown on the last dilution of PCA, *Pseudomonas* Agar Base, APT and STAA were collected, purified and stored in 20% (vol/vol) glycerol at -20°C. The isolates were tested for morphology, motility, Gram staining by optical microscopy (1200X), catalase and oxidase productions and oxidative/fermentative metabolism (OF test, Hugh *et al.*, 1953) for a preliminary biochemical characterisation.

5.2.4 DNA extraction and PCR protocols

Genomic DNA from isolates was extracted from 300µl of an overnight culture diluted with 400µl of 1X TE buffer (10mM Tris-HCl and 1mM Na2EDTA, pH 8.0) as described by Mora et al. (2000).
All PCR reactions were performed in a volume containing approximately 50-100ng of bacterial genomic DNA solution, 5µl of 10X PCR reaction buffer, 200µM of each dNTP, 2mM of $MgCl_2$, 0.5µM of each primer and 0.5U of Taq Polymerase (Amersham- Pharmacia).

The primers and the amplification conditions used are shown in table 5.1, except for 16S rDNA amplification that was performed using the following thermal profile: 2 min at 94 °C; 5 cycles consisting of 94 °C for 45s, 55 °C for 1min, 72 °C for 2min; 35 cycles consisting of 92 °C for 45s, 60 °C for 45s, 72 °C for 2min; final extension of 72 °C for 2min; and final cooling at 4 °C. All the amplifications were performed in a DNA thermal cycle (Biometra T gradient, Germany). Following the amplification, 7µl of each amplificate was analysed by electrophoresis at 100V (1% agarose gel, 0.2µg of ethidium bromide ml-1) in TAE buffer.
For *Brochothrix* genus new forward primer was created based on the 16S gene. The sequences of two *B. thermosphacta* (ATCC 11509) and *B. campestris* (ATCC 43754) were aligned with the sequences of other bacteria using *ClustalW*. The optimization of the amplification was made by conducing a gradient of the annealing temperature between 45 and 55°C and 52°C appeared the optimal temperature.

5.2.5 Partial sequencing of the 16S rDNA gene

After amplification of the 16S rDNA gene from extracted DNA, the PCR product was purified according to the instructions of a commercial kit (Qiaquick, Qiagen), and the strains were sequenced with the 16S forward primer in a model 310 automatic DNA sequencer (Applied Biosystem, Foster City, CA). The obtained sequences were elaborated by using the software Chromas 2.13 (Technelysium Pty Ltd. Helensvale, Queensland, Australia). Sequence data of type strains were retrieved from NCBI sequence database and pairwise comparison was conduced using BLAST program.

5.2.6 Challenge test

The aim of a challenge test is to assess the growth of a pathogen in food by artificially inoculation with a known concentration of the microorganism target, followed by the determination of its growth during a defined period (shelf life) at given temperatures.
Challenge testing was performed on hamburgers with the following method: three *Listeria monocytogenes* strains (ATCC 20600^T and two strains isolated from meat products) were grown overnight at 30 °C in Tryptone Soya Broth (Oxoid, Basingstoke, Hampshire, England), diluted and mixed to inoculate the surface of the meat in order to obtain inoculum levels of about 2 and 4log cfu g^{-1}. The total count of the inoculum was determined by microscopy quantification, and the concentration of the alive cells of the inoculum was quantified by culture dilutions plating on Tryptone Soya Agar (Oxoid, Basingstoke, Hampshire, UK).
For each sampling day, two inoculated hamburgers were prepared. They were re-packed in PVC overlapped trays and in master bag conditions. The analyses were conduced during storage of traditionally packaged samples and at the same opening times reported in 5.2.1.
The challenge test permits to establish the growth potential, which is calculated as difference between the $\log_{10}$ counts at the end of shelf life and the $\log_{10}$ of the initial concentration. If the difference between the counts at "day end of shelf life" and "day 0" did not exceed $0.5\log_{10}$, the count values changes may be attributed to the uncertainty of microbiological measurement and hence this was not identify as significant growth of the pathogen (EU CRL *L. monocytogenes*, 2008) (Uyttendaele et al., 2009).

5.2.7 Statistical analysis

The obtained counts were subjected to one-way analysis of variance (ANOVA). In case of statistically significant differences the Tukey multiple comparison test was performed ($p < 0.05$ and $p < 0.01$).

Table 5.1 PCR primers and annealing temperature condition used.

Gene	Primers pair (5'-3')		Annealing temperature
Intergenic spacer region (ITS) (Mora et al., 2003)	ITSF: ITSR:	GTCGTAACAAGGTAGCCGTA CAAGGCATCCACCGT	54°C
16S rDNA region (Lane et al., 1985)	16SF: 16SR:	AGAGTTTGATCCTGGCTCAG CTACGGCTACCTTGTTACGA	See in the text
Brochotrix spp. (F: this study; R: Turner et al., 1999)	BrocT: 1100R:	TGTGCTGAACATCAT AGGGTTGCGCTCGTTG	52°C

5.3 RESULTS AND DISCUSSION

5.3.1 Beef and hamburger quality control and master bag conservation analysis.

The analysed beefs and hamburgers appeared of good hygienic quality: *Salmonella* spp. and *Listeria monocytogenes* were always absents in 25g, while *E. coli* was found with values less than 1log cfu g^{-1}, the accepted level according to the European Regulation (Reg. 1441/2007). Table 5.2 shows the evolution of the quality microbial indices recognized in meat never stored in master bag. At time zero, before packaging, the Total Bacterial Count showed good values of about 3.4log cfu g^{-1} for slices and 5.4log cfu g^{-1} for hamburgers, and seemed mostly represented by Lactic Acid Bacteria, Pseudomonadaceae and *Brochothrix thermosphacta*. The last two indices grew during the two/three days of conservation, according to their metabolism in the traditional storage conditions (packaged in PVC wrapped trays, in air and stored at 4±2°C). Especially *Pseudomonas* spp., which is the most important responsible of spoilage of meat, under aerobic condition represented the dominant microbiota. In particular in hamburgers it passed from 4.8 to 6.7log cfu g^{-1} during three days of storage, never reaching the limit value (7log cfu g^{-1}) attributed to slime and off-odours formation (Nychas et al., 2008). LAB grew slowly, reaching values similar to *Pseudomonas* spp. when the oxygen availability decreased.
The master bag conservation instead for the slices of beef seemed to have a bacteriostatic activity decelerating the TBC especially during the first two weeks, and limiting the growths of *Pseudomonas* spp. and *B. thermosphacta* that showed constant and low concentration values until the end of the experimentation (around 3log cfu g^{-1}). The Lactic Acid Bacteria are the microbial population favourite in these conditions: they have a very rapid growth especially in the first seven days, after which they represent the biggest microbial component of the microbiota. Similar but more pronounced trends were identified for hamburgers: TBC slowly grew during the first four days reaching a stable value around 6.3log cfu g^{-1}, *Pseudomonas* spp. and *Brochothrix* spp. showed stable levels until the end and LAB growth was favourite (Table 5.3).
The explanation of those trends is the hypothesis that *Pseudomonas* spp. and *Brochotrix* spp. were inhibited by the master bag conditions (combination of low oxygen concentrations with high carbon dioxide levels) and also by the acidification induced by Lactic Acid Bacteria (Lee and Yoon, 2001). The pH of the slices of beef, for example, decreased from 5.75±0.03 to 5.50±0.05 in three weeks of master bag storage.

5.3.2 Display-life of slices of beef after master bag storages.

The results of the aerobic display-life analysis after 7 and 14 days in master bag are shown in table 5.4.
For the first master bag opening (after 7 days of storage), Total Bacterial Count and *Pseudomonas* spp. seemed to be stable during the three days of conservation with low values. The other two indexes showed an important deceleration during the first 24 hours, after which Lactic Acid Bacteria became stable and *B. thermosphacta* started to grow, reaching values similar to the TBC.
For the second master bag opening (after 14 days of storage), Total Bacterial Count and Lactic Acid Bacteria showed a similar fluctuant trend: during the first 24 hours they grew of 1log cfu g^{-1}, then they came back to the initial values and during the last day they grew again. *Pseudomonas* spp. and *B. thermosphacta* instead showed the same attitude found after the first opening of master bag: the first stayed always stable and the second had an initial deceleration after which it grew again.

The explanation of those trends is different for each index: the deceleration of Lactic Acid Bacteria during the display life of the samples was due to the aerobic conditions, which appeared less favorable for their growth than the anoxic master bag storage; *Pseudomonas* spp. maintained constant concentrations during the display-life in air, probably for the residual effect of carbon dioxide of master bag; *B. thermosphacta* instead showed a trend inversely correlated to the LAB concentrations, in accordance to Russo et al. (2006) in fact a decrease was recognized when the LAB level increased.

5.3.3 Display-life of hamburgers after 4 and 8 days in master bag.

The results of the aerobic display-life analysis after 4 and 8 days in master bag are shown in table 5.5.
For the first master bag opening, after an initial adaptation of about one day during which they showed a decrease, all the indices slowly grew, except for *Pseudomonas* spp., which rapidly increased reaching important values close to the TBC.
Similar trends were recognized after the second master bag opening, even if the adaptation appeared less important especially for *Pseudomonas* spp. and *Brochothrix* spp., which rapidly increased of one log during the first day.
In hamburgers it seemed that the residual effect of carbon dioxide of master bag less affected *Pseudomonas* spp., probably because of the feature of the matrix: hamburgers made by minced meat are characterized by a large surface area in contact with the external environment that allow a rapid replacement of the atmosphere, quickly reducing the carbon dioxide concentration at the mater bag opening.

TABLE 5.2. Evolution of the most interesting microbial indices during traditional storage.

Sample	Time (days)	TBC	*Pseudomonas* spp.	Lactic Acid Bacteria	*Brochothrix thermosphacta*
Slices of beef	0	3.4±0.1	3,0±0.1	2.3±0.1	3.0±0.1
	1	4.2±0.2	3.7±0.1	2.5±0.1	3.1±0.1
	2	4.8±0.1	4.2±0.2	2.3±0.2	4.0±0.2
Hamburgers	0	5.0±0.2	4.8±0.2	5.7±0.2	4.2±0.1
	1	5.2±0.1	5.4±0.1	6.1±0.2	4.2±0.2
	2	5.8±0.3	5.6±0.3	6.2±0.1	5.4±0.1
	3	7.6±0.2	6.7±0.1	6.5±0.1	5.7±0.1

TABLE 5.3. Evolution of the most interesting microbial indices during storage in master bag.

Sample	Time (days)	TBC	*Pseudomonas* spp.	Lactic Acid Bacteria	*Brochothrix thermosphacta*
Slices of beef	0	3.4±0.1	3,0±0.1	2.3±0.1	3.0±0.1
	7	4.4±0.2	3.3±0.2	5.5±0.3	3.1±0.1
	14	5.1±0.2	3.0±0.1	5.3±0.1	3.0±0.2
	21	6.9±0.2	2.8±0.3	6.6±0.2	3.3±0.1
Hamburgers	0	5.0±0.2	4.8±0.2	5.7±0.2	4.2±0.1
	4	6.3±0.1	5.1±0.1	6.1±0.1	5.1±0.1
	8	6.3±0.2	5.0±0.1	6.7±0.2	4.0±0.2
	13	6.4±0.2	5.2±0.1	6.7±0.3	4.0±0.1

TABLE 5.4. Evolution of the most interesting microbial indices in beef during aerobic display-life after 7 and 14 days in master bag.

	Time (hours)	TBC	*Pseudomonas* spp.	Lactic Acid Bacteria	*Brochothrix thermosphacta*
First opening	7	4.4±0.2	3.3±0.2	5.5±0.3	3.1±0.1
	8	4.4±0.1	3.4±0.1	4.5±0.2	2.8±0.2
	9	4.6±0.2	3.5±0.1	4.8±0.1	4.4±0.2
	10	4.7±0.3	3.5±0.2	4.9±0.2	4.5±0.3
Second opening	14	5.1±0.2	3.0±0.1	5.3±0.1	3.0±0.2
	15	6.2±0.1	3.2±0.1	6.3±0.2	3.2±0.2
	16	5.1±0.2	3.3±0.2	5.6±0.1	4.2±0.2
	17	5.6±0.2	3.8±0.3	6.6±0.2	4.4±0.1

TABLE 5.5. Evolution of the most interesting microbial indices in hamburgers during aerobic display-life after 4 and 8 days in master bag.

	Time (hours)	TBC	*Pseudomonas* spp.	Lactic Acid Bacteria	*Brochothrix thermosphacta*
First opening	4	6.3±0.1	5.1±0.1	6.1±0.1	5.1±0.1
	5	6.6±0.1	5.0±0.2	6.1±0.2	4.5±0.2
	6	6.7±0.2	6.6±0.1	6.5±0.1	4.6±0.2
Second opening	8	6.3±0.2	5.0±0.1	6.7±0.2	4.0±0.2
	9	6.6±0.1	6.1±0.2	5.6±0.2	5.1±0.1
	10	6.8±0.2	6.7±0.1	6.0±0.1	5.3±0.2

5.3.4 Identification of the strains.

A total of 270 strains were isolates from PCA, MRS, PAB and STAA media. From PCA the 70% was Gram negative, aerobic rod, the 15% was facultatively anaerobic Gram negative rod and 15% Gram positive, among which only the 3.5% was spores producer. Among the LAB from MRS the most important group (80%) was the hetero – fermentative rod. The strains isolated from PAB and STAA appeared preliminarily belonged from *Pseudomonas* spp. and *Brochothrix* spp. respectively; in fact the firsts were aerobic, Gram negative, oxidase positive rods and the seconds were facultatively anaerobic, Gram positive, catalase positive irregular rods.
Microbial diversity investigation of all isolates was performed using the ITS-PCR assay with the universal primers. ITS profiles were used to create different groupings, and no less than two representatives from each group were identified by partial 16S rDNA sequencing.
The sequencing results of the isolates form beef are reported in table 5.5. According to literature (Ercolini et al., 2006; Pennacchia et al., 2011), the Gram negative isolates appeared almost divided between Pseudomonadaceae and Enterobacteriaceae families; all the isolates from PAB belonged to *Pseudomonas* genus. Among Gram positives bacteria, the most represented genus were *Carnobacterium* and *Brochothrix*, they are typical microorganisms of meat.
The sequencing results of hamburgers isolates are reported in table 5.6. In hamburgers less variability was found and the microbiota appeared almost equally divided between Gram positive and Gram negative strains. Among the positives, *Carnobacterium* spp. and *B. thermosphactha* appeared the numerically more important groups. *Psedumonas fragi* instead was the dominant specie in the Gram negative group and it represented the 50% of the total population.

5.3.5 *Brochothrix thermosphacta*

Seeing the importance of *Brochothrix* spp. in spoilage of meat (Nychas et al., 2008; Russo et al., 2006; Ercolini et al., 2009; Nowak et al., 2012a; Gribble et al., 2013) and its detected presence in the analysed meat, we created a primer specific for this genus. In particular the 16S gene sequences of the two species *B. thermosphacta* (ATCC 11509) and *B. campestris* (ATCC 43754) were aligned with the sequences of other bacteria commonly encountered in meat products (figure 5.1). The alignments were performed using *ClustalW*. The portion underlined in yellow appeared typical of *Brochothrix* and it was used as forward primer; a universal primer was used as reverse. The gradient of the annealing temperature, conduced for the optimization of the amplification, indicated the optimal temperature at 52°C (results not shown), and the electrophoresis confirmed that the amplification was present only for *Brochothrix* genus (figure 5.2).

TABLE 5.5. Identification of isolates from beef, obtained by 16S rDNA partial sequencing.

Group	Identification	Accession number	Isolates number
A	*Carnobacterium maltaromaticum*	AB680942.1	59
B	*Carnobacterium divergens*	HM244940.1	3
C	*Brochothrix thermosphacta*	AB680248.1	20
D	*Lactobacillus sakei*	GQ222408.1	1
E	*Streptococcus* sp.	X78826.1	16
F	*Bacillus* sp.	AJ000648.1	4
G	*Weissella beninensis*	EU439435	1
H	*Staphylococcus* sp.	X84731.1	3
1	*Pseudomonas fluorescens*	JF327452.1	30
2	*Pseudomonas putida*	EU118779.1	13
3	*Pseudomonas fragi*	AB680088.1	4
4	*Pseudomonas* sp.	GQ280063.1	5
5	*Stenotrophomonas maltophila*	HQ671069.1	30
6	*Pantoea agglomerans*	FJ756348.1	2
7	*Serratia* sp.	FM178865.2	3
8	*Rahnella aquatilis*	DQ440548.1	4
9	*Hafnia alvei*	FM179944	3

TABLE 5.6. Identification of isolates from hamburgers, obtained by 16S rDNA partial sequencing.

Group	Identification	Accession number	Isolates number
A	*Carnobacterium maltaromaticum*	AB680942.1	7
B	*Carnobacterium divergens*	HM244940.1	9
C	*Brochothrix thermosphacta*	AB680248.1	9
D	*Lactobacillus sakei*	GQ222408.1	8
1	*Pseudomonas fluorescens*	JF327452.1	2
2	*Pseudomonas fragi*	HM032860.1	10
3	*Pseudomonas fragi*	AB680088.1	13
4	*Pseudomonas fragi*	AB685607.1	11

Fig. 5.1 Sequence alignment of 16S gene sequences of *B. thermosphacta*, *B. campestris* and other species including meat products associated bacteria, showing the region where the primer BrocT was designed. Alignment was performed using *ClustalW*.

Brochothrix thermosphacta	...AATACCGNAATGTGCTGAACATCATAAGATGTTCAAGTG...
Brochothrix campestris	...AATACCGAA - TGTGCTGAACATCATAAGATGTTCAAGTG...
Acinetobacter baumanii	...AATACCGCATA- - - - - - -CGTCCTACGGGAGAAAGCAGG...
Bacillus coagulans	...AATACCAGATAGTTTTTTCCTCCGCATGGAGGAAAAAGG...
Carnobacterium maltaromaticum	...AATACCGCATAGTTTCAGGAATCGCATGATTCTTGAAGG...
Carnobacterium divergens	...AATACCGCATATTTCAAGTGACCGCATGGTCGCTTGATG...
Citrobacter freundii	...AATACCGCATA- - - - - - -AYGTCGCAAGACCAAAGAGGG...
Enterobacter ludwigii	...CTTACCCCGTA- - - - - - - TCATCTCACGACCCCAAAGAGG...
Hafnia alvei	...AATACCGCATG- - - - - - -AYGTCTTCGGACCAAAGTGGGG...
Lactobacillus curvatus	...AATACCGCATAAAACCTAGCACCGCATGGTGCAAGGTTG...
Leuconostoc mesenteroides	...AATACCGAATAAAACTTAGTGTCGCATGACACAAAGTTAA...
Pseudomonas fluorescens	...AATACCGCATA - - - - - - - CGTCCTACGGGAGAAAGCAGG...
Psedumonas fragi	...AATACCGCATA - - - - - - - CGTCCTACGGGAGAAAGCAGG...
Psedumonas putida	...AATACCGCATA - - - - - - - CGTCCTACGGGAGAAAGCAGG...
Staphylococcus epidermidis	...AATACCGGATAATATATTGAACCGCATGGTTCAATAGTGA...
Streptococcus thermophilus	...AATACCGCATAACAATGGATGACACATGTCATTTATTTGA...
Klebsiella oxytoca	...AATACCGCATA - - - - - - - ACGTCGCAAGACCAAAGAGGG...

Fig. 5.2 Electrophoretic gel of the amplification with primers specific for *Brochothrix* genus.

5.3.6 Challenge test analysis

In figure 5.3 the evolution of the microbial indices of traditionally packaged samples is shown. Under aerobic condition *Pseudomonas* spp. represented the dominant microbiota. Both LAB and *Pseudomonas* spp. faster increased in presence of higher *Listeria* concentration (Figure 5.3b) showing similar trends. These conditions didn't represent a limit for *L. monocytogenes* growth: starting at low concentration, *L. monocytogenes* increased of two orders in one day, then it reached a plateau and remained stable until the end of experimentation (Figure 5.3a); when the initial inoculum was high (figure 5.3b), it grew more slowly, taking three days to increase of two orders.
The master bag storage instead favored the LAB growth (figures 5.4 and 5.5). After 7 days of storage in master bags, in presence of low content of *L. monocytogenes* (fig. 5.4a) Lactic Acid Bacteria increased from 5 to 6log cfu g^{-1} and *Pseudomonas* spp., inhibited by the anaerobic condition, remained constant. On the contrary, in presence of high *L. monocytogenes* concentration (fig. 5.5a), *Pseudomonas* spp. increased of more than one log, as if the presence of *L. monocytogenes* favored *Pseudomonas* growth. After the opening of master bags, during display life at both pathogen concentrations, the aerobic condition promoted a rapid increasing of *Pseudomonas* spp.
The storage in master bag limited the growth of *L. monocytogenes* that remains stable at 2 and 4log cfu g^{-1} respectively. However the effects of the master bag are only bacteriostatic, and after the opening also this index showed a rapid growth especially during the first day of exposure.
The results obtained after 10 days of storage in master bag and during display life (figures 5.4b and 5.5b) confirmed the previous results.

5.3.6.1 Calculation of potential growth

The obtained results allow to calculate the growth potential (δ) (Table 5.6), which represents an useful parameter to evaluate how much the investigated food promote the growth of the pathogen. The limit indicated by Reg. CE 1441/2007 upon which the food supports the growth of the pathogen is $\delta \geq 0.5\log_{10}$.
Form these results it appeared clear that master bag solution doesn't create favorable conditions for *L. monocytogenes*, showing δ lower $0.5\log_{10}$. However due to the bacteriostatic effects of the storage in master bag, after the opening the environment become again suitable for *L. monocytogenes*, showing δ values similar to that of the traditionally air packaged samples.

Fig. 5.3 Evolution of the most important indices of traditionally packaged hamburgers in presence of a) low inoculum concentration and b) high *Listeria monocytogenes* inoculum.

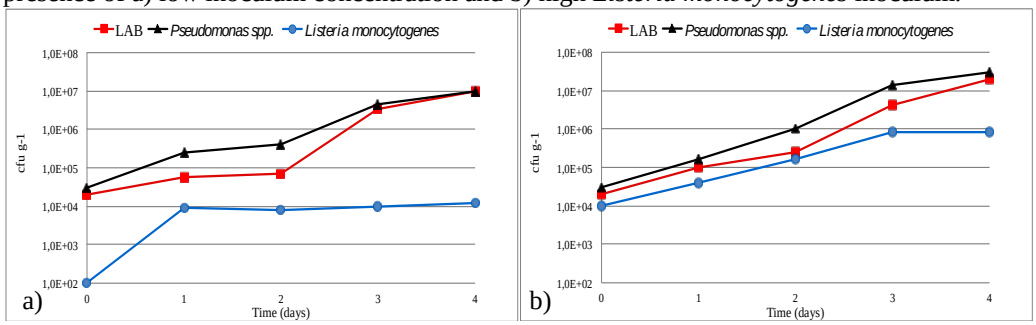


Fig. 5.4 Evolution of the most important indices in hamburgers after storage in master bag for a) 7 days and b) 10 days, in presence of low inoculum concentration of *Listeria monocytogenes.*

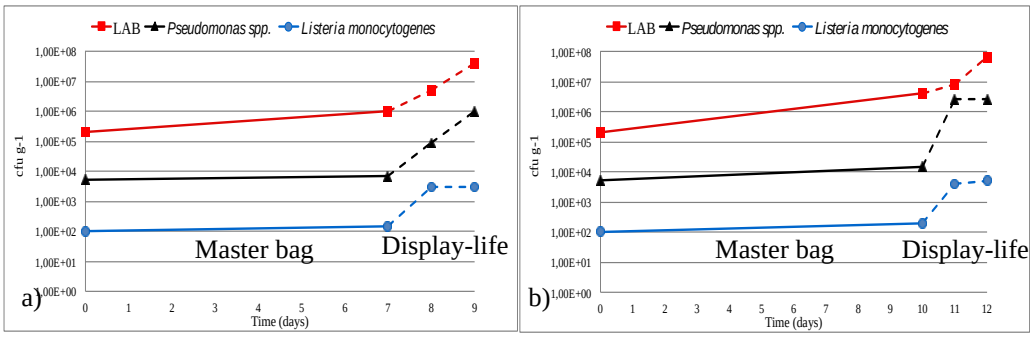


Fig. 5.5 Evolution of the most important indices in hamburgers after storage in master bag for a) 7 days and b) 10 days, in presence of high inoculum concentration of *Listeria monocytogenes.*

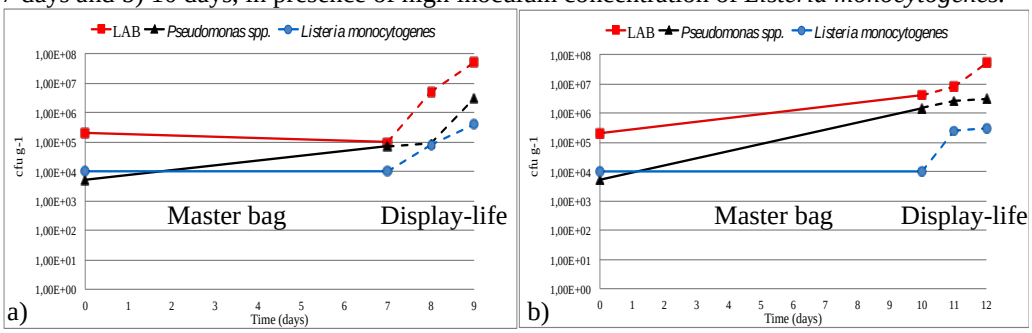


TABLE 5.6. Potential growth (δ) calculated for hamburger stored in air or master bag and during shelf life; * low inoculation (2log cfu g^{-1}), ** high inoculation (4log cfu g^{-1}).

Samples: hamburgers		δ	Suitability for the growth of *L. monocytogenes*
Inoculation	Packaging conditions		
Low *	Air 4 days	2.08	YES
High **	Air 4 days	1.91	YES
Low *	Master bag 7 days	0.09	NO
High **	Master bag 7 days	0.04	NO
Low *	Master bag 10 days	0.03	NO
High **	Master bag 10 days	0.30	NO
Low *	Master bag 7 days, air 2 days	1.30	YES
High **	Master bag 7 days, air 2 days	0.90	YES
Low *	Master bag 10 days, air 2 days	1.48	YES
High **	Master bag 10 days, air 2 days	1.70	YES

5.4 CONCLUSIONS

In conclusion the investigated products' quality was good. Total Bacterial Count, Lactic Acid Bacteria and *Pseudomonas* spp. were the most important indices to define the quality of the final product.
The composition of the microbiota appeared similar in slices of beef and hamburger, although less biodiversity was found in hamburgers. The relevance of Pseudomonadaceae (in particular *P. fluorescens* and *P. fragi*) and Lactic Acid Bacteria (especially *Carnobacterium* spp. and *Brochothrix* spp.) was detected.
The storage conditions affected the evolution of the microbiota, favoring different microbial groups in dependence on the atmosphere inside the package. Anyway also the feature of the matrix influences the effects of conservation, in fact in hamburgers, which have also a initial microbial contamination higher then the slices, it seemed that storage in master bag has a better bacteriostatic activity, even if during the following display life the effects faster disappeared. Those events are explained by the larger surface exposed by minced meat of hamburgers which allow a faster replacement of the atmosphere, rapidly reducing the oxygen tension at the closing of master bag and the carbon dioxide concentration at the opening of mater bag.
The evaluation of the growth of *L. monocytogenes* showed that the master bag doesn't represent a favorable environment and the pathogen, if present, remains stable on initial values without growing or decreasing. However, after the opening of master bag, during the exposure on the bench (display-life), meat has to be considered as fresh meat: when *L. monocytogenes* is present, finding a favorable environment, it quickly grows.

5.5 REFERENCES

Angelotti R, Hall HE, Foter MJ, Lewis KH. 1962. Quantitation of *Clostridium perfringens* in foods. Journal of Applied Microbiology, 10: 193–199.

Dainty RH, Hibbard CM. 1980. Aerobic metabolism of *Brochothrix thermosphacta* growing on meat surfaces and in laboratory media. Journal of Applied Microbiology, 48(3): 387–396.

Ercolini E, Russo F, Torrieri E, Masi P, Villani F. 2006. Changes in the spoilage-related microbiota of beef during refrigeration storage under different packaging conditions. Applied and Environmental Microbiology, 72 (1): 4663-4671.

Ercolini D, Russo F, Nasi A, Ferranti P, Villani F. 2009. Mesophilic and psychrotrophic bacteria from meat and their spoilage potential in vitro and in beef. Applied and Environmental Microbiology, 75(7): 1990-2001.

Ercolini D, Ferrocino I, La Storia A, Mauriello G, Gigli S, Masi P, Villani F. 2010. Development of spoilage microbiota in beef stored in nisin activated packaging. Food Microbiology, 27 (1): 137 - 143.

EU Community Reference Lab for *L. monocytogenes*. 2008. Working document November 2008. Technical guidance document on shelf-life studies for *Listeria monocytogenes* in ready-to-eat foods.

Faber JM. 1991. Microbiological aspect of modified atmosphere packaging thecnology. Journal of Food Prot., 54: 58-70.

Galli Volonterio A. 2005. Microbiologia degli alimenti Casa editrice Ambrosiana, Milano 285-300.

Gram L, Ravn L, Rasch M, Bruhn JB, Christensen AB, Givskov M. 2002. Food spoilage and interactions between food spoilage bacteria. International Journal of Food Microbiology, 78: 79-97.

Gribble A, Brightwell G. 2013. Spoilage characteristics of *Brochothrix themrosphacta* and *campestris* in chilled vacuum packaged lamb, and their detection and identification by real time PCR. Meat Science, 94: 361-368.

Grill CO. 2003. Active packaging in practice: meat. P. 378-396. In H. Ahvenainem (ed.) Novel food packaging technology. Woodhead Publishing Limited and CRC Press LLC, Boca Raton, Fla.

Hugh R, Leifson E. 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram negative bacteria. Journal of Bacteriology, 66: 24-26.

Jeremiah LE. 2001. Packaging alternatives to deliver fresh meats using short- or long-term distribution. Food Research International, 34: 749-772.

Koutsoumanis KP, Stamatiou AP, Drosinos EH, Nychas GJE. 2008. Control of spoilage microorganisms in minced pork by a self-developed modified atmosphere induced by the respiratory activity of meat microflora. Food Microbiology, 25: 915-921.

Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML, Pace NR. 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc. Natl. Acad. Sci USA. 82: 6955-6959.

Lee KT, Yoon CS. 2001. Quality changes and shelf life of imported vacuum-packaged beef chuck during storage at 0 °C. Meat Science, 59: 71–77.

Limbo S, Torri L, Sinelli N, Franzetti L, Casiraghi E. 2010. Evaluation and predictive modeling of shelf life of minced beef stored in high-oxygen modified atmosphere packaging at different temperatures. Meat Science, 84: 129–136.

Limbo S, Uboldi E, Adobati A, Iametti S, Bonomi F, Mascheroni E, Santagostino S, Powers TH, Franzetti L, Piergiovanni L. 2013. Shelf life of case-ready beef steaks (*Semitendinosus*

muscle) stored in oxygen-depleted master bag system with oxygen scavengers and CO_2/N_2 modified atmosphere packaging. Meat Science, 93: 477-484.

Marshall DL, Bal'a MFA. 2001. Microbiology of Meat. In: Hui YH, editor. Meat Science and Applications. New York: Marcel Dekker Inc. 160–181.

Mauriello G, Ercolini D, La Storia A, Casaburi A, Villani F. 2004. Developemnt of polyethylene films for food packaging activated with an antilisterial bacteriocin from *Lactobacillus curvatis* 32Y. Journal of Applied Microbiology, 97: 314- 322.

Mora D, Fortina MG, Parini C, Daffonchio D, Manachini PL. 2000. Genomic subpopulations within the species *Pediococcus acidilactici* detected by multilocus typing analysis: relationship between pediocin AcH/PA-1 producing and non- producing strains. Microbiology, 146: 2027 - 2038.

Mora D, Ricci G, Guglielmetti S, Daniele Daffonchio D, Fortina MG. 2003. 16S–23S rRNA intergenic spacer region sequence variation in *Streptococcus thermophilus* and related dairy streptococci and development of a multiplex ITS-SSCP analysis for their identification. Microbiology, 149: 807–813.

Nychas GJE, Skandamis PN, Tassou CC, Koutsoumanis KP. 2008. Meat spoilage during distribution. Meat Science, 78: 77-89.

Nowak A, Piotrowska M. 2012a. Biochemical activities of *Brochothirx thermosphacta*. Meat Science, 90: 410-413.

Nowak A, Rygala A, Oltuszak-Walczak E, Walczak P. 2012b. The prevalence and some metabolic traits of *Brochothrix themrosphacta* in meat and meat products packaged in different ways. Journal of Science Food Agriculture, 92: 1304-1310.

Olsson C, Ahrne S, Pettersson B, Molin G. 2003. The bacterial flora of fresh and chill- stored pork: analysis by cloning and sequencing of 16S rRNA genes. International Journal of Food Microbiology, 83(3): 245–252.

Pennacchia C, Ercolini D, Villani F. 2011. Spoilage-related microbiota associated with chilled beef stored in air or vacuum pack. Food Microbiology, 28: 84-93.

Russo F, Ercolini D, Mauriello G, Villani F. 2006. Behaviour of *Brochothrix thermosphacta* in presence of other meat spoilage microbial groups. Food Microbiology, 23: 797-802.

Skandamis PN, Nychas GJE. 2001. Effect of oregano essential oil on microbiological and physic-chemical attributes on minced meat stored in air and modified atmosphere. Journal of Applied Microbiology, 91: 1011-1022.

Turner S, Pryer KM, Miao VPW, Palmer JD. 1999. Investigating Deep Phylogenetic Relationships among *Cyanobacteria* and Plastids by Small Subunit rRNA Sequence Analysis Journal of Eukaryotic Microbiology, 46: 327-338.

Uyttendaele M, Busschaert P, Valero A, Geeraerd AH, Vermeulen A, Jacxsens L, Goh KK, De Loy A, Van Impe JF, Devlieghere F. 2009. Prevalence and challenge tests of *Listeria monocytogenes* in Belgian produced and retailed mayonnaise-based deli-salads, cooked meat products and smoked fish between 2005 and 2007. International Journal of Food Microbiology, 133: 94-104.

Venter P, Shale K, Lues JFR, Buys EM. 2006. Microbial proliferation and mathematical indices of vacuum-packaged bovine meat. Journal of Food Processes and Preservation, 30: 433-448.

6. Identification, enzymatic characterization and proteolytic activity quantification of *Pseudomonas* spp. isolated from different foods

6.1 INTRODUCTION

The genus *Pseudomonas* comprises a heterogeneous group of microorganisms of the Pseudomonadaceae. It includes ubiquitous Gram negative, aerobic, non-fermentative, catalase and oxidase positive, mesophilic and/or psychrotrophic, non-spore forming rods.
The present classification of the genus is complex and needs a profound revision based on molecular, genotypic and phenotypic data. Comparative 16S rRNA gene sequence analysis is used as taxonomic frame, but the high conservative nature of ribosomal genes makes them not suitable for species differentiation (Janda *et al.*, 2007; Mellmann *et al.*, 2008). Sequencing of several less conservative housekeeping genes was gradually included to obtain a more discriminatory phylogenetic evaluation (Ait Tayeb *et al.*, 2005; Mulet *et al.*, 2010; Rees-George *et al.*, 2010; Yamamoto *et al.*, 2000).
Pseudomonas members are adapted to various conditions and therefore are found in a wide range of niches as soil, water, plants, animal tissues, foods, etc. (Franzetti *et al.*, 2007) thanks to their complex enzymatic systems. Members of this genus are frequently implicated in the degradation and spoilage of a wide range of foods deriving from plants or animals. It is important to notice that *Pseudomonas* members produce many enzymes. In particular heat-resistant proteases result in consolidation of activity also after heat treatment processes used to eradicate microorganisms in certain matrices. This phenomenon is very important in UHT milk and dairy products in which the protease can cause coagulation and instability phenomena, respectively (Datta *et al.*, 2001).
The mechanisms of these spoilage processes are not completely known but are strain dependent and related to temperature and environmental conditions (Chabeaud *et al.*, 2001; Woods *et al.*, 2001; Nicodème *et al.*, 2005).
However, it is known that the responsible protease is an extracellular alkaline metallo-protease belonging to the AprX protein family, which has been extensively studied in *Pseudomonas aeruginosa* (Okuda *et al.*, 1990, Duong *et al.*, 2001; Blevesa *et al.*, 2010). This protein is codified by the *aprX* gene, located in an operon that contains also the *lipA* gene, coding for a lipase, a protease inhibitor (*inh*), a secretion system (*aprDEF*) and two auto-secreted serine-protease homologues (*prtAB*) (Woods *et al.*, 2001).

6.1.1 Aims of the study

The aims of this work are *i)* the characterization and identification of *Pseudomonas* isolates from different food products (ready-to-eat vegetables, meat, milk and dairy products); and *ii)* the study of the heterogeneity in protease-activity of the isolates, in terms of quantification of the extracellular proteolytic activity, and genetic heterogeneity of the *aprX* gene.

6.2 MATERIALS AND METHODS

6.2.1. Microbial strains

A total of 66 isolates of putative *Pseudomonas* spp. were examined for phenotypic and genotypic characteristics. The strains have been isolated from different food matrices; their origin is reported in Table 6.1 as well as eight type and reference strains, obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. All the strains were maintained on Tryptic Soy Agar slopes at 5°C and frozen at -24°C in Tryptic Soy Broth with 20% of glycerol.

TABLE 6.1. Tested strains and their origin. AP salad: Air-Packaged green salad; AP carrots: Air-Packaged cut carrots; MAP salad: Modified-Atmosphere Packaged green salad.

Reference strain	**DSM Number**
P. aeruginosa	DSM 50071
P. cichorii	DSM 50259
P. fluorescens biotype A, bv.I	DSM 50090
P. fluorescens biotype C, bv. III	DSM 50120
P. fluorescens biotype C, bv. III	DSM 50124
P. fluoresens biotype G	DSM 50148
P. mendocina	DSM 50017
P. putida	DSM 291

	Isolates	**Origin**		**Isolates**	**Origin**
Ready-to-eat vegetable strains	P.cic1	AP salad	Dairy products isolates	P.spp1	Soft cheese (Crescenza)
	P.flu13	AP salad		P.spp2	Soft cheese (Crescenza)
	P.flu17	AP salad		P.spp3	Soft cheese (Crescenza)
	P.flu20	AP salad		P.spp4	Soft cheese (Crescenza)
	P.flu21	AP salad		M9	Mozzarella cheese
	P.flu22	AP salad		M5	Mozzarella cheese
	P.flu23	AP salad		M3	Mozzarella cheese
	P.flu24	AP salad		M6	Mozzarella cheese
	S2	AP salad		CC	Brown mozzarella cheese
	S31	AP salad		LUCY	Mozzarella cheese
	S32	AP salad		H1	Blue mozzarella cheese
	S34	AP salad		H2	Blue mozzarella cheese
	S38	AP salad		H6	Blue mozzarella cheese
	I15	MAP salad		H9	Blue mozzarella cheese
	I20	MAP salad		H11	Blue mozzarella cheese
	I27	MAP salad		M240	Pink mozzarella cheese
	I43	MAP salad		M241	Pink mozzarella cheese
	LC28	MAP salad		M243	Pink mozzarella cheese
	FQ52	MAP salad		M244	Pink mozzarella cheese
	C15	AP carrots		M260	Blue mozzarella cheese
	C58	AP carrots		M261	Blue mozzarella cheese
	C61	AP carrots		M266	Blue mozzarella cheese
	C126	AP carrots		CPM	Mozzarella cheese
	C131	AP carrots	Meat products isolates	1B2	Beef hamburger
Mik isolates	LA1	Raw milk		2B1	Beef hamburger
	LA2	Raw milk		2B4	Beef hamburger
	LA3	Raw milk		5B1	Beef hamburger
	LA4	Raw milk		1D2	Beef hamburger
	LA6	Raw milk		3D5	Beef hamburger
	LA7	Pasteurized milk		8A	Speck
	LA8	Pasteurized milk		8B	Speck
	LA9	Raw milk		8C	Speck
	LA10	Pasteurized milk		8D	Speck

6.2.2 Phenotypic characterization of the strains

Each isolate was observed by optical microscope for its morphology, motility (480X), and Gram stain (1200X). Catalase and oxidase tests, growth in Tryptic Soy Broth at 4 and 10°C for ten days and 37, 40 and 45 °C for seven days, and oxidative/fermentative metabolism (OF test, Hugh *et al.*, 1953) were used for preliminary biochemical characterization. The production of fluorescent pigments on King B media (King *et al.*, 1954) and diffusible pigments on Triptic Soy Agar (TSA, VWR International), Pigment Producing Media (PPMD) (Puopolo, 2006) and Agar Mascarpone (500g mascarpone, 5g/l yeast extract, 500ml water, 15g/l agar) were evaluated. The following enzymatic activities were tested: lipase activity (Sierra, 1957), pectinolytic activity (Sands *et al.*, 1972), starch hydrolysis (Stanier *et al.*, 1966), lecithinase activity (Nutrient Agar plates plus 5% v/v egg-yolk emulsion) and proteolytic activity (Nutrient Agar plates containing 10% w/v skim milk powder). According to Scarpellini *et al.* (2004), the production of levans (fructose polymers) was verified.

6.2.3 Molecular characterization and identification of the strains

Total DNA of each strain was extracted as described by Mora *et al.* (2000). If not specified otherwise below, the different PCR assays were performed in a mixture containing approximately 50ng of bacterial genomic DNA, 5µl of 10X PCR reaction buffer (Amersham-Pharmacia), 200µM of each dNTP, 2mM of $MgCl_2$, each primer (Table 6.2) at a concentration of 0.5µM and 0.5U of Taq Polymerase (Amersham-Pharmacia). After amplification, 8µl of each sample were subjected to gel electrophoresis on 2% Seakem LE agarose gel in 0.5X TAE buffer, at 100V for 30 min, and the banding pattern was visualized under ultraviolet light after 15min in Ethidium Bromide (2µg ml^{-1}).

In the case of BOX-PCR, the amplification was conducted in a mixture containing 50ng of DNA solution, 5µl of 5x Gitschier – buffer (83mM $(NH_4)_2SO_4$, 335mM Tris-HCl pH 8.8, 33.5mM $MgCl_2$, 33.5µM EDTA and 150mM ß-mercapto-ethanol), 32mM of each dNTP, 0.4µl of BSA (Roche, 20mg/ml), 2.5µl DMSO (Sigma, 100%), BOX-A1R primer at a concentration of 1µM and 1.5U of Red Diamond Taq (Eurogentec). The thermal program was 7 min at 95°C; 30 cycles of 94°C for 1min, 53°C for 1min, 65°C for 8min; final extension at 65°C for 16min. Four µl of each amplicon was subjected to agarose (1.5% Seakem LE agarose) gel electrophoresis (120V for 4hours at room temperature) in 1XTBE buffer and the banding pattern visualized after EtBr staining (30 min). The resulting fingerprintings were analyzed by BioNumerics 6.6 software package (Applied Maths Inc). Similarities were calculated using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and Pearson correlation.

Allocation of the isolates at the genus level. In order to confirm the allocation of the isolates to the genus *Pseudomonas*, a *Pseudomonas* specific 16S rRNA gene fragment (Calisti, 2008) was amplified with the following thermal program: 2 min at 94°C; 35 cycles of 95°C for 10sec, 62,4°C for 30sec, 72°C for 30sec; final extension at 72°C for 7min.

16S rRNA gene analysis. Amplification of a nearly complete 16S rRNA gene was realized with universal primers (Lane et al., 1985) and the following thermal program: 2 min at 94°C; 5 cycles of 94°C for 45sec, 55°C for 1min, 72°C for 2min; 35 cycles of 92°C for 45sec, 60°C for 45sec, 72°C for 2min and final extension at 72°C for 2min. The obtained amplicons were sequenced with 16S forward primer using a 310 automatic DNA Sequencer (Applied Biosystem, Foster City, CA, USA) and the sequences were elaborated by Chromas 2.13 software (Technelysium Pty Ltd. Helensvale, Queensland, Australia).

RpoB analysis. For *rpoB* amplification, the following thermal profile was used: 1.5min at 94°C, 32 cycles of 94°C for 10sec, 50°C for 20sec, 72°C for 50sec and final extension at 72°C for 5min. Sequencing was performed using a ABI 3730 XL DNA Sequencer (Applied Biosystem, Foster City, CA, USA) and the sequences were elaborated by Kodon 3.6 software (Applied Maths, Sint-Martens-Latem, Belgium).

GyrB analysis. For *gyrB* amplification the following program was used: 2 min at 94°C; 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1min and final extension of 72°C for 7 min. The obtained amplicons were sequenced using a 310 automatic DNA Sequencer (Applied Biosystem, Foster City, CA, USA) and Chromas 2.13 software (Technelysium Pty Ltd. Helensvale, Queensland, Australia) was used to analyse the sequences.

RFLP analysis of the ITS1 rDNA and of the 16S rDNA region *(Scarpellini et al., 2004).* The RFLP approach was used to allocate the putative *Pseudomonas fluorescens* strains at the biovar/biotype level. The *16S-ITS-23S region* (ITS1) amplification was performed using the following thermal program: 5 min at 94°C; 30 cycles of 94°C for 1min, 55°C for 1min, 72°C for 2min; final extension of 72°C for 2 min.

Restriction digestion of ITS1 amplicons was carried out for 16 hours at 65 °C in 25µl reaction mixture containing 15µl template, 2.5µl of 10X restriction digestion buffer, 18.75U of *Taq*I (Amersham Pharmacia Biotech). The restriction digestions were then analysed by agarose gel electrophoresis (3% w/v) (Guasp *et al*., 2000) and the results were interpreted using Quantity One 4.6 software package (Bio-Rad Laboratories, Milan, Italy).

The 16S rRNA part for restriction analysis was amplified using universal primers (Table 2) and following the above described protocol for 16S rRNA gene analysis.

Restriction digestion of 16S rDNA was carried out for 16 hours at 30°C in 25µl reaction mixture containing 15µl of template, 2.5µl of 10X restriction digestion buffer, 18.75U of restriction enzymes *Hae*III or *Vsp*I (Amersham Pharmacia Biotech) and the results were interpreted using Quantity One 4.6 software package (Bio-Rad Laboratories, Milan, Italy).

aprX gene analysis. The amplification of the *aprX* gene of *Pseudomonas* spp. was performed using the SM2F and SM3R primers (Table 2) and following thermal program: 3 min at 94°C, 30 cycles of 95°C for 15s, 60°C for 15s; 72°C for 30sec, final extension at 72°C for 8min. Sequencing was performed using a ABI 3730 XL DNA Sequencer (Applied Biosystem, Foster City, CA, USA) and the sequences were elaborated by Kodon 3.6 software (Applied Maths, Sint-Martens-Latem, Belgium). The EMBL database was used to assess the most similar sequences. These database sequences were then aligned with the new generated sequences using *ClustalW*. A phylogenetic tree was constructed using MEGA 5.1 software, applying the neighbour joining algorithm without corrections. Tree statistical evaluation was performed by bootstrap analysis with 1000 replications and the rooted tree was created with *Pseudomonas aeruginosa* PAO1 as outgroup.

Putative identification based on molecular data. Sequence data of type strains were either retrieved from NCBI sequence database or from the in house LMG database for 16S rRNA, *gyrB* and *rpoB* genes. They were used as frame for putative identification of the *Pseudomonas* isolates. For 16S rRNA and *gyrB* gene, pairwise comparison was conduced using BLAST and FASTA programs respectively. For *rpoB*, the putative identifications were obtained via comparative sequence analysis based on UPGMA program generated similarity matrix and clustering; the cut-off levels for species delineation were arbitrary chosen on the basis of the smallest dissimilarities observed between an isolate and two type strains of different closely related species.

6.2.4 Quantification of proteolytic activity in milk

Firstly each frozen isolate was recovered in Brain Heart Infusion broth (BHI) (OXOID) (room temperature until visible growth), then 100µl of this overnight culture in BHI were inoculated in 10ml of commercial UHT milk and incubated overnight at room temperature. 100µl of the overnight grown UHT milk culture was transferred to10 ml fresh UHT milk and incubated again over night at room temperature. From this second UHT culture (this step was included to permit the isolates to adapt to milk medium), 100µl of the grown cultures were added to 10ml fresh UHT milk to a final concentration of about 10^3cfu/ml and stored at 5°C for 5 days. After 20 min centrifugation (Eppendorf centrifuge 5810-R) at 6000g, bronopol (Merck, Schushardt, Germany) and sodium azide (Merck, Darmstadt, Germany) were added to the supernatans up to a concentration of 0.025% and 0.01 % respectively, to prevent further bacterial growth.
Then, 1ml of the supernatant was added to 9ml of fresh UHT milk in duplo. One tube was frozen at -24°C (T_0) and the other was incubated at 37°C for 14 days.
The quantification of the proteolytic activity is based on the determination of the α-amino groups reacting with the TNBS reagent (Sigma-Aldrich) at pH 9.2 in the dark. The intensity of the developed yellow colour is measured by the spectrophotometric absorbance at 420nm (Biotek instruments, Uvikon XL). As described by Marchand *et al.* (2009a) the proteolytic activity was calculated as the difference between the free amino groups at the T_0 and after 14 days expressed as µmol of glycine equivalent ml^{-1} milk.

TABLE 6.2. Utilized primers.

Primer	Sequence (5'→3')	Amplification portion	Reference
BOX A1R	CTACGGCAAGGCGACGCTGACG	Repetitive extragenic palindromic (BOX-PCR)	Rademaker *et al.*, 2000
P94F	CGGACGGGTGAGTAATGCCTAG	16S gene signature for *Pseudomonas* spp.	Calisti, 2008
P649R	CAGGAAATTCCACCACCCTCTACC		
16S945	GGGCCCGCACAAGCGGTGG	16S-ITS-23S region (ITS1)	Guasp *et al.*, 2000
23R458	CTTTCCCTCACGGTAC		
16SF	AGAGTTTGATCCTGGCTCAG	16S gene (Universal primers)	Lane *et al.*, 1985
16SR	CTACGGCTACCTTGTTACGA		
LAPS	TGGCCGAGAACCAGTTCCGCGT	*rpoB* gene	Ait Tayeb et al., 2005
LAPS27	CGGCTTCGTCCAGCTTGTTCAG		
UP1S	GAAGTCATCATGACCGTTCTGCA	*gyrB* gene	Yamamoto *et al.*, 1995
UP2R	AGCAGGGTACGGATGTGCGAGCC		
SM2F	AAATCGATAGCTTCAGCCAT	*aprX* gene of *Pseudomonas* spp.	Marchand *et al*., 2009b
SM3R	TTGAGGTTGATCTTCTGGTT		

6.3 RESULTS

6.3.1 Phenotypic classification of the strains

The results of the phenotypic investigation are compiled in Table 1. The growth of the isolates was temperature dependent. All isolates grew at 4 and 10°C; whereas at 40°C growth was observed for only 80% of the meat and dairy products isolates, 70% of the isolates from vegetables and 12% of the milk isolates, respectively. Raising the incubation temperature to 45°C resulted in growth of 40% of the isolates overall.
The major part of the isolates (71%) produced fluorescent pigments, especially the vegetables (92%) and cheese isolates (83%). However, the production of diffusible pigments was medium dependent. On TSA, the pigment producers (about 55% of the total) exhibited different colours (brown, yellow, green, orange and pink). On PPMD around 50% of the pigment producers on TSA appeared colourless and others showed less intense pigment production. On Mascarpone agar, a blue pigment appeared but all producers were isolated from milk and dairy products (26% and 44% of the respective isolates). The pectinolytic activity, known as responsible of vegetables browning (Membré *et al.*, 1994), was demonstrated for more than 70% of the vegetables isolates and for around 65% of the meat isolates. Amylolytic activity was less frequently present and was demonstrated for 33% of the vegetables isolates and 10% of the meat isolates, respectively.
Around 40% of the isolates overall showed lipolytic activity, whereas the lecithinase hydrolysis was found especially in vegetables (67%) and milk - dairy products (72%) isolates.
The extracellular protease activity occurred amongst the isolates from every matrix. At 5°C, 50% of the vegetables isolates, 48% of the dairy products isolates, 20% of the milk isolates and 33% of the meat isolates were positive, while at 25°C protease production was observed for 58% of the vegetables, 65% of the dairy products, 70% of the milk and 10% of the meat isolates.

6.3.2 Molecular classification and identification

All isolates showed the specific 16S rDNA amplicon of 550bp with the primers P94F-P649R, confirming their assignment at the genus level (data not shown).
In Table 6.3 identifications are given for each isolate based on comparative analysis of the sequences of the housekeeping genes as well as the biotype and biovar differentiation based on RFLP of 16S rDNA and ITS1.
For the decision on the species allocation, the putative identification was established combining the single gene based identification with the RFLP results; when this combination produced indecisive identification we indicated the isolates as *Pseudomonas* sp.
Among the isolates from milk, 45% was identified as *Pseudomonas fluorescens*, principally biotype C, and about 20% was considered *Pseudomonas* sp. Similar indications were found for the isolates from mozzarella: *P. fluorescens* appeared the dominant specie (44%), especially biotype C, followed by *P. fragi* (17%), and a large number of the isolates was indicated as *Pseudomonas* sp. (around 30%). The numerically more important species found in meat were *P. fragi* and *P. putida* (50% and 40% respectively). More biodiversity was recognized in vegetables isolates: *P. fluorescens* (17%) and *P. marginalis* (13%) were surrounded by various species in very low percentage (*P. cichorii*, *P. fragi*, *P. jessenii*, *P. putida*, *P. veronii*, *P. viridiflava*, *P. grimmonti*).

In the BOX analysis, BOX patterns of previous work (Marchand *et al.* 2009b) were added for comparison. The obtained dendrogram (Figure 6.1) shows the existence of high genetic diversity among the isolates and at a delineation level of 60% similarity, 16 clusters were observed. In particular seven clusters grouped isolates with different putative identifications (clusters 1, 3, 4, 7, 11, 14 and 16); the others instead were characterized by grouping the isolates with a similar putative identification: cluster 2 grouped *P. fluorescens* Biotype C biovar III, cluster 5 grouped *P. fluorescens* biotype C, cluster 6 grouped *P. fluorescens* biotype C biovar III, cluster 8 grouped *P. cichorii*, cluster 9 grouped *P. veronii*, cluster 10 grouped *P. grimmonti*, cluster 12 grouped *P. rhodesiae*, and cluster 13 and 15 both grouped *P. fragi*.

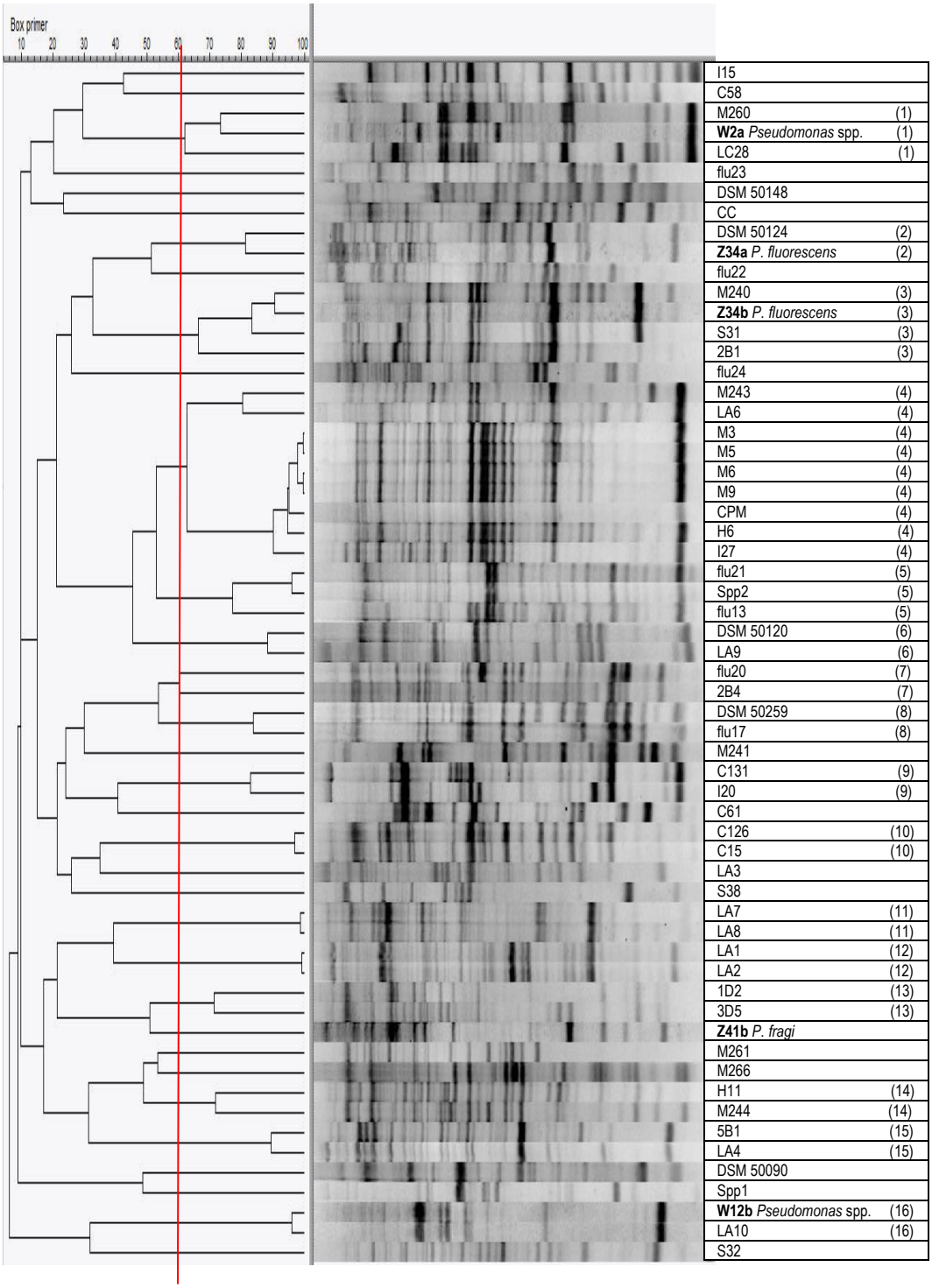
TABLE 6.3. 16S, *rpoB* and *gyrB* genes sequence based identifications and biotype/biovar analysis. The isolates are grouped for food matrix and the numbers in parenthesis refer to the clusters based on BOX-PCR. ND: Non-determined.

Isolate and BOX-PCR cluster (where applicable)			16S sequence based identification	*rpoB* sequence based identification	*gyrB* sequence based identification	Putative identification	Biotype / biovar (where applicable)
Ready-to-eat vegetable isolates	cic1		*P. cichorii*	*P. cichorii*	*P. cichorii*	*P. cichorii*	ND
	flu13	(5)	*P. fluorescens*	Close to *P. gessardii*	*P. fluorescens*	*P. fluorescens*	Biotype C
	flu17	(8)	*P. fluorescens*	Close to *P. cichorii*	*P. cichorii*	*P. cichorii*	ND
	flu20	(7)	*P. fluorescens*	*P. marginalis*	*P. marginalis*	*P. marginalis*	ND
	flu21	(5)	*P. fluorescens*	*Pseudomonas* sp.	*P. fluorescens*	*P. fluorescens*	Biotype C
	flu22		*P. fluorescens*	Close to *P. proteolytica*	*P. fluorescens*	*Pseudomonas* sp.	ND
	flu23		*P. fluorescens*	Close to *P. mediterranea*	*P. fluorescens*	*Pseudomonas* sp.	ND
	flu24		*P. fluorescens*	ND	*Pseudomonas sp.*	*Pseudomonas* sp.	ND
	S2		*P.frederiksbergensis*	*Pseudomonas* sp.	*Pseudomonas* sp.	*Pseudomonas* sp.	ND
	S31	(3)	*P. synxantha*	Close to *P. gessardii*	*P. fluorescens*	*Pseudomonas* sp.	ND
	S32		*P. kilonensis*	*Pseudomonas* sp.	*Pseudomonas sp.*	*Pseudomonas* sp.	ND
	S34		*P. argentiniensis*	ND	*Pseudomonas sp.*	*Pseudomonas* sp.	ND
	S38		*P. viridiflava*	*P. viridiflava*	*P. viridiflava*	*P. viridiflava*	ND
	I15		*P. fluorescens*	Close to *P. tolaassi*	*P. fluorescens*	*P. fluorescens*	Biotype G
	I20	(9)	*P. meridiana*	*P. veronii*	*P. veronii*	*P. veronii*	ND
	I27	(4)	*P. koreensis*	Close to *P. gessardii*	*Pseudomonas* sp.	*Pseudomonas* sp.	ND
	I43		*P. fragi*	ND	*P. fragi*	*P. fragi*	ND
	LC28	(1)	*P. jessenii*	ND	*P. jessenii*	*P. jessenii*	ND
	FQ52		*P. putida*	ND	*P. putida*	*P. putida*	ND

	C15	(10)	*P. grimmonti*	*P. grimmonti*	*P. grimmonti*	*P. grimmonti*	ND
	C58		*P. fluorescens*	Close to *P. proteolytica*	*P. fluorescens*	*P. fluorescens*	Biotype G
	C61		*P. fluorescens*	*P. fluorescens*	*Pseudomonas sp.*	*P. fluorescens*	Biotype G
	C126	(10)	*P. fluorescens*	*P. grimmonti*	*P. grimmonti*	*P. grimmonti*	ND
	C131	(9)	*P. veronii*	*P. veronii*	*P. veronii*	*P. veronii*	ND
Meat products isolates	1B2		*P. fragi*	ND	*P. fragi*	*P. fragi*	ND
	2B1	(3)	*P. fluorescens*	Close to *P. gessardii*	*Pseudomonas sp.*	*Pseudomonas* sp.	ND
	2B4	(7)	*P. fragi*	*Pseudomonas* sp.	*P. fragi*	*P. fragi*	ND
	5B1	(15)	*P. fragi*	*P. fragi*	*P. fragi*	*P. fragi*	ND
	1D2	(13)	*P. fragi*	*P. fragi*	*P. fragi*	*P. fragi*	ND
	3D5	(13)	*P. fragi*	*P. fragi*	*P. fragi*	*P. fragi*	ND
	8A		*P. putida*	ND	*P. putida*	*P. putida*	ND
	8B		*P. putida*	ND	*P. putida*	*P. putida*	ND
	8C		*P. putida*	ND	*P. putida*	*P. putida*	ND
	8D		*P. putida*	ND	*P. putida*	*P. putida*	ND
Milk isolates	LA1	(12)	*P. fluorescens*	Close to *P. rhodesiae*	*P. rhodesiae*	*P. rhodesiae*	ND
	LA2	(12)	*P. fluorescens*	Close to *P. rhodesiae*	*P. rhodesiae*	*P. rhodesiae*	ND
	LA3		*P. fluorescens*	*P. proteolytica*	*P. fluorescens*	*P. fluorescens*	Biotype C bv. III
	LA4	(15)	*P. fragi*	*P. fragi*	*P. fragi*	*P. fragi*	ND
	LA6	(4)	*P. fluorescens*	Close to *P. gessardii*	*P. fluorescens*	*P. fluorescens*	Biotype C
	LA7	(11)	*P. fluorescens*	Close to *P. peli*	*P. fluorescens*	*Pseudomonas* sp.	ND
	LA8	(11)	*P. fluorescens*	Close to *P. peli*	*P. fluorescens*	*Pseudomonas* sp.	ND
	LA9	(6)	*P. fluorescens*	*P. brenneri*	*P. fluorescens*	*P. fluorescens*	Biotype C bv. III
	LA10		*P. fluorescens*	ND	*P. fluorescens*	*P. fluorescens*	Biotype G

Dairy products isolates	spp 1		*P. fragi*	Close to *P. fragi*	*P. fragi*	*P. fragi*	ND
	spp 2	(5)	*P. fluorescens*	Close to *P. fragi* or to *P. psychrophila*	*P. fluorescens*	*P. fluorescens*	Biotype C
	spp 3		*P. fragi*	ND	*P. fragi*	*P. fragi*	ND
	spp 4		*P. fragi*	ND	*P. fragi*	*P. fragi*	ND
	M9	(4)	*P. fluorescens*	Close to *P. gesardii*	*P. fluorescens*	*P. fluorescens*	Biotype C
	M5	(4)	*P. fluorescens*	Close to *P. gesardii*	*P. fluorescens*	*P. fluorescens*	Biotype C
	M3	(4)	*P. fluorescens*	Close to *P. gesardii*	*P. fluorescens*	*P. fluorescens*	Biotype C
	M6	(4)	*P. fluorescens*	Close to *P. gesardii*	*P. fluorescens*	*P. fluorescens*	Biotype C
	CC		*P. koreensis*	Close to *P. moraviensis*	*P. fluorescens*	*Pseudomonas* sp.	ND
	Lucy		*P. fragi*	ND	*P. fragi*	*P. fragi*	ND
	H1		*P. poae / fluorescens*	ND	*P. fluorescens*	*P. fluorescens*	Biotype B
	H2		*P. poae / fluorescens*	ND	*P. fluorescens*	*P. fluorescens*	Biotype B
	H6	(4)	*P. fluorescens*	Close to *P. gesardii*	*Pseudomonas sp.*	*Pseudomonas* sp.	ND
	H9		*P. putida*	ND	*P. putida*	*P. putida*	ND
	H11	(14)	*P. fluorescens*	Close to *P. proteolytica*	*P. fluorescens*	*Pseudomonas* sp.	ND
	M240	(3)	*P. fluorescens / synxantha*	Close to *P. gesardii*	*P. fluorescens*	*P. fluorescens*	Biotype C
	M241		*P. koreensis / putida*	Close to *P. koreensis*	*P. koreensis*	*P. koreensis*	ND
	M243	(4)	*P. fluorescens*	Close to *P. gessardii*	*P. fluorescens*	*P. fluorescens*	Biotype C
	M244	(14)	*fluorescens*	*P. lurida*	*P. fluorescens*	*P. fluorescens*	Biotype C
	M260	(1)	*P. fluorescens*	Close to *P. proteolytica*	*P. fluorescens*	*Pseudomonas* sp.	ND
	M261		*P. fluorescens*	Close to *P. proteolytica*	*P. fluorescens*	*Pseudomonas* sp.	ND
	M266		*P. veronii*	Close to *P. proteolytica*	*P. fluorescens*	*Pseudomonas* sp.	ND
	CPM	(4)	*P. fluorescens*	*P. gessardii*	*Pseudomonas* sp.	*Pseudomonas* sp.	ND

Figure 6.1. Dendrogram based on BOX-PCR, constructed using UPGMA and Pearson coefficient. BOX-cluster numbers are in parentheses. In bold are the comparative strains (Marchand *et al.*, 2009b).



6.3.3 Proteolytic activity quantification

According to the overall protease evaluation on skimmed milk plates more than 50% of vegetables and dairy products isolates were positive, and around 20 and 25% of milk and meat isolates, respectively. For all isolates, protease activity was quantified in milk. In our approach, the *Pseudomonas* proteases were first produced in UHT milk at 5°C (storage condition of the analysed foods). In the second step, their proteolytic activity was assessed with the TNBS method after incubation for two weeks at 37°C (optimal temperature for the enzymes). The results are shown in Table 6.4 and in Figures 6.2, 6.3 and 6.4. The strains were considered active if the value of the protease activity was higher than 2µmol glycin equivalent ml^{-1}. The values of growth and proteolytic activity reported in Table 6.4 are means of cfu values (all isolates grew in milk) and of proteolytic activities of the positive isolates, respetively. The meat isolates showed the highest (9.74±2.22µmol glycin equivalent ml^{-1}) activity, although proteolytic activity was found in only 25% of the meat isolates. All mean values of proteolytic activity showed very high standard deviations, indicating important differences for various isolates from the same food matrix. This variation can be due to the behavior of different isolates: none of the *Pseudomonas putida* isolates showed proteolytic activity; *P. veronii* had values between 3 and 6 µmol glycin equivalent ml^{-1}; when present in *P. marginalis*, the proteolytic activity ranged between 2 and 4µmol glycin equivalent ml^{-1}. *Pseudomonas cichorii* (both isolate and type strain DSM 50259^{T}) showed high activity (15 and 12µmol glycin equivalent ml^{-1} respectively) at rather low cell density (7.65 and 7.85 log cfu ml^{-1} respectively). For *P. fragi* and *P. fluorescens* we found more variability depending on the strains.

TABLE 6.4. Isolation source, % of proteolytic isolates, means growth density (log cfu ml^{-1}) and quantified proteolytic activity (µmol glycin equivalent ml^{-1}) in UHT milk.

Isolates origin	**Percentage of positive isolates**	**Growth**		**Proteolytic activity**	
		Mean	**Standard deviation**	**Mean**	**Standard deviation**
Vegetables	54%	8.06	0.34	8.23	4.49
Meat	25%	7.95	0.47	9.74	2.22
Dairy products	57%	8.67	0.80	8.93	4.18
Milk	20%	9.36	0.24	8.26	5.95

Figure 6.2. Quantification of the proteolytic activity in milk of milk and dairy products isolates.

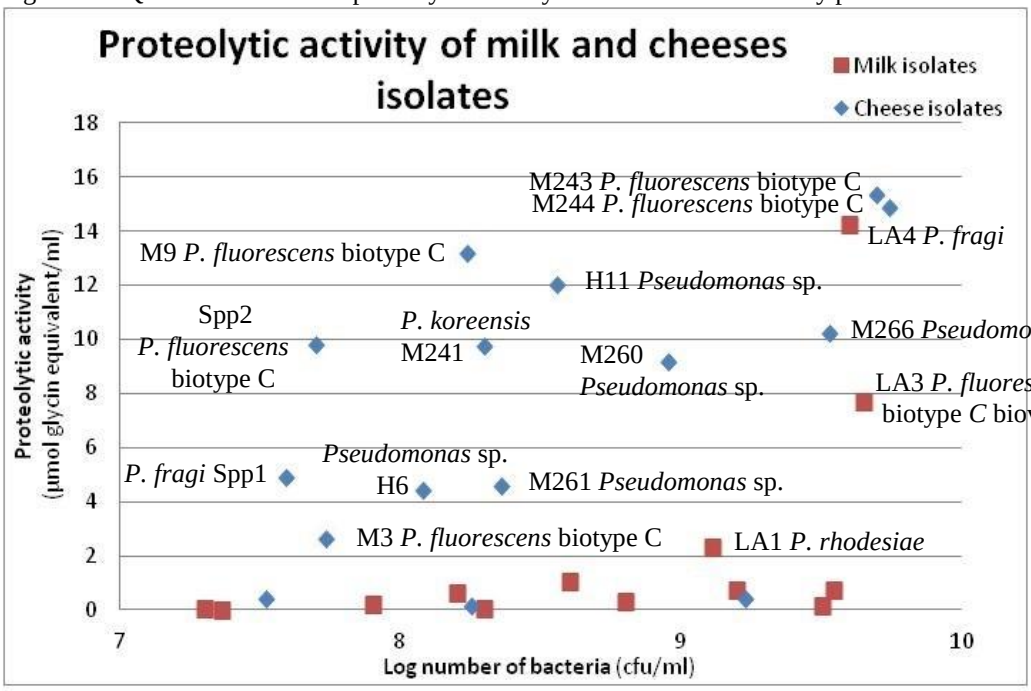


Figure 6.3. Quantification of the proteolytic activity in milk of meat isolates and reference strains.

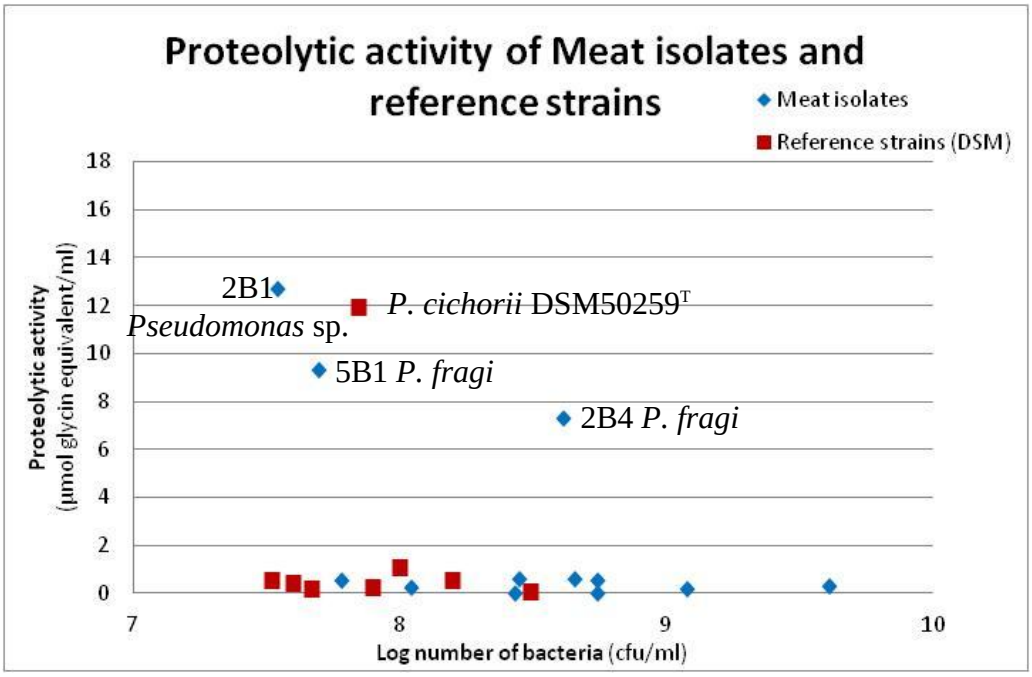
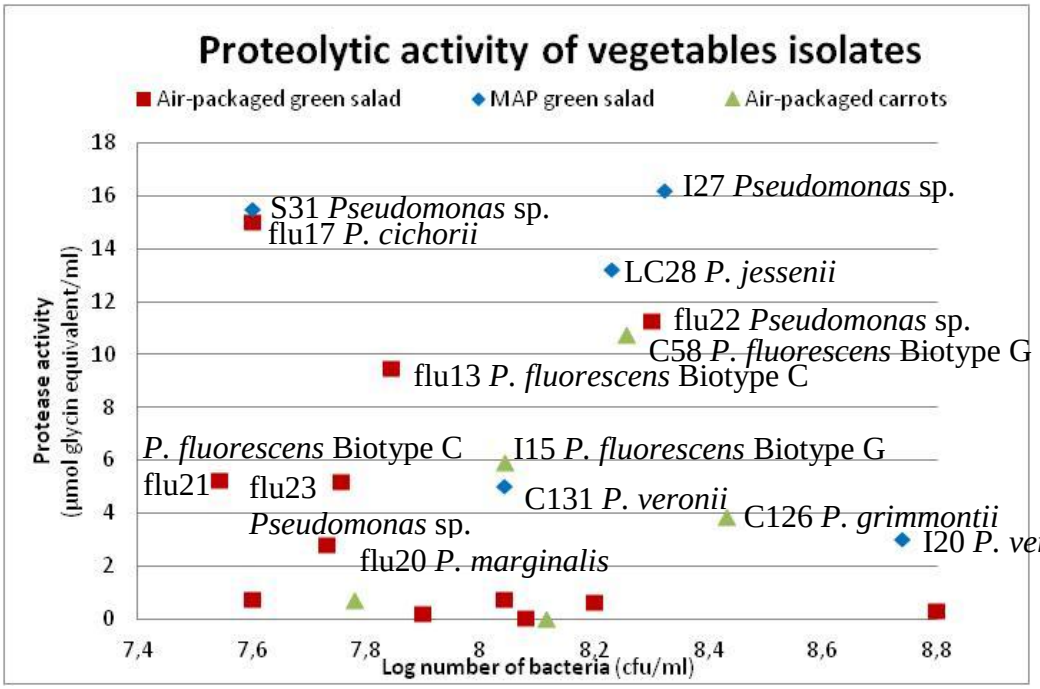


Figure 6.4. Quantification of the proteolytic activity in milk of vegetables isolates.



6.3.4 *aprX* gene analysis

All the isolates and the reference strains were subjected to the specific amplification of the *aprX* gene. Forty-seven *Pseudomonas* isolates and 4 reference strains rendered an amplicon of the expected size (±850bp), for the other twenty-three strains (19 isolates and 4 references), which did not show a protease production on plates, no amplification was observed.
The heterogeneity of *aprX* gene was shown in a comparative alignment and phylogenetic analysis of the sequences (Figures 6.5 and 6.6) in which the sequences of Marchand *et al.* (2009b) were also included.
On the basis of this analysis five groups (A, B, C, D and E) were identified supported by high bootstrap values. In group C, two sub-groups (C1 and C2) can be delineated, though not supported by high bootstrap values. It is interesting to observe that generally each group included the isolates from one specific food matrix; group A consists of milk isolates, groups B and E contains mostly mozzarella strains, while group C2 is mostly composed of vegetables isolates; group D contains only meat isolates. The only mixed group is C1 that consists of isolates from different matrices.
Observing the *aprX* sequences we noticed that all the strains of A and B groups showed an insertion of more then twenty bases, having two different sequences (figure 6.6). Interesting was to notice that all the isolates belonged to A group resulted as *P. fluorescens* biotype C biovar III. The absence of this insertion in all the *P. fluorescens* biotype C non-biovar III suggests that *aprX* gene, when present, could be a target gene to identify *P. fluorescens* biotype C biovar III, favouring its discrimination from *P. fluorescens* biotype C non-biovar III.

Fig. 6.5. *aprX*-phylogenetic analysis; values higher than 65% are given.

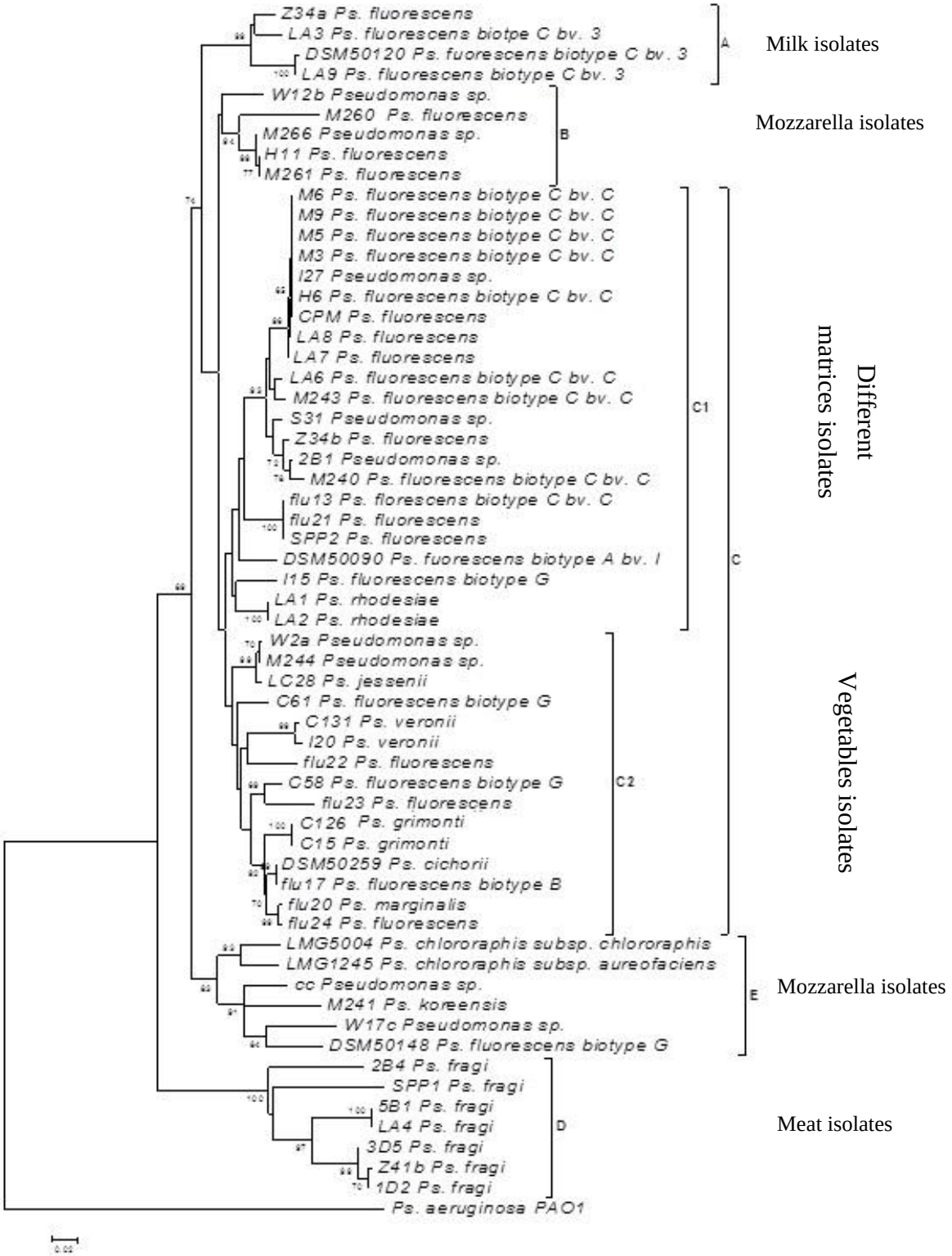
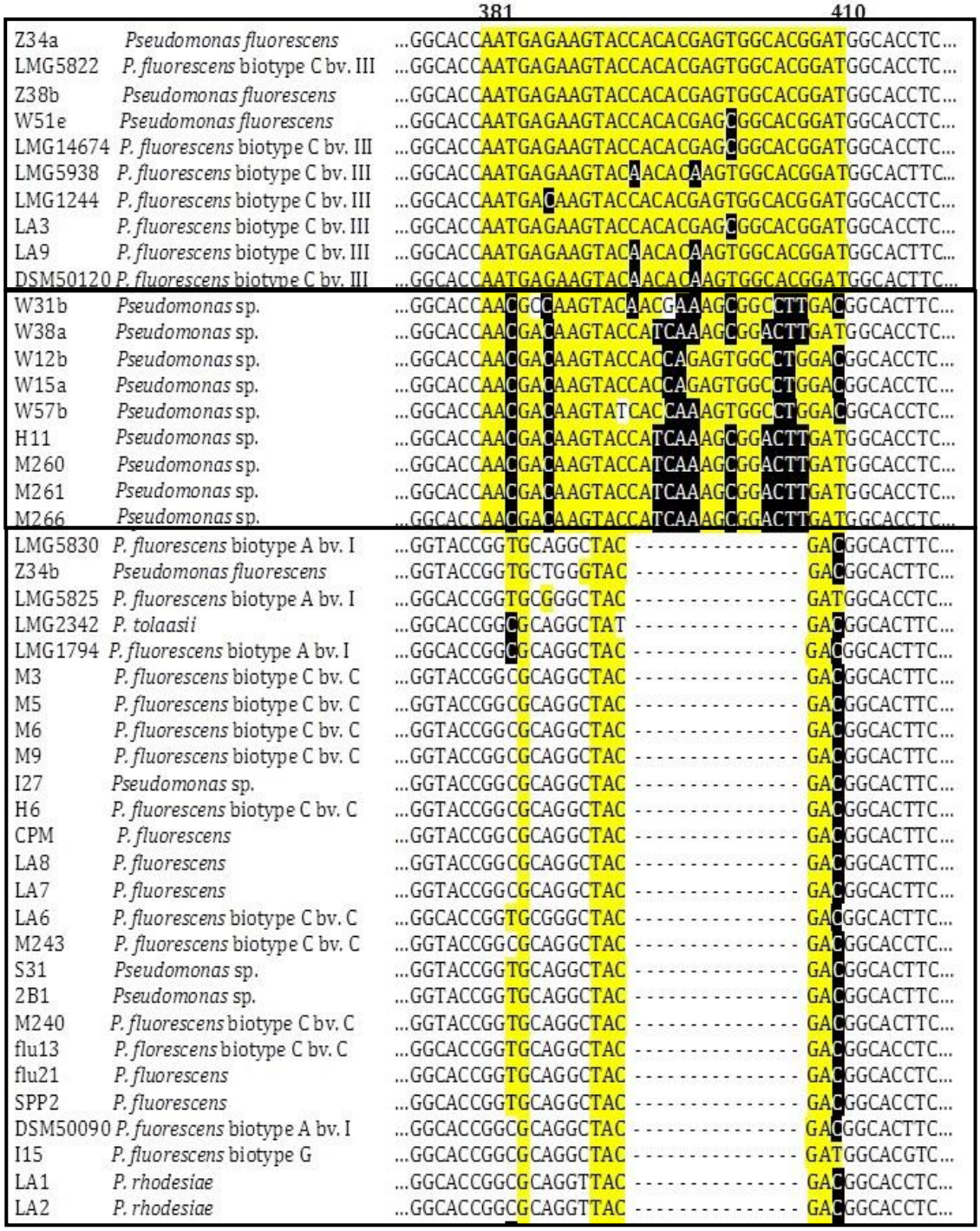
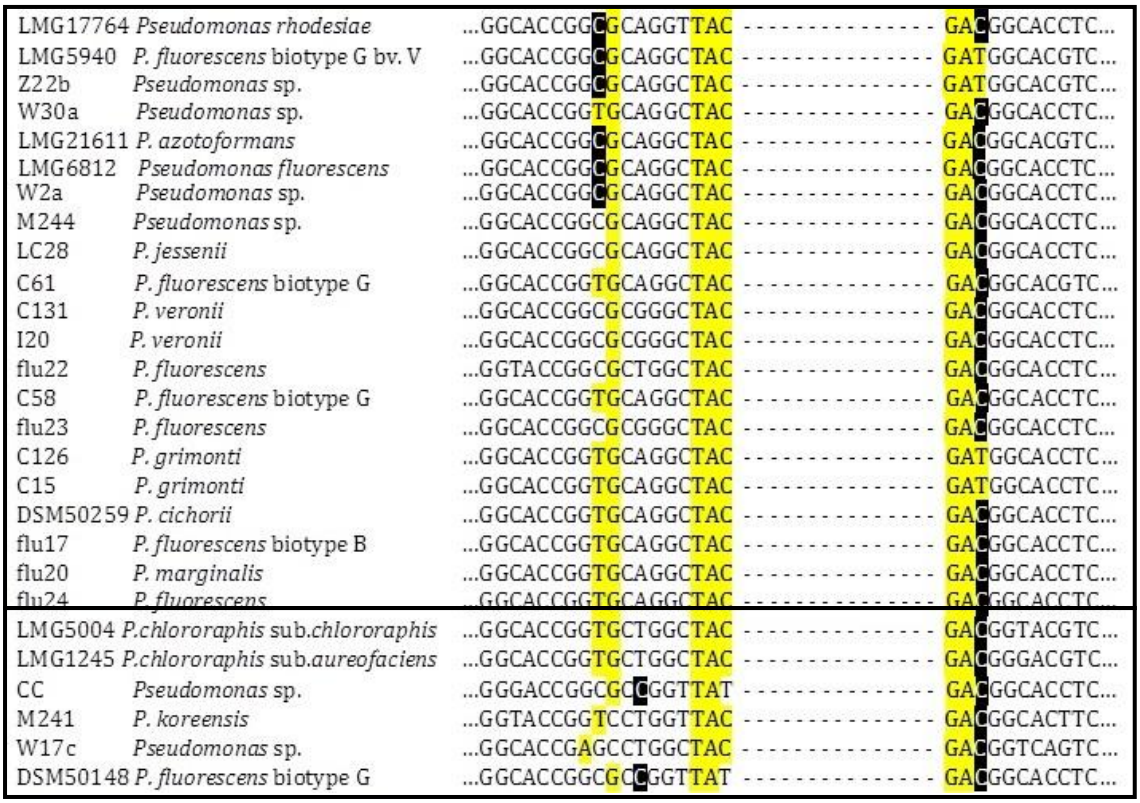


Figure 6.6. *aprX* sequences of the strains. Group A and group B showed an insertion.



Group C, sub-group C2

Group E



6.4 DISCUSSION AND CONCLUSION

The goals of this study were to identify the species level and evaluate the enzymatic spoilage activities of *Pseudomonas* spp. isolated from different food matrices (ready-to-eat vegetables, meat, milk and dairy products). *Pseudomonas* members are known to be the most common microbiota involved in spoilage of many kinds of foods, due to their very simple nutritional requirements and their metabolic versatility that allows them to thrive in various environments. In this work, the proteolytic activity was studied qualitatively as well as quantitatively in combination with the presence and heterogeneity of the *aprX* gene. The *aprX* gene codes for the most common extracellular protease of *Pseudomonas* spp.; it is a heat-resistant metallo-protease that can survive also after UHT processing, and therefore may induce the spoilage of fresh, pasteurized and sterilized products.

After a molecular confirmation of the genus level, the isolates were tested for their enzymatic activities. The results showed that all the isolates were capable of producing enzymes and pigments that can affect food products. According to the features of the isolation matrix, the isolates showed different enzymatic activities. The isolates from vegetables were characterized by pectinolytic, lecithinase and proteolytic activities, the meat isolates induced proteolysis, pectinolysis and lipolysis especially, and the dairy products isolates produced proteolytic and lecitinase enzymes.

The complex and disordered taxonomy of the *Pseudomonas* genus explains the difficulty that we found in the identification of the isolates. Only for eight strains the three genes showed the highest homology with the same species giving indubitable identification, for the major part of the isolates the sequencing of the housekeeping genes didn't always show the same results (Table 6.3).

The qualitative approach of proteolytic activity was confirmed by quantitative analysis, showing significant values of glycin equivalent ml^{-1} in UHT milk. Seeing that the food matrices of origin were mostly fresh food, not subjected to heat- treatment, we decided to quantify with this analysis the total proteolytic activity and not only the heat-resistant proteolytic activity as Marchand et al. (2009a) did. Relatively more cheese isolates had protease activity compared to the milk isolates (figure 2), and they also showed a higher activity (table 6.4). A minority (25%) of the meat isolates showed, protease activity but when present the activity was very high (9.74 ± 2.22 μmol glycin equivalent ml^{-1}). In contrast, proteolytic activity was more commonly present among the vegetables isolates.

The molecular analysis of the *aprX* gene showed five groups (A, B, C, D and E) and two subgroups (C1 and C2). In each group the strains appeared isolated from principally one food matrix.

Four groups (A, B, C and D) were the same as obtained by Marchand *et al.* (2009b) and in addition, we were able to recognize another group (E), containing principally mozzarella isolates and some outsiders of Marchand *et al.* (2009b). From comparative analysis of the *aprX* sequences it can be deduced that all isolates of A and B groups showed an insertion between around 381 and 410 bases on the sequence, having two different sequences (Figure 6.6). Groups A and D are the most homogeneous ones, containing isolates putative identified as *Pseudomonas fluorescens* biotype C biovar III and *Pseudomonas fragi*, respectively.

6.5 REFERENCES

Ait Tayeb L, Ageron E, Grimont F, Grimont PAD. 2005. Molecular phylogeny of the genus *Pseudomonas* based on *rpoB* sequences and application for the identification of isolates. Research in Microbiology, 156: 763-773.

Blevesa S, Viarrea V, Salachaa R, Michela GPF, Filloux A, Voulhouxa R. 2010. Protein secretion systems in *Pseudomonas aeruginosa*: A wealth of pathogenic weapons. International Journal of Medical Microbiology, 300: 534-543.

Calisti C. 2008. Sviluppo di metodiche molecolari per il rilevamento rapido di *Pseudomonas* in matrici alimentari e ambientali. *PhD thesis in Foods Biotechnology, Università degli studi della Tuscia, Viterbo*.

Chabeaud P, de Groot A, Bitter W, Tommassen J, Heulin T, Achouak W. 2001. Phase-variable expression of an operon encoding extracellular alkaline protease, a serine protease homolog, and lipase in *Pseudomonas brassicacearum*. Journal of Bacteriology, 183(6): 2117-2120.

Datta N, Deeth HC. 2001. Age gelation of UHT milk - a review. Food And Bioproducts Processing, 79: 197-210.

Duong F, Bonnet E, Géli V, Lazdunski A, Murgier M, Filloux A. 2001. The AprX protein of *Pseudomonas aeruginosa*: a new substrate for the Apr type I secretion system. Gene, 262: 147-153.

Franzetti L, Scarpellini M. 2007. Characterization of *Pseudomonas* spp. isolated from Foods. Annals of Microbiology, 57 (1): 39-47.

Guasp C, Moore ERB, Lalucat J, Bennasar A. 2000. Utility of internally transcribed 16S-23S DNA spacer regions for the definition of *Pseudomonas stutzeri* genomovars and other *Pseudomonas* species. International Journal of Systematic and Evolutionary Microbiology, 50: 1629 - 1639.

Hugh R, Leifson E. 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram negative bacteria. Journal of Bacteriology, 66: 24-26.

Janda JM, Abbott SL. 2007. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. Journal of Clinical Microbiology, 45(9): 2761-2764.

King EO, Ward WK, Raney DE. 1954. Two simple media for the determination of pyocyanine and fluorescine. Journal of Laboratory and Clinical Medicine, 44: 301-307.

Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML, Pace NR. 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc Natl Acad Sci USA*.82: 6955-6959.

Lelliot RA, Billing E, Hayaward AC. 1966. A determinative for the fluorescent plant pathogenic *Pseudomonas*. Journal of Applied Bacteriology, 29: 470-489.

Marchand S, Heylen K, Messens W, Coudijzer K, De Vos P, Dewettinck K, Herman L, De Block J, Heyndrickx M. 2009a. Seasonal influence on heat-resistant proteolytic capacity of *Pseudomonas lundensis* and *Pseudomonas fragi*, predominant milk spoilers isolated from Belgian raw milk samples. Environmental Microbiology, 11: 467-482.

Marchand S, Vandriesche G, Coorevits A, Coudijzer K, De Jonghe V, Dewettinck K, De Vos P, Devreese B, Heyndrickx M, De Block J. 2009b. Heterogeneity of heat-resistant proteases from milk *Pseudomonas* species. International Journal of Food Microbiology, 133: 68-77.

Mellmann A, Cloud J, Maier T, Keckevoet U, Ramminger I, Iwen P, Dunn J, Hall G, Wilson D, LaSala P, Kostrzewa M,Harmsen D. 2008. Evaluation of matrix-assisted laser desorption Ionization-Time-of-Flight mass spectrometry in comparison to 16S rRNA gene sequencing for species identification of nonfermenting bacteria. Journal of Clinical Microbiology, 36(6): 1946-1954.

Membré JM, Burlot PM. 1994. Effects of Temperature, pH, and NaCl on Growth and

Pectinolytic Activity of *Pseudomonas marginalis*. Applied and Environmental Microbiology, 60 (6): 2017-2022.

Mora D, Fortina MG, Parini C, Daffonchio D, Manachini PL. 2000.Genomic subpopulations within the species *Pediococcus acidilactici* detected by multilocus typing analysis: relationship between pediocin AcH/PA-1 producing and non- producing strains. Microbiology, 146: 2027-2038.

Mulet M, Lalucat J, Garcìa-Valdés E. 2010. DNA sequence-based analysis of the *Pseudomonas* specie. Environmental Microbiology, 12 (6): 1513-1530.

Nicodème M, Grill JP, Humbert G, Gaillard JL. 2005. Extracellular protease activity of different *Pseudomonas* strains: dependence of proteolytic activity on culture conditions. Journal of Applied Microbiology, 99: 641-648.

Okuda K, Morihara K, Atsumi Y, Takeuchi H, Kawamoto S, Kawasaki H, Suzuki K, Fukushima J. 1990. Complete nucleotide sequence of the structural gene for alkaline proteinase from *Pseuodmonas aeruginosa* IF03455. Infection and immunity, 58: 4083 – 4088.

Puopolo G. 2006. Regulation of phenazine biosynthesis in a fluorescent pseudomonad: *P. aureofaciens* 30-84. *PhD in Plant Pathology, University of Naples*.

Rademaker JLW, Hoste B, Louws FJ, Kersters K, Swings J, Vauterin L, Vauterin P, de Bruijn FJ. 2000. Comparison of AFLP and rep-PCR genomic fingerprinting with DNA–DNA homology studies: *Xanthomonas* as a model system. International Journal of Systematic and Evolutionary Microbiology, 50: 665-677.

Rees-Georgea J, Vannesteb JL, Cornishb DA, Pushparajaha IPS, Yub J, Templetona MD, Everett KR. 2010. Detection of *Pseudomonas syringae* pv. *actinidiae* using polymerase chain reaction (PCR) primers based on the 16S–23S rDNA intertranscribed spacer region and comparison with PCR primers based on other gene regions. Plant Pathology, 59: 453-464.

Sands DC, Hankin L, Zucker M. 1972. A selective medium for pectolytic fluorescent *Pseudomonas*. Phytopatology, 62: 998-1000.

Scarpellini M, Franzetti L, Galli A. 2004. Development of PCR assay to identify *Pseudomonas fluorescens*and its biotype. FEMS Microbiology Letters, 236: 257-260.

Sierra G. 1957. A simple method for the detection of lypolitic activity of microorganisms and some observations on the influence of the contact between cells and fatty substrates. Anton Van Leeuwenhoek Journal, 23: 115-122.

Stanier RY, Palleroni NJ, Doudoroff M. 1966. The aerobic pseudomonads: a taxonomic study. Journal of General Microbiology, 43: 159-271.

Woods RG, Burger M, Beven CA, Beacham, IR. 2001. The aprX–lipA operon of *Pseudomonas fluorescens* B52: a molecular analysis of metalloprotease and lipase production. Microbiology, 147: 345-354.

Yamamoto S, Harayama S. 1995. PCR amplification and direct sequencing of *gyrB* genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. Applied and Environmental Microbiology, 61: 1104-1109.

Appendix 1

APPENDIX 1. COPIES OF ABSTRACTS OF PAPERS, ORAL COMMUNICATIONS AND POSTERS

1. Caldera L, Franzetti L. 2013a. Effect of storage temperature on the microbial composition of ready-to-use vegetables. Current Microbiology, DOI 10.1007/s00284-013-0430-6.

2. Caldera L, Van Coillie E, De Vos P, Heyndrickx M, Franzetti L. 2013b. Identification and genetic heterogeneity of *Pseudomonas* spp. isolated from different foods, ABSTRACTS FEMS 2013, 5th Congress of European Microbiologists, Leipzig Germany, 21-25 luglio 2013, 2393.

3. Caldera L, Van Coillie E, De Block J, Heyndrickx M, Franzetti L. 2013c. Quantification of proteolytic activity of *Pseudomonas* spp. in milk. ABSTRACTS FEMS 2013, 5th Congress of European Microbiologists, Leipzig Germany, 21-25 luglio 2013, 2418.

4. Caldera L, Franzetti L. 2013d. Specific spoilage bacteria in different type of packaged fresh foods, Microbial Spoilers in Food 2013 - International congress July 1st to 3rd, 2013 Quimper France, ISBN: 978-2-916248-45-5, p14.

5. Caldera L. Franzetti L. 2013e. Alterazione degli alimenti e Specific Spoilage Organisms, Ascca News (Associazione per lo Studio e il Controllo della Contaminazione Ambientale), gennaio 2013.

6. Franzetti L, Caldera L, Tinelli L. 2011. Valutazione dell'attività antimicrobica di disinfettanti naturali, Igiene alimenti, disinfezione & igiene ambientale 28-3: 8-12

7. Caldera L, Franzetti L, Limbo S, Rollini M, Scarpellini M. 2011. Identificazione di microrganismi indicatori della qualità isolati da vegetali minimamente trattati diversamente confezionati. Atti del convegno 10° CISETA, Milano 9 e 10 maggio 2011.

8. Rollini M, Musatti A, Caldera L, Manzoni M. 2011. Attività antimicrobica di sostanze di origine naturale impiegabili in imballaggi plastici funzionali di vegetali minimamente trattati (IV gamma). Atti del convegno 10° CISETA, Milano 9 e 10 maggio 2011.

9. Caldera L. 2011. Research and isolation of Specific Spoilage Organisms (SSOs) from different food matrices. 16th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Lodi (Italy), 21-23 September, pp. 297-298.

10. Caldera L. 2012. Detection and characterization of specific spoilage organisms (SSOs) in different food matrices. 17th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Cesena (Italy), 19-21 September, pp. 191-192.

11. Caldera L. 2013. Specific spoilage organisms (SSOs) in different food matrices. 18th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Conegliano Veneto (Italy), 25-27 September, pp. 35-39.

Appendix 1

Curr Microbiol
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Effect of Storage Temperature on the Microbial Composition of Ready-to-Use Vegetables

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Abstract Four different salad preparations were investigated from microbiological point of view: two were packaged in air and two under Modified Atmosphere. The samples were stored at 4 and 10 °C, and analysed at established times. Total bacterial count (TBC) was taken as the most relevant index to define their hygiene and quality at both temperatures. Lactic acid bacteria, yeasts and moulds were found only occasionally. In general, the most important factor was the packaging technique: TBC was lower when the product is packed under modified conditions. The packaging technique also influences the microbial population: Gram-negative aerobic rods are dominant in air-packaged products, whilst the presence of Enterobacteriaceae becomes important in salads packaged under Modified Atmosphere. *Pseudomonas fluorescens*, with all its biovars, was the most frequently found species amongst the aerobic isolates, whilst for the Enterobacteriaceae strains, there was no dominant species.

Appendix 1

ABSTRACTS FEMS 2013, 5th Congress of European Microbiologists, Leipzig Germany, 21-25 luglio 2013.

IDENTIFICATION AND GENETIC HETEROGENEITY OF *PSEUDOMONAS* SPP. ISOLATED FROM DIFFERENT FOODS

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Background: The genus *Pseudomonas* is an heterogeneous and ecologically significant bacterial group, composed by Gram negative aerobic rods. This microbial genus is widespread throughout nature and is characterized by a high metabolic versatility. In food science *Pseudomonas* spp. have an important place seeing its active role in spoilage processes.

The taxonomy of *Pseudomonas* genus is in continuous evolution and the classification is not yet fully established. The 16S rRNA gene sequence is frequently used, but the high conservative nature of ribosomal genes makes it not suitable for species identification. Several additional (housekeeping) genes of a less conservative nature were gradually included to obtain a more discriminatory phylogenetic evaluation.

Objectives: This work was focused on the analysis of the genetic heterogeneity and on the identification of *Pseudomonas* members isolated from different food products (vegetables, meat, milk and dairy products). In particular the *aprX* gene, responsible of the production of an extracellular alkaline metallo-protease, was used to conduct a phylogenetic analysis.

Methods: The sixty-seven *Pseudomonas* strains were identified by 16S rRNA and *rpoB* gene sequencing. Genetic heterogeneity was analysed by BOX-PCR and a*prX* gene sequencing. The phylogenetic heterogeneity within the *aprX* gene was examined by comparing partial (about 750) nucleotide sequences.

Conclusions: The 16S rRNA gene sequencing didn't allow an identification at the species level.

The BOX-PCR showed a high diversity among the strains. On the basis of *aprX* gene six clusters were identified, each cluster was characterized by a predominant food isolation matrix which suggests a food matrix specific *Pseudomonas* microbiota.

Appendix 1

ABSTRACTS FEMS 2013, 5th Congress of European Microbiologists, Leipzig Germany, 21-25 luglio 2013.

QUANTIFICATION OF PROTEOLYTIC ACTIVITY OF PSEUDOMONAS SPP. IN MILK

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Background: The genus *Pseudomonas* harbours many complex enzymatic systems that allow it to grow on different substrates. It is known that different species of this genus cause spoilage of food products. The most important enzymatic spoiling mechanism is the proteolytic activity, that can alter many kinds of fresh foods. Anyway the major effect of this alteration is showed on milk; in fact since that these proteases are heat-resistant, they can affect both raw and heat processed milk products. These enzymes cause coagulation and instability phenomena in UHT milk and dairy products, respectively. However, the mechanisms of these processes are not completely known and depend on the strain, temperature and environmental conditions.

Objectives: The aims of this work were

i) evaluation of proteolytic activity at different temperatures;

ii) quantification of the extracellular proteolytic activity of *Pseudomonas* species isolated from milk and dairy products.

Methods: Thirty-five strains of *Pseudomonas* spp. isolated from milk and dairy products (raw and pasteurized milk, crescenza and mozzarella cheeses) were tested for their proteolytic activity on plates (milk agar) at 5 and 30°C. Quantification of the activity in milk at 5°C was performed using the trinitrobenzenesulphonic acid (TNBS) method.

Conclusions: The major part of the isolates showed proteolytic extracellular activity on milk agar. The mozzarella isolates appeared as the most active strains, especially at 5°C.

Quantification of the proteolytic activity was performed at 5°C. The isolates from cheeses (mozzarella and crescenza) showed higher activity than these from milk.

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Microbial Spoilers in Food 2013 - International congress July 1st to 3rd, 2013 Quimper France

Specific spoilage bacteria in different type of packed fresh foods

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Introduction: The Specific Spoilage Organism is the fraction of the microbiota responsible of the spoilage of food. Generally this fraction is composed by different taxonomic groups that, at the beginning are only a minority, then during the storage become numerically more important, growing faster then the other microorganisms, and produce the metabolites and the enzymes responsible of the spoilage. The SSO is represented by different microbial species depending on the characteristics of the food, the type of packaging and the storage conditions. In this work we studied the microbiota of different fresh foods to identify the bacterial fraction of their SSO.

Material and methods: In this study, two ready-to-use green salads packaged in air and under Modified Atmosphere respectively, ready-to-use stick carrots, mozzarella cheese and beef meat in different packaging conditions, were used. All the products were analyzed during the shelf-life with traditional plate count method, using generic and differential / selective media. The isolates were classified and identified until specie level by molecular techniques. For the *Pseudomonas* genus the molecular analysis was conduced until biovar identification, and an enzymatic characterization was made.

Results: In carrots we recognized *Pseudomonas* spp. and *Leuconostoc* spp., in particular *L. mesenteroides sub. mesenteroides* and *L. pseudomesenteroides*. This genus is responsible of exudation and losses in texture of carrots, thanks to its obligatory hetero-fermentative metabolism. The SSOs of salads were related to the internal atmosphere of the packages: the salads packaged under Modified Atmosphere were characterized by the presence of facultative aerobic and anaerobic bacteria, such as Enterobacteriaceae and Lactic Acid Bacteria, even if the dominant genus was always *Pseudomonas*, in particular *Ps. fluorescens* and other species belonged to group I, known as *Pseudomonas in sensu stricto*. All these isolates were able to produce fluorescence and diffusible pigments, causing the browning of green salad. In meat, especially packaged in low oxygen concentration, we identified as SSO *Pseudomonas* spp. (dominant specie *Ps. fragi*), while in air packaged products *Brochothrix thermosphacta* produce undesirable odors. In mozzarella *Pseudomonas* spp. was the most important spoilage organism, in particular the specie *fluorescens*, able to colonized mozzarella and create blue and yellow spots on the surface. The most part of *Pseudomonas* were characterized by proteolytic, pectinolytic and lecithinasic activities according to the isolation matrix and were mostly represented by *Ps. fluorescens* biovar G.

Significance: This work shows that each analyzed food has, depending on the storage conditions, its own spoilage bacteria characterized by different activities. We also noticed the relevance of *Pseudomonas* genus, as the most common spoilage bacteria in packed fresh foods.

Keywords: Specific Spoilage Bacteria; fresh foods; packaging conditions; *Pseudomonas* spp.

Appendix 1

Articolo Tecnico

Alterazione degli alimenti e Specific Spoilage Organisms

L'idoneità al consumo di un alimento dipende da diversi eventi di natura chimica, fisica, enzimatica ed infine, ma non per importanza, microbiologica. Sarà proprio lo sviluppo microbico quello che maggiormente influirà non solo sulla qualità ma soprattutto sulla shelf-life di un alimento. In questo articolo ci si soffermerà sull'attività di alcuni SSOs (Specific Spoilage Organisms), responsabili della degradazione degli alimenti

Parole chiave: Specific Spoilage Organisms • Alterazione microbiche • Shelf-life • Genere ***Pseudomonas*** • Prodotti caseari • Prodotti da forno • Vegetali di IV gamma • Prodotti ittici • Biofilm

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Introduzione

Gli alimenti sono composti da principi nutritivi che possono essere trasformati in energia, fungere da materiale per la formazione di tessuti biologici, catalizzare reazioni chimiche, oppure essere accumulati nell'organismo con funzione di riserva; si definisce alimento pertanto qualunque sostanza solida o liquida che, assimilata da un organismo vivente, possa svolgere funzione nutritive, energetiche, plastiche, bio-rigeneranti e protettive.

L'idoneità al consumo di un alimento è funzione di numerosi eventi di natura chimica, fisica, enzimatica ed infine, ma non per importanza, microbiologica. Sarà proprio lo sviluppo microbico quello che maggiormente influirà sulla qualità e soprattutto avrà un effetto determinante sulla conservazione ovvero shelf-life di un alimento. La velocità di deterioramento dipende dalla composizione dell'alimento, dalla tecnologia di trasformazione, dalle modalità di conservazione, dalla distribuzione e dal consumo (vedi Tabella 1).

Tabella 1 Principali reazioni coinvolte nei fenomeni di alterazione degli alimenti

Reazione	Alterazione	Conseguenze
Modificazioni fisiche	Variazione del contenuto in acqua	Disidratazione/Idratazione Fenomeni di aggregazione
Modificazioni chimiche	Ossidazione	Rancidità
	Reazioni di Maillard	Modificazioni di colore Imbrunimento
Modificazioni enzimatiche	Polifenolossidasi	Imbrunimento enzimatico
	Lipossigenasi e lipasi	Rancidità
	Proteasi	Gelatinizzazione Modificazioni di struttura e colore
	Amilasi	Cambiamenti di struttura
Modificazioni microbiche	Sviluppo di microrganismi alteranti	Perdita delle caratteristiche sensoriali e riduzione shelf-life
	Sviluppo di microrganismi patogeni	Rischi per la salute del consumatore
	Sviluppo di microrganismi protecnologici	Modificazioni positive a carico di struttura, aromi

La presenza di un microrganismo, a qualsiasi stadio della produzione dell'alimento, è sempre indice di una contaminazione, tuttavia non tutto il microbiota (insieme dei microrganismi che vive in uno stesso ambiente) presente agisce negativamente sul prodotto. Infatti rispetto ai ruoli che i microrganismi svolgono su un alimento si distinguono in:

microrganismi pro-tecnologici la loro presenza è richiesta ed è indispensabile per la produzione e maturazione dell'alimento. I batteri lattici sono i microrganismi pro-tecnologici per eccellenza, tuttavia non sono i soli, importanti funzioni svolgono anche lieviti, batteri acetici ecc. Il loro numero, inizialmente elevato, in quanto responsabili delle caratteristiche sensoriali e molto spesso anche strutturali del prodotto, con il tempo andrà riducendosi sino a scomparire;

microrganismi indicatori di qualità, o secondo la normativa europea indicatori di processo. Si tratta di microrganismi che variano in funzione dell'alimento e forniscono informazioni sulla sua qualità globale e ne influenzano la shelf-life. Il loro numero, espresso come ufc/ml o g di prodotto, deve essere molto contenuto già a livello iniziale ed aumentare il meno possibile durante la conservazione, pena lo scadimento delle proprietà sensoriali che nella pratica si traduce in una riduzione del tempo di conservazione. Tutte le tecniche di conservazione, di volta in volta messe a punto hanno la finalità di contenere e

Appendix 1

Atti del convegno 10° CISETA, Milano 9 e 10 maggio 2011

Identificazione di microrganismi indicatori della qualità isolati da vegetali minimamente trattati diversamente confezionati

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I vegetali di quarta gamma sono prodotti con incisive prestazioni di servizio (convenience foods) e marcate caratteristiche di freschezza e vengono genericamente raggruppati sotto la denominazione di “alimenti minimamente trattati”. Questi prodotti prima di essere commercializzati e consumati subiscono infatti una serie di trattamenti tecnologici di blanda intensità tali da non comprometterne le caratteristiche naturali di freschezza e al tempo stesso volte ad aumentare il loro valore e convenienza d’uso.
Numerose sono però le alterazioni che possono rapidamente comprometterne la qualità igienica e sensoriale. La loro degradazione coinvolge processi di natura chimico-fisica, biochimica e microbiologica. In questo lavoro è stata seguita la crescita microbica in sei verdure di IV gamma, tre (carote, songino e lattughino) confezionate in aria, e tre (lattuga iceberg, lattuga foglia quercia e lattuga cappuccio) in atmosfera modificata, conservate a 5°C e 10° C. L’indice microbico di maggior interesse è apparso la CBT, costituita in particolare da bastoncini Gram negativi aerobi/anaerobi facoltativi, mentre i batteri lattici e lieviti rappresentano una componente minoritaria, fatta eccezione per le carote. Tutti gli li isolati sono stati identificati fino al livello di specie, con prove tecniche biochimiche e molecolari.

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Atti del convegno 10° CISETA, Milano 9 e 10 maggio 2011

Attività antimicrobica di sostanze di origine naturale impiegabili in imballaggi plastici funzionali di vegetali minimamente trattati (IV gamma)

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La ricerca, inserita nell'ambito del progetto Vegapack, relativo alla qualità e sicurezza dei prodotti vegetali minimamente trattati (IV gamma) attraverso imballaggi plastici funzionali, ha previsto la determinazione dell'attività antimicrobica di carvacrolo e timolo, oli essenziali (EO), unitamente al LAE (etil-lauroil-arginato), solubile in acqua. L'attività antimicrobica è stata inizialmente determinata nei confronti di microrganismi di collezione. I batteri Gram positivi (*Listeria*, *Staphylococcus* e *Bacillus*) sono risultati più sensibili al LAE, con MIC pari a 12mg/L coltura, mentre i Gram negativi (*E. coli* e *Pseudomonas*) e *S. cerevisiae* hanno presentato sensibilità inferiore, con MIC da 24mg/L. Il timolo non ha presentato attività significativa, anche ad elevate concentrazioni (30g/L), mentre per il carvacrolo la MIC è risultata compresa tra 17 e 100µl /L. L'impiego dei due antimicrobici in associazione ha prodotto un effetto sinergico, con MIC dell'ordine di 12mg/L per LAE e 50µl /L per carvacrolo.

L'attività antimicrobica dei due composti è stata inoltre valutata nei confronti di microrganismi isolati da prodotti vegetali minimamente trattati, e i risultati ottenuti hanno confermato l'efficacia delle molecole allo studio. L'approccio proposto di associazione delle due attività antimicrobiche, una attiva in fase vapore e una in fase liquida, potrebbero costituire quindi un'innovazione nel settore del packaging alimentare.

Appendix 1

Valutazione dell'attività antimicrobica di disinfettanti naturali

L. Franzetti, L. Caldera, L. Tinelli

Introduzione

Le problematiche della disinfezione sono da sempre di estrema importanza in campo alimentare. Lavorare in ambienti e con prodotti puliti è fondamentale per l'ottenimento di un prodotto finito in grado di soddisfare i criteri di qualità e di sicurezza d'uso richiesti dai consumatori.

Numerose sono le sostanze chimiche ad azione disinfettante reperibili sul mercato, tuttavia a seguito dei problemi derivanti da un non sempre corretto utilizzo negli ultimi anni l'interesse si è rivolto verso disinfettanti naturali, atossici e che non lasciano residui.

Si tratta di prodotti composti interamente da ingredienti naturali, estratti di agrumi acerbi e che prevedono la combinazione di acidi organici (acido citrico) e polifenoli (bioflavonoidi). Sono biodegradabili (98%), non tossici e non corrosivi.

Il loro utilizzo, inoltre, non modifica le caratteristiche sensoriali del prodotto finale. Sono efficaci a basse concentrazioni contro un ampio range di microrganismi patogeni e non: gram positivi, gram negativi, muffe, lieviti e virus. Questo effetto antimicrobico è duraturo nel tempo e positivo anche contro i biofilm, molto difficili da eliminare altrimenti.

La presenza degli acidi organici è fondamentale in quanto facilitano la rimozione di ioni (necessaria per esempio in caso di acque dure), realizzando così un ambiente più favorevole ai bioflavonoidi, importanti e stabili composti antiossidanti. Inoltre, possiedono un'azione chelante, acidificano l'ambiente (3.5-5.0) e migliorano la shelf-life dei prodotti. I bioflavonoidi sono caratterizzati da un'elevata stabilità, sia come molecole di piccole che grandi dimensioni.

L'azione antimicrobica è dovuta, da un lato, all'ambiente acido e dall'altro all'inibizione di sistemi enzimatici in seguito alla attività ossidante o attraverso interazioni aspecifiche con le proteine di membrana. Sulla base di queste osservazioni è stata verificata l'azione antimicrobica di due disinfettanti naturali recentemente comparsi anche sul mercato italiano.

Materiali e metodi

Campioni. È stata verificata l'attività antimicrobica di due disinfettanti di nuova generazione, prodotti denominati A e B. La Tabella 1 riporta i prodotti oggetto della sperimentazione e le loro caratteristiche.

Possono essere applicati su molti alimenti, da frutta e verdura a prodotti caseari, salse, prodotti ittici e carnei, bevande a base di latte e succhi di frutta. Nello specifico il prodotto A è consigliato per frutti con la buccia, mentre B per prodotti vegetali di IV gamma.

Microrganismi test. Per le prove sono stati utilizzati microrganismi provenienti da collezioni nazionali ed internazionali:

- *Pseudomonas fluorescens* DSM50106, alterante di prodotti vegetali;

Tabella 1 - Composizione percentuale dei prodotti commerciali oggetto della sperimentazione

Prodotto	Composizione (%W/W)					
	Citrus aurantium amara extract	Acqua (demineralizzata)	Acido citrico	Acido glicolico	Poliglucoside alchilico	Glicerina
A	0.35	87.25	1.40	9.00	3.00	-
B	2.50	73.00	19.50	-	-	5.00

Tabella 2 - Condizioni di impiego dei disinfettanti utilizzati nella sperimentazione

Prodotto	Concentrazioni	Tempi di contatto
A	1%	0 5 minuti 10 minuti
	2%	
B	0.5	
	1%	
	2%	

Appendix 1

16th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Lodi (Italy), 21-23 September 2011.

Research and isolation of Specific Spoilage Organisms (SSOs) from different food matrices

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This PhD thesis research project is aimed at identify and characterize microorganisms responsible for the spoilage of food. The purpose of the work is to study the enzymatic activity of microorganisms, search for rapid methods for the quantification, assess the relationship between these microorganisms and food spoilage and study the relationships between different microbial species through the study of chemicals (autoinducers) issued by the microorganisms present in the food.

Appendix 1

17th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Cesena (Italy), 19-21 September 2012

Detection and Characterization of Specific Spoilage Organisms (SSOs) in Different Food Matrices

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The aim of this PhD thesis is to study the microorganisms responsible of foods spoilage. The work was carried out following the points indicated last year (Caldera, 2011). Here are presented the first three points of the research. Firstly, the microbiological quality of different food matrices was investigated and the SSOs of each product were isolated, characterized and identified until specie level. Then the total DNA and RNA of some foods were extracted and the SSOs were quantified with molecular techniques. After that, the gene coding for a spoilage enzyme was detected directly in foods.

Appendix 1

18th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Conegliano Veneto (Italy), 25-27 September 2013

Specific spoilage organisms (SSOs) in different food matrices

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This PhD thesis dealt with the study of the main microorganisms responsible of foods spoilage. For each food, the microbial quality was verified and the SSOs were isolated. The phenotypical and genotypical classification of the isolates was studied: the strains were identified until specie, biovar and biotype level and the most interesting enzymatic activities (in particular the proteolytic one) were analyzed with qualitative and/or quantitative methods. The concentration of the SSOs into the foods was quantified with molecular techniques (qPCR and RT-PCR) and the gene aprX, coding for a spoilage enzyme, was detected directly in foods.