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Bacterial enzymatic activities as potential markers for assessing the technological properties of (un)processed milk

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To my parents and
my grandmother Pina

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ABSTRACT

Psychrotrophic bacteria are responsible for the highest spoilage of unprocessed or heated milk during storage because of their capacity to synthesize thermostable extracellular proteases and lipases. The activities of these enzymes lead to formation of off-odours/flavours, gelation of milk, lowering of milk foaming properties, loss of sensory quality and shortening of the shelf life. To date, still little is known about the specific proteolytic and lipolytic pathways of these thermostable enzymes.

Initially we evaluated the enzymatic traits of 80 raw milk-associated psychrotrophic strains. Among psychrotrophic isolates, *Pseudomonas* were the most commonly occurring contaminants (78.75%) being *Pseudomonas fluorescens* the predominant isolated species (30.16 %), along with Enterobacteriaceae (21.25%), primarily *Serratia marcescens* (52.94 %). Forty-one of the psychrotrophic strains were positive for all the enzymatic activities. The highest number of positive strains for all incubation temperatures was found for the lipolytic activity (59), followed by proteolytic (31) and lecithinase (28) activities. The enzymatic traits varied among the *Pseudomonas* and Enterobacteriaceae strains and were markedly influenced by incubation temperature being 30 °C the optimal one. The *apr*X gene was detected in 19 out of 80 psychrotrohic strains and it resulted widespread among *P. fluorescens* strains (15 out of 18).

The second part of the research was focused on the evaluation of spoilage potential of psychrotrophic strains by analyzing the production of volatile organic compounds (VOCs) and the release of free fatty acids (FFAs). From results of SPME-GC/MS analysis, different species of the genus *Pseudomonas* and *Serratia marcescens* produced a complex and strain-dependent VOCs profiles in UHT milk samples at different storage and time conditions. Fifty-six VOCs belonging to 7 chemical groups (aldehydes, ketones, fatty acids, esters, alcohols, sulphur compounds and hydrocarbons) were identified. Generally, the VOCs went to increase during the storage time both in the control and contaminated milk samples, some compounds being detected only in the latter samples. Compounds such as 3-methylbutan-2-ol, 3-methylhexan-2-ol, pentan-1-ol and 3,3-dimethylhexane were detectable only for *P. fragi*. *P. rhodesiae* was the only species producing pentane-2,3-dione, heptane and 3-methylhexane while hexane was released only by *P. fluorescens*. *P. mosselii* and *P. fragi* produced the highest number of sulphur compounds and alcohols, respectively. The highest number of FFAs and ketons was detected in the headspace of milk samples contaminated by *P. rhodesiae* and *S. marcescens*. *P. fluorescens* provided the lowest development of VOCs. 3-methylbutan-1-ol, 2 methylpropan-1-ol, 3-hydroxybutan-2-one, butane-2,3-dione and butanoic and hexanoic acids could be regarded as potential markers of psycrotrophic contamination useful for the early detection of milk bacterial spoilage. Regarding the release of FFAs, different quantities of these compounds have been released from milk fat by tested bacteria, between and within species, in relation to diverse capacity for production of lipolytic enzymes. Palmitic (16:0), oleic (18:1) and linoleic (18:2) acids levels were found to be the highest among the SFAs, MUFAs and PUFAs, respectively. *P. fluorescens* PS73 and *P. fluorescens* PS81 were the major FFAs producers, at 24 h and 4 days of incubation, respectively while *H. alvei* PS57 and *P. fragi* PS55 were the less active in lipid breakdown at both the incubation conditions.
Lipases from psychrotrophic strains showed a variable range of specificity toward fatty acid esters with different fatty acid chain lengths, being *P. fragi* PS55, *P. putida* PS17, *P. fluorescens* PS14 and *P. fulva* PS10 the more active to hydrolyse triglycerides. Lipase from *P. rhodesiae* PS62 showed the highest hydrolytic resistance toward all tested fatty acid triglycerides.

Finally, proteomic characterization of extracellular proteases of *P. fluorescens* strains has been performed. One thermostable protease of approximately 45 kDa was detected in each of the cell-free supernatant of the selected strains on a casein zymogram gel. After concentration by ultrafiltration (10 kDa), the protease extract of *P. fluorescens* PS19 showed a high proteolytic activity and two additional proteolytic bands with molecular masses of approximately 15 and 25 kDa on casein zymography. This extract was subjected to proteomic characterization by nLC/MS/MS analysis of both *in gel* and *in solution* digestion. Results showed the protease of 45 kDa to correspond to *P. fluorescens* AprX metalloprotease (acc. no. C9WKP6, UniProt). In addition, the same results leaded to recognize the 15 kDa protease as a fragment of this AprX metalloprotease. On the contrary, the 25 kDa protease showed no homology to any known protein of *Pseudomonas* spp.
The characterization by LC/MS of the peptides profile generated by the action of thermostable proteases of the same strain on milk caseins is still under investigation.
Overall, this study provides a better understanding of the enzymatic activities of psychrotrophic bacteria in milk.

RIASSUNTO

I batteri psicrotrofi sono i principali responsabili del deterioramento del latte crudo o termotrattato poiché sono in grado di sintetizzare proteasi e lipasi extracellulari termostabili, causa di formazione di odori e sapori sgradevoli, fenomeni di gelificazione, riduzione delle proprietà schiumogene del latte, perdita di qualità sensoriale e riduzione della *shelf-life*. Ad oggi, non esiste ancora una sufficiente conoscenza degli specifici *pathways* proteolitici e lipolitici di questi enzimi termostabili.

Inizialmente questo lavoro ha riguardato la caratterizzazione dell'attività enzimatica di 80 ceppi di psicrotrofi isolati da latte crudo. I batteri appartenenti al genere *Pseudomonas* sono stati i più isolati (78.75%) e *Pseudomonas fluorescens* è risultata la specie predominante (30.16%); tra le Enterobacteriaceae (21.25%), *Serratia marcescens* è stata la specie più frequentemente isolata (52.94%). Quarantuno ceppi di psicrotrofi mostravano tutte le attività enzimatiche. Il più alto numero di ceppi positivi a tutte le temperature di incubazione è stato osservato per l'attività lipolitica (59) e, a seguire, proteolitica (31) e lecitinasica; i tratti enzimatici variavano tra i ceppi di *Pseudomonas* e Enterobacteriaceae ed erano marcatamente influenzati dalla temperature di incubazione, essendo 30 °C quella ottimale. Il gene *apr*X è stato ritrovato in 19 ceppi di *Pseudomonas* ed è risultato esser diffuso tra i ceppi di *P. fluorescens* (15 su18).

La seconda parte della ricerca è stata focalizzata sulla valutazione della produzione di composti organici volatili (VOCs) e del rilascio di acidi grassi liberi (FFAs) da ceppi di batteri psicrotrofi. Diverse specie del genere *Pseudomonas* e il ceppo *Serratia marcescens* hanno mostrato profili di VOCs complessi e dipendenti dal ceppo batterico inoculato in campioni di latte UHT, durante le differenti condizioni di incubazione. In particolare, sono stati identificati 56 VOCs appartenenti a aldeidi, chetoni, acidi grassi, alcoli, composti solforati e idrocarburi. Generalmente, il numero di VOCs tendeva ad incrementare con il progredire del tempo di incubazione, sia nel latte controllo non inoculato sia nei campioni di latte contaminati. Tra i VOCs rilevati, alcune molecole sono state individuate solo quando il latte era contaminato da uno specifico ceppo microbico. Nel dettaglio, 3-metilbutan-2-olo, 3-metilesan-2-olo, pentan-1-olo e 3,3-dimetilesano sono stati rilevati solo a seguito dello sviluppo di *P. fragi*. *P. rhodesiae* è stata l'unica specie a produrre pentano-2,3-dione, eptano e 3-metilesano, mentre l'esano è stato rilasciato solo in campioni di latte contaminati con *P. fluorescens*. La maggior produzione di composti solforati e alcoli è stata individuata nello spazio di testa del latte contaminato con *P. mosselii* e *P. fragi*, rispettivamente. Lo sviluppo e l'attività di *P. rhodesiae* e *S. marcescens* sono risultati associati ad un più alto numero di acidi grassi e chetoni mentre *P. fluorescens* ha mostrato la più scarsa produzione di composti volatili. Alcuni VOCs come 3-metilbutan-1-olo, 2-metilpropan-1-olo, 3-idrossibutan-2-one, butano-2,3-dione, acido butanoico ed acido esanoico potrebbero perciò rappresentare potenziali *marker* per il riconoscimento dell'attività enzimatica di batteri psicrotrofi e per la precoce individuazione del deterioramento del prodotto.
Per quanto riguarda il rilascio di FFAs, diverse quantità di questi composti sono state rilasciate dai batteri psicrotrofi appartenenti a specie diverse e alla stessa specie conseguentemente alla diversa capacità di produrre lipasi. Gli acidi palmitico (16:0), oleico (18:1) e linoleico (18:2) sono risultati i più presenti tra gli acidi grassi saturi, monoinsaturi e polinsaturi. *P. fluorescens* PS73 e *P. fluorescens* PS81 sono state le specie che hanno prodotto la maggior quantità di FFAs, a 24 h e 4 giorni di incubazione, rispettivamente. Al contrario, *H. alvei* PS57 e *P. fragi* PS55 hanno rilasciato la minor quantità di FFAs ad entrambi i tempi di incubazione.
Le lipasi dei ceppi di psicrotrofi hanno mostrato una specificità variabile nei riguardi degli esteri degli acidi grassi con diversa lunghezza della catena carboniosa. *P. fragi* PS55, *P. putida* PS17, *P. fluorescens* PS14 and *P. fulva* PS10 sono risultate le specie più attive nell'idrolisi dei

trigliceridi. La lipasi del ceppo di *P. rhodesiae* PS62 ha mostrato la più scarsa capacità idrolitica verso tutti i trigliceridi testati.

Infine, è stata effettuata la caratterizzazione proteomica di proteasi extracellulari di ceppi di *P. fluorescens*. Una proteasi termostabile di circa 45 kDa è stata individuata su *casein zymogram* gel in ciascun surnatante dei ceppi batterici selezionati. L'estratto enzimatico del ceppo *P. fluorescens* PS19, concentrato per ultrafiltrazione (10 kDa), ha mostrato un'elevata attività proteolitica e due ulteriori bande proteolitiche di circa 15 e 25 kDa. I risultati delle analisi nLC/MS/MS dopo digestione sia *in gel* che *in solution* hanno evidenziato che la proteasi di 45 kDa corrisponde ad una AprX metalloproteasi di *P. fluorescens* (acc. no. C9WKP6, UniProt). La proteasi di 15 kDa è stata riconosciuta come un frammento della stessa AprX metalloproteasi, mentre la proteasi di 25 kDa non ha mostrato nessuna omologia con alcuna delle proteine note di *Pseudomonas*.
La caratterizzazione tramite LC/MS del profilo peptidico generato dall'azione delle proteasi termostabili dello stesso ceppo sulle frazioni caseiniche del latte è in corso di studio.
In generale, questo studio fornisce ulteriori conoscenze per la lo studio delle attività enzimatiche di batteri psicrotrofi nel latte.

0. PREFACE

0.1 Milk and sources of microbial contamination

Raw milk, due to its high nutritional value, water content and near neutral pH, is an environment that contains a diverse and complex microbial population (Quigley et al., 2011; Vacheyrou et al., 2011). The microbial composition of raw milk is variable according to a variety of sources, including the teat apex, milking equipment, air, water, feed, grass, soil, and other environments (Lejeune & Rajala-Schultz 2009; Vacheyrou et al., 2011).

The mammary glands of very young cows yield no bacteria in aseptically collected milk samples, but as numbers of lactations increase, so do the chances of isolating bacteria in milk drawn aseptically from the teats. The stresses placed on the cow's teats and mammary glands by the very large amounts of milk produced and the actions of the milking machine cause teat canals to become more open and teat ends to become misshapen as time passes. These stresses may open the teat canal for the entry of bacteria capable of infecting the glands and consequently to enter in milk. (Ledenbach & Marshall, 2009). Microbial populations contaminating the raw milk can include bacteria of technological relevance such as lactic acid bacteria that are fundamental if the milk is employed in cheese-making (Coppola et al., 2008); however, spoilage bacteria including both Gram-positive and Gram-negative microorganisms occur sometimes with considerable effects on the quality of milk and derived products (Cousin, 1982).

Contamination of milk by spoilage microorganisms also occurs due to inadequately sanitized surfaces of milking and milk storage equipment. The organisms grow in milk residues present in crevices, joints, rubber gaskets, and dead ends of badly cleaned milking plants. Although many different bacterial types can be introduced into milk from milk mineral deposits present in milking equipment, the most important of these are the Gram-negative psychrotrophs, which predominate among the microflora that adhere to stainless-steel milk transfer pipelines. Ancillary equipment such as agitators, dipsticks, outlet plugs, and cocks can be difficult to clean and these may be a possible source of contamination (Mac Phee & Griffiths, 2002).

The combination of longer storage times and lower temperatures of raw milk throughout the dairy chain prior to heat treatment creates selective conditions for the growth of psychrotrophic bacteria, particularly *Pseudomonas* spp., altering the structural and sensory properties of the finished dairy product (Chen et al., 2003; Lafarge et al., 2004). Milk may be collected from farms on alternate days, or even longer in some instances. Thus, at collection, part of the milk in the bulk tank may be 48 h old or more. Although alternate-day collection may have little effect on the bacteriological quality of milk rapidly cooled to 4 °C or below before addition to the tank, the growth potential of the raw milk microflora is significantly affected. Thus, milk collected on alternate days will contain a greater number of bacteria that are entering the exponential phase of growth when the milk arrives at the processing site, and the amount of time that this milk can be subsequently stored will be reduced. For example, it has been shown that *Pseudomonas* spp. isolated from milk that had been stored at 7 °C for 3 days grew 10 times faster at this temperature, had 1000-fold more proteolytic activity, and were 280-fold more lipolytic than pseudomonads isolated from freshly drawn milk. Moreover, changes in dairy industry practices, such as the introduction of a 5-day working week and milk shortages at certain times of the year due to the adoption of quota systems, have led to milk being stored for longer times before processing. Thus, the temperature at which milk is stored becomes critical. It has been recommended that milk is cooled to, and maintained at, 3 °C on receipt at the processing plant before storage. When the milks were stored for a further 48 h at 6 °C, the psychrotroph count increased by 2 log cycles to 1.3×10^7 cfu ml^{-1}. The majority of bacteria

present were pseudomonads (70.2%) but Enterobacteriaceae (7.7%), Gram-positive bacteria (6.9%), and other Gram-negative, rod-shaped organisms were also isolated.
Milk is usually transported in insulated tanks or in refrigerated tankers, and may be transferred to larger vehicles for longer journeys. During transportation, the main cause of increased bacterial count is inadequately cleaned vehicles and growth of bacteria already present in the milk. The latter is dependent on the milk temperature and journey time. A twofold increase in count is common during transportation of milk from the farm to the processing site and this is due primarily to the growth of psychrotrophic bacteria, including pseudomonads. Critical sites in the milk tanker for cleaning have been identified as the air separator, the milk meter, the milk sieve, and the suction hose, and factors that contribute to inadequate cleaning include blockage of the cleaning-in-place (CIP) spray system and low water pressure and flow rate. These can lead to buildup of milk stone on the inner surface of the tanker.
Although Gram-negative psychrotrophic bacteria present in raw milk do not survive pasteurization, these organisms are commonly isolated from pasteurized milk and cream, again with *Pseudomonas* spp. being the most frequently encountered. Thus, the shelf life of pasteurized products is limited by post-pasteurization contamination. A likely cause of post-pasteurization contamination in the processing plant is shedding of bacteria from biofilms formed on gaskets in pasteurized milk pipelines. The biofilm is generally defined as a complex structural, heterogeneous, genetically divergent community of microorganisms that exist on a solid surface in the form of an extracellular matrix composed of polymeric compounds (exopolysaccharides and/or lipopeptides). The nature of the extracellular three-dimensional matrix, the ratio of proliferation and interaction between cells within the biofilm is determined by the available conditions for growth, the medium and substrate (Watnik & Kolter, 2000; Constantin, 2009). Bacteria in biofilms (sessile form) are more resistant to chemical sanitizers and the majority of antibiotics than are the same bacteria in suspension (planktonic form) (Mosteller & Bishop, 1993), leaving viable bacteria to be dislodged into the milk product. Gram-negative bacteria like *Pseudomonas* are largely recognized by their capability to produce large amounts of exopolysaccharides, which contribute to adhesion and biofilm growth (Drenkard & Ausubel, 2002). *Pseudomonas* biofilms can develop on the sides of gaskets, despite operation of CIP systems and represent a long-lasting source of permanent product contamination. There is substantial evidence that the filling operation has the greatest influence on the potential shelf life of pasteurized milk.

0.2 Psychrotrophic bacteria

Psychrotrophic bacteria are defined as group of different bacterial species that are able to grow at 7 °C or less regardless of their optimal temperature of growth (IDF Bulletins, 1976).
Psychrotrophic microorganisms are rod-shaped and forced aerobic and include the Gram-negative genera (*Pseudomonas*, *Alcaligenes*, *Acchromobacter*, *Aeromonas*, *Serratia*, *Chromobacterium*, *Flavobacterium*), and the Gram positive genera (*Bacillus*, *Clostridium*, *Corynebacterium*, *Streptococcus*, *Lactobacillus*, *Microbacterium*).
Psychrotrophic bacteria are ubiquitous in nature, primarily in water and soil, including vegetation and a small number can also be present in the air. Ubiquitous distribution of these bacteria indicates a remarkable degree of their physiological and genetic adaptability (Spiers et al., 2000). Their optimal metabolic activity is expressed at temperatures between 20 and 30 °C. However, they can grow and multiply at low temperatures through an enrichment of polyunsaturated fatty acid in their membrane lipids. In other words, the altered cell membrane secures sufficient permeability for membrane fluidity and transport activity of metabolites necessary for growth and reproduction of bacteria at low temperatures (Schinik, 1999).

During cold storage after milk collection, psychrotrophic bacteria replaced Gram-positive mesophilic aerobic bacteria and dominate the flora, leading to many quality problems in dairy products (Cousin & Bramley, 1985; Lafarge et al., 2004; Ercolini et al., 2009). Under sanitary conditions, <10% of the total microflora are psychrotrophs, compared to >75% under unsanitary conditions (Suhren, 1989). The numbers of psychrotrophs that develop after milk collection depend on the storage temperature and time.

Typically, 65–70% of the psychrotrophs isolated from raw milk are *Pseudomonas* species (Griffiths et al., 1987; García et al., 1989). Other important associated-raw milk psychrotrophs include members of the genera *Bacillus*, *Micrococcus*, *Aerococcus*, and *Lactococcus* and of the family Enterobacteriaceae (genera *Serratia*) (Nörnberg et al., 2010).

Pseudomonads are Gram-negative, straight or curved rods, which are motile by polar flagella. They are aerobic and their metabolism is never fermentative. They are catalase-positive and the majority of species are oxidase-positive. Some of the species show distinguishable colony morphologies or pigmentation (i.e., the blue-green derivative of phenazine, pyocyanin, and the yellow-green fluorescing pigments; Kıska & Gilligan, 1999). *Pseudomonas fluorescens* is found predominantly in soil and water and it produces a diffusible fluorescent pigment, pyoverdin. The taxonomy of the genus *Pseudomonas* is complex and there is extensive genetic heterogeneity among its members. In comparison to other psychrotrophic bacteria, *Pseudomonas* spp. are characterised by a short generation time (<4 h) at 0-7 °C and the lowest theoretical minimum growth temperature of -10 °C, which is close to that of typical psychrophiles (Chandler & McMeekin, 1985; Sorhaug, 1992). *Pseudomonas* spp., with predominance of *P. fluorescens*, are the most commonly isolated bacteria in raw and pasteurized milk at the time of spoilage (Cousin, 1982; Sørhaug and Stepaniak, 1997). Spoilage is occurred as the change of flavour, undesirable coagulation of milk proteins, and the increased concentration of free fatty and amino acids. In addition, depending on the type of dairy product, the atypical texture and proportion of certain undesirable organic compounds are occurred (Cox, 1993; Boor & Murphy, 2002; McPhee & Griffiths, 2002; Cempírková & Mikulová, 2009). With regard to other quality aspects, such as suitability of milk for the production of dairy products, psychrotophs have a significant negative effects on yields as well as on limiting shelf life of dairy products (Cousin, 1982).

In addition to the ability to grow and multiply at low temperatures, psychrotophic bacteria have the ability to produce heat-stable extracellular hydrolytic enzymes (Cousin, 1982; Chen et al., 2003).

0.3 Enzymes in milk

0.3.1 Indigenous milk enzymes

Bovine milk is a biologically active product. Fifty to sixty different indigenous enzyme activities have been reported in clean, freshly drawn milk (Andrews, 1991; Muir, 1996a). The levels of these enzymes are not constant. Factors that influence this variability in enzyme levels include the breed and age, stage of lactation, diet and nutrition, health status of the cow, season (Andrews, 1991; Deeth & Fitz-Gerald, 1994). However, only a few of the indigenous milk enzymes (i.e. enzymes in fresh unprocessed milk) have a substantial impact on the quality and shelf life of milk and milk products (Muir,1996a). The most important of these are proteases and lipases.

Two particular indigenous milk proteases have been studied in detail: milk alkaline proteinase (MAP, now referred to as ‘‘plasmin’’, a serine protease; Reimerdes, 1981) and milk acidic proteinase (cathepsin D, an aspartic protease; Kaminogawa & Yamauchi, 1972; Larsen et al.,

1996). These indigenous proteases arise from mammary tissue cells, blood plasma or leucocytes (Andrews, 1991).
The most studied indigenous milk lipase was milk lipoprotein lipase (LPL), a dimer of glycoprotein chains, each of 42 kDa, containing 8.3% carbohydrate. LPL is synthesized in mammary gland secretory cells (Deeth & Fitz-Gerald, 1976).
Although indigenous milk enzymes may affect the shelf-life of milk, the accumulation and action of extracellular proteolytic and lipolytic enzymes, produced by psychrotrophic bacteria, are mainly correlated with quality deterioration in milk and dairy products.

0.3.2 Bacterial extracellular enzymes

Apart from indigenous enzymes, milk (raw or processed) also contains enzymes originating from contaminating psychrotrophic bacteria, responsible for the highest spoilage of milk and dairy products during storage.
In addition to being able to grow rapidly in refrigerated milk, psychrotrophs produce extracellular enzymes (proteases, lipases and lecithinases) that can degrade milk components, producing functional and flavor defects. Populations of psychrotrophs ranging from 10^6 to 10^7 cfu/ml^{-1} can produce sufficient amounts of extracellular enzymes to cause defects in milk that are detectable by sensory tests (Fairbairn & Law, 1987). Extracellular enzyme production by psychrotrophs is normally in the late exponential or early stationary phase of growth (Fox & Stepaniak, 1983; Griffiths, 1989; Kohlmann et al., 1991). Optimal enzyme synthesis occurs in majority of psychrotrophs at 20-30 °C, but considerable synthesis occurs at lower temperatures. For example, production of extracellular protease by *Pseudomonas fluorescens* at 5 °C was 55% of that produced at 20 °C (Mckellar, 1982).
Although most psychrotrophs present in milk do not survive pasteurization (Varnam & Sutherland, 1994; Muir, 1996a) or ultra high heat treatment (UHT, 135-150°C for 1-4 s) regimes (Griffiths et al.,1981; Suhren, 1989), in many cases the extracellular enzymes that they produce are unlikely to be destroyed by the heat processes and remain active in the final treated product (Muir, 1996c). Both proteases and lipases produced by psychrotrophs, representative of a number of genera, retained 60–70% of their activity after heating at 77 °C for 17 s and about 30–40% of their activity remained after UHT treatment at 140 °C for 5 s. For example, proteases from *Pseudomonas* species isolated from raw milk retained 55–65% of the initial activity after a heat treatment at 77 °C for 17 s and 20–40% activity after heat treatment at 140 °C for 5 s in buffers at pH 7 (Griffiths et al., 1981). The crude lipase produced by a psychrotrophic *Pseudomonas* species retained 75–100% activity after heat treatment at 100°C for 30 s in skim milk (Fitz-Gerald et al., 1982). Lipases are produced concomitantly with proteases by the same bacterium and are generally more heat-stable than the proteases (Driessen & Stadhouders, 1974; Griffiths et al., 1981; Fox & Stepaniak, 1983; Chen, 2000). Among the features that stabilize heat-resistant enzymes from psychrotrophic microorganisms are additional salt bridges and hydrogen bonds, tighter Ca^{2+} binding sites, maximed packing, shorter loops and an expanded hydrophobic core (Stepaniak & Sorhaug, 1995). Psychrotrophic enzymes are not active (heat –labile) above 50-60 °C, in particular, the low temperature instability of *Pseudomonas* protease was in the 50-60 °C range while the temperature of minimum stability for lipases from *Pseudomonas* was 60-80 °C (McKellar, 1982; Stepaniak & Sorhaug, 1995). The inactivation of most extracellular enzymes from psychrotrophs would thus require heat treatments that are completely unacceptable in the dairy industry.
A variety of factors, including quorum sensing, growth phase, environmental and nutritional factors (such as iron availability), are involved in the regulation of synthesis of extracellular enzymes. For example, pH, temperature, oxygen tension, adenosine triphosphate pools, presence of ions, organic nutrients, triglycerides, and many more have been found to influence

enzyme synthesis. In a Belgian study (Marchand et al., 2009), the incidence of proteolytic psychrotrophs was lower in milks collected in winter than in summer but the strains isolated in winter exhibited greater proteolytic activity than their summer counterparts. Surprisingly, the regulation of synthesis of extracellular enzymes by *Pseudomonas* is not unequivocally established, and probably involves a variety of regulatory mechanisms acting in concert. This fact further highlights the complexity and diversity of *Pseudomonas* extracellular enzymes. Understanding the complex mechanisms that direct enzyme synthesis will provide strategies to target for control (McPhee & Griffiths, 2002).
An interesting consequence of enzyme activity from psychrotrophs is the stimulation of the growth of starter lactic acid bacteria (LAB) in milk. Presumably this is because LAB can utilize peptides, amino acids and ammonia that accumulate in milk and are produced by psychrotrophic proteases. On the other hand, free fatty acids releases by lipases may inhibit the growth of LAB (Sørhaug & Stepaniak, 1991; Jaspe et al., 1995).

0.3.2.1 Proteases

In general, the proteases of psychrotrophic bacteria degrade milk casein (producing different peptides with altered compositional, structural and physical characteristics), leading to the formation of a gel structure or coagulation of sterilized milk during storage (Harwalker et al., 1993; Datta & Deeth, 2003) Development of astringency in some raw milk and in pasteurised or ultra-high temperature (UHT)-sterilised milk samples during storage has been related to the production of polypeptides by proteases that survive UHT treatment (Sørhaug & Stepaniak, 1997). In cheese making, proteases (which are not extracted by whey) cause a significant yield loss (Cousin, 1982; Mitchell & Marshall, 1989). Furthermore, proteolysis caused by psychrotrophic bacteria has a negative effect on the products flavour, which has been described as bitter, foreign, unclean, fruity, yeasty or metallic (Marshall, 1982). Most of the proteases isolated from *Pseudomonas* are metalloenzymes with molecular mass between 40-50 kDa, containing at least 1 zinc atom and up to 16 calcium atoms per molecule. They are rich in alanine and glycine residues and poor in cysteine and methionine residues and calcium is essential for the activity and stability of these proteases (Mitchell et al., 1986).The optimum pH of the *Pseudomonas* proteases are neutral (~7) or alkaline (7-9) and temperature optima range from 30-45 °C; in all cases, activity decreases sharply at temperatures above the optimum but the proteases retain partially activity also at lower temperatures (4 °C) (Mitchell & Ewings, 1985).
In milk, *Pseudomonas* proteases preferentially hydrolyze κ-casein > β-casein > α_{S1}-casein. The proteins of the lactoserum are very few hydrolyzed (Cousin, 1982; Fairbairn & Law, 1986; Koka & Weimer, 2000; Rajmohan et al., 2002). One extracellular metalloprotease called AprX, which belongs to the serralysin family, has been characterized in *P. fluorescens* and its gene was identified in several strains (Martins et al., 2005).

0.3.2.2 Lipases and Phospholipases

Psychrotrophic lipases, catalysing the hydrolysis of triacylglycerols, lead to the accumulation of free (non-esterified) fatty acids, partial glycerol esters (monoacylglycerols, diacylglycerols) and even glycerol in some cases in milk (Deeth, 2006). The products of lipolysis are highly detrimental to the formation and stability of milk foams that are colloidal systems in which air bubbles are stabilized by a matrix composed of milk components. The depression of milk foaming, caused by lipolysis, is due to the partial glycerides, which are surface active and displace the foam-stabilizing proteins at the air-water interface of the foam bubbles. Besides, FFAs are the primary cause for the changes in product flavour that is described as rancid,

unclean, soapy, butyric, astringent or bitter, which could influence consumer acceptance of milk and dairy products. The lipolytic flavours defects are particularly pronounced in cream, butter, cheese and sterilised (UHT) milk (Stead, 1986; Champagne et al., 1994).
In general, psychrotrophic lipases have molecular masses ranging from 30 to 50 kDa, pH optima between 7 and 9 and temperature optima range from 22 to 55 °C. From the patterns of hydrolysis of triacylglycerols bacterial lipases are divided into two major groups, I and II. Group I (non-specific) lipases catalyse the production of glycerol by releasing fatty acids from all the three positions; group II (1,3-specific) lipases catalyse the formation of 1,2- and 2,3-diacylglycerols and 2-monoacylglycerols with the release of fatty acids from positions 1 and 3 of the triglyceride. In the case of group II lipase products, acyl migration leads to the production of 1,3-diacylglycerols and 1-monoacylglycerol and subsequently the production of glycerol over an extended period (Macrae, 1983).
Lecithinases are important groups of phospholipases of psychrotrophic bacteria that are able to disrupt the protective membrane structure of fat globules and milk fat become available to the native milk lipases resulting in physical degradation of the emulsion in milk (Shah, 1994).
The ability of psychrotrophic bacteria to produce lipases and phospholipases varies considerably across genera, as well as among species of the same genera (Witter, 1969).

0.4 Control of psychrotrophs and related enzymes

The negative impacts of psychrotrophic bacteria on the quality of milk and dairy products are unquestionable. Their ubiquitous nature in the production environment , the ability for rapid growth under low temperatures and the capacity to synthesize thermo-stable extracellular enzymes, have made this group of bacteria the leading cause of spoilage of milk and dairy products. Contamination of raw milk with psychrotrophs, even under the best manufacturing practices, cannot be completely avoided. However, cooling of milk at a temperature of 2 °C, instead of at temperatures between 4 and 6 °C (which is the most commonly applied cooling temperature), can significantly slow their growth as well as their proteolytic and lipolytic activity, only in cases where their initial number is $\leq 10^3$ cfu mL^{-1} (Kumarsan et al., 2007). The heat treatment of raw milk (65-69 °C/15 s) in the dairy plant prior to pasteurization can reduce the number of Gram-negative psychrotrophic bacteria by 77-97 % (Champagne et al., 1994). Other examples of methods currently proposed to control psychrotrophic bacteria and/or their extracellular enzymes include additives (CO_2, nitrogen), high-pressure treatment, modified atmosphere storage of raw milk, microbial antagonism, activation of the lactoperoxidase system in milk, addition of enzyme inhibitors, addition of bacteriocin-producing lactic acid bacteria, and low temperature inactivation of enzymes, to name a few (Champagne et al., 1993; Shah, 1994; Sørhaug & Stepaniak, 1997).
However, adequate cleaning and permanent hygiene control in all aspects of milk handling, transport and processing, strict maintenance of refrigeration at 4 °C or lower, minimization of the storage time of raw milk, and a suitable method to kill or remove psychrotrophic bacteria, followed up by an effective HACCP system, are all primary concerns for quality assurance in the dairy industry. Most practices currently employed in the industry focus on elimination of the bacteria by heat processing but it is clear that bacterial numbers (i.e. viable bacteria) do not correlate with enzyme levels since the enzymes survive treatments that kill the bacteria, and cannot give an accurate indication of product quality. Therefore it would be useful to develop a quick and simple enzyme assay method that can provide a direct correlation between enzyme activities and levels and keeping quality of dairy products. The dairy industry would be able to concentrate its efforts on control of the most problematical bacteria (and consequently control of the levels of enzyme introduced into products) and therefore maximize processing efficiency.

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1. STATE OF THE ART

Milk is a rich growth medium for microorganisms, which can increase rapidly during storage thus affecting the technological properties of raw milk. Refrigeration has an important impact on the microbiological quality of raw milk by decreasing the presence of mesophilic bacteria. However, cold storage of unprocessed milk creates selective conditions for the development of psychrotrophic bacteria, that are able to grow below 7 °C (Champagne et al., 1994). The psychrotrophs from refrigerated milk include both Gram-negative and Gram-positive bacteria, belonging to numerous genera. *Pseudomonas* spp. dominate the psychrotrophic microflora of raw or pasteurized milk at the time of spoilage (Sørhaug & Stepaniak, 1997); for this reason up to now most of the research has been focused on *Pseudomonas* genus, which includes the predominant microorganisms affecting the shelf-life of processed fluid milk at 4 °C. *P. fluorescens*, *P. putida*, *P. fragi*, *P. putrefaciens*, are the most isolated species and less frequently *P. aeruginosa* (Gilmour & Rowe, 1990). Significant contaminations by psychrotrophs occur due to inadequately sanitized surfaces of milking, storage and transporting equipments. Furthermore post-pasteurization contaminations may happen at the filling operation (Mac Phee & Griffiths, 2002).

Psychrotrophs are responsible for the highest spoilage of milk because of their capacity to produce thermostable extracellular enzymes (proteases, lipases and lecithinases), which have major effects on the quality of raw milk and milk products (Sørhaug & Stepaniak, 1997).

Although most psychrotrophic bacteria are destroyed by the conventional thermal treatments (pasteurization and UHT processing) employed in the dairy industry, such treatments have minor effects on their enzymes; they can resist and continue to degrade milk in the absence of viable bacterial cells, reducing the shelf-life of milk and dairy products (Cousin, 1982; Koka & Weimer, 2000; Chen et al., 2003).

Enzymes can also be detrimental to the quality of cheese by causing bitter or rancid flavors and by impairing the coagulation properties of the milk (Richter & Vedamuthu, 2001). Proteases degrade casein, resulting in bitterness in milk, gelation of UHT-sterilized milk and decreased yields of soft cheese. Lipases by hydrolyzing triglycerides to free fatty acids and glycerol, produce flavor defects (rancid, butyric, astringent, soapy) in cream, butter, cheese and UHT products. *Pseudomonas* spp. are the primary concern with regard to lipolytic degradation of milk fat (MacPhee & Griffiths, 2002). Lecithinases are able to disrupt the integrity of milk fat globule membrane (MFGM), increasing the susceptibility of milk fat to the action of lipases (Herrera, 2001; Ray, 2004).

Most of the research has been focused on *Pseudomonas* spp. (Eneroth et al., 1998; Martins et al., 2006), but there is growing evidence that other genera, such as Enterobacteriaceae, may have similar relevance by producing strong lipo-proteolytic activity (Nörnberg et al., 2010).

The enzymes are generally good indicator of the keeping quality of protein and lipid-rich foods and the knowledge of their impact and deleterious effects on products may lead to the definition of pertinent indicators and possible markers that can be used for quality control during processing (Cousin et al., 2001).

Over the past years, although many studies have assessed the microbial diversity in raw milk, just a few have provided more information on the development of off-flavors, on the release and levels of FFAs from milk fat and on identification of peptides, related to the lipo-proteolytic activity of psychrotrophs, that can be directly linked to functional and sensory properties and quality of milk.

Moreover, scant information about other specific psychrotrophic microorganisms and the effects of their enzymes on milk quality is available.

1.1 References

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2. AIMS OF THE STUDY

Hygiene in all aspects of milk handling, reduction of storage times, use of effective refrigeration temperatures, availability of methods for reduction of pathogenic and spoilage microbial flora, and an effective employment of HACCP systems are principal issues for the dairy industry.
In recent years, also in Lombardy, frequent technological and sensory troubles arose from processing of milk containing psychrotrophic bacteria, responsible for the highest spoilage of raw or heated milk during storage. Indeed, nowadays, these bacteria represent a constant and major cause of concern for the dairy industry with regard to their capacity to synthesize thermostable extracellular enzymes. Such enzymes can hydrolyse milk fats and proteins leading to development of gelation, off-odours/flavours, loss of sensory quality and shelf life. For these reasons, this research project aims to acquire further knowledge on the enzymatic traits of raw milk-associated psychrotrophic bacteria. On these bases, the research also deals with the detection of specific molecules and metabolites derived from by bacterial enzymatic degradation of milk components. In particular, in this study, we attempted to assess the hydrolytic activities of 80 psychrotrophic bacteria, belonging to different genera, isolated from raw bovine milk samples collected at different farms located in Lombardy. Moreover, we evaluated their spoilage potential by analyzing the production of VOCs and the release of FFAs in milk and then we characterize their extracellular proteolytic activity by means the evaluation of caseinolytic potential, identification of secreted proteases and characterization of peptide profile generated by the action of bacterial protease.
Overall, the final aim of this PhD thesis was to recognize particular molecules and metabolites derived from bacterial enzymatic degradation of milk components useful as a good markers to early recognize the activity for psychrotrophic strains in raw milk.

3. RESULTS AND DISCUSSION

3.1 Characterization of Gram-negative psychrotrophic bacteria isolated from raw milk

The combination of longer storage times and lower temperatures of raw milk throughout the dairy chain prior to heat treatment creates selective conditions for growth of thermo-resistant enzyme-producing psychrotrophic bacteria, altering the structural and sensory properties of the finished dairy product (Chen et al., 2003).
Psychrotrophs are bacteria that can grow at temperatures between 0°C and 7°C, although their optimal growth temperature is higher (Champagne et al., 1994; Sørhaug & Stepaniak, 1997) and can constitute up to 70–90% of the microbial population in raw milk stored at low temperatures (Cousin, 1982; Sørhaug & Stepaniak, 1997). Psychrotrophic bacteria from numerous genera have been isolated from milk, the genus *Pseudomonas* generally dominating the microbiota of refrigerated raw milk, (Eneroth et al., 2000; Ercolini et al., 2009; Rasolofo et al., 2010; De Jonghe et al., 2011; Raats et al., 2011;), but also reported to be prevalent are the Enterobacteriaceae (Nörnberg et al., 2009). Psychrotrophic pseudomonads are recognized as major spoilage microorganisms of milk and dairy products due to the production of heat-stable extracellular enzyme, proteases, lipases and lecithinases (Cousin, 2001; Herrera, 2001). Although psychrotrophic bacteria are easily destroyed by the heating settings applied by the dairy industry, their enzymes can survive and remain active in processed milk and derived dairy products (Deeth, 2002; Ray, 2004; Bhunia, 2008). Proteases are predominantly active towards the casein fraction, producing a grey colour and bitter off-flavours in milk, gelation of UHT sterilized milk and decreased yields of soft cheese. Most proteases are metalloproteases, which are rich in alanine and glycine residues and poor in cysteine and methionine residues; calcium is essential for the activity and stability of these proteases (Mitchell et al., 1986). In milk, they preferentially hydrolyze casein κ, then casein β, then casein α_{S1}.
The *apr*X gene, which encodes a heat-resistant alkaline metalloprotease, belongs to the serralysin family, and has been characterized in several strains of *Pseudomonas* spp. (Duong et al., 1992; Liao & McCallus, 1998; Kawai et al., 1999; Kumeta et al., 1999; Chessa et al., 2000; Chabeaud et al., 2001), and extensively studied only in *P. aeruginosa* and *P. fluorescens*. AprX is believed to be responsible for the spoilage of milk, and has been proposed as a marker for milk bacterial contamination, with high protein degrading effects (Dufour et al., 2008).
Lipases catalyze the hydrolysis of triglycerides, which leads to rancid, butyric, astringent, soapy flavours and the depression of milk foaming properties (De Jonghe et al., 2011). Lipolytic degradation of milk is not as predominant as proteolytic degradation, but the free fatty acids released from milk fat hydrolysis, caused by psychrotrophic lipase activity, are the primary cause of changes in product flavor, described as rancid, unclean, soapy or bitter, making the product barely acceptable (Ma et al., 2000; Deeth, 2006).
Psychrotrophic bacteria are particularly troublesome in milk, also for their lecithinase enzymes that act on the phospholipids of milk fat globule membranes (MFG); the disruption of the MFG results in an unstable fat emulsion (flocks on the milk and cream surfaces) and the triacylglycerols are then easy targets for bacterial lipases (Shah, 1994; Herrera, 2001; Fox, 2002, Ray, 2004).
In recent years in Italy, there have been frequent technological and sensory troubles caused by both the proteolytic and the lipolytic activity of psychrotrophic bacteria in UHT milk and also in pasteurized milk.
Due to the relevance of the aforementioned issues, and as the psychrotrophic microbiota is the most important in the process of enzymatic milk spoilage, a more detailed evaluation on proteolytic, lipolytic and lecithinase activities is crucial for the development of control strategies, increasing both the sensory quality and the shelf life of milk.

Thus the first study of this PhD thesis aimed to (i) isolate and identify Gram-negative psychrotrophic bacterial strains from unprocessed bulk milk collected from different farms located in Lombardy; (ii) assess the bacterial lipolytic, proteolytic and lecithinase traits that could influence the milk itself, and the dairy product shelf life in different conditions; iii) evaluate the presence of *apr*X gene in psychrotrophic strains.

3.2 Material and methods

3.2.1 Psychrotrophic bacterial counts and isolation of gram-negative psychrotrophic (GNP) strains

Eighty psychrotrophic strains were isolated from 56 summer and winter samples of unprocessed bulk milk collected from 28 different farms in the Lombardy region of Northern Italy; the total production was destined to produce Grana Padano P.D.O. cheese. Bulk milk was refrigerated at 11°C after the two daily milkings, following the indications of Grana Padano P.D.O. production rules. At each farm, raw milk was sampled twice over a 6 month period, in January and July.
The milk samples were transported to the laboratory in sterile boxes, stored at 4°C, and analyzed within 24h. Decimal progressive dilutions were carried out with quarter-strength Ringer's solution (Scharlau, Barcelona, Spain). Psychrotrophic bacteria were isolated on Plate Count Agar (Oxoid, Ltd., Basingstoke, UK) at 7°C after 10 days of incubation. Two colonies with different and distinguishable morphologies were picked out from each sample, and subcultured on Brain Heart Infusion (BHI) Agar (Biolife, Milan, Italy) to obtain pure cultures. The presumptive *Pseudomonas* colonies were preliminarily confirmed either on Pseudomonas Selective Agar (Biolife), after incubation at 37°C during 18-42 h, and on Penicillin-Pimaricin (Biolife) supplemented Pseudomonas agar base (Oxoid) plates according to ISO-13720:2000, incubated at 25°C for 48 h. The isolates were maintained and propagated in BHI broth (Biolife) and incubated under aerobic conditions at 30°C overnight. The strains were stored at -20°C in litmus milk until use.

3.2.2 Phenotypic characterization and biochemical tests

Pure cultures were Gram-stained and examined for growth at different temperatures, motility, pigment production, oxidase and catalase activity, gas and extracellular enzymes production. The bacterial isolates were incubated in Brain Heart Infusion broth (Biolife) at 7, 22 and 30 °C until growth occurred. Motility was measured as the diameter of zone travelled by bacteria point-inoculated into Soft-Luria-Bertani (SOFT-LB) agar plates (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract , 10 g L^{-1} NaCl, 3 g L^{-1} agar). Plates were incubated 18–20 h at 30 °C and halo diameter measurements were recorded. Bacterial motility was negative for halo diameter < 0,5 cm. The colony pigmentation was visualized on “Mascarpone agar” plates (1 kg L^{-1} mascarpone cheese 30 g L^{-1} agar, 10 g L^{-1} yeast extract) incubated at 30 °C for 24 h to 72 h. The oxidase reaction was determined using BBLDrySlide oxidase slides (Becton Dickinson Company, Sparks, Md. USA), according to the manufacturer’s instructions. For catalase activity, 1.5% H_2O_2 was dropped onto a bacterial smear that had been placed on a glass slide. Gas production indicated a positive reaction. All isolates were checked for gas production from glucose in BHI broth (Biolife) containing Durham tubes.
All the strains were tested for their proteolytic, lipolytic and lecithinase activities by agar diffusion method assay at 7, 22 and 30 °C up to 20 days (Tables 3.3.3 and 3.3.4). Proteolytic enzyme production was visualized on skim milk agar (5% skim milk powder, 3% agar). After incubation, the presence of a clear zone around the colonies was indicative of proteolysis. Lipolytic strains were screened using Tributyrin Agar plates (Oxoid), and the activity of the

lipase was observed as a zone of hydrolysis around the bacterial colonies (Meghwanshi et al., 2006). The production of extracellular phospholipases (lecithinases) was determined on Plate Count Agar (Oxoid) supplemented with 10% egg yolk emulsion (Biolife), as described by Dogan and Boor (2003). Colonies surrounded by an opaque ring were deemed to evince lecithinase activity (Bates & Liu, 1963).

3.2.3 DNA extraction

DNA extraction was carried out using 1 ml of a BHI broth overnight culture containing approximately 1 -3 * 10^8 cells. DNA was extracted using the Microlysis kit (Labogen, Rho, Italy) following the manufacturer's instructions.

3.2.4 Identification of psychrotrophic isolates by 16S rRNA and *rpo*B genes sequencing

The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the universal primers p8FLP (5'-AGTTTGATCCTGGCTCAG-3')/p806R (5' GGACTACCAGGGTATCTAAT-3') (Mc Cabe et al., 1995) to generate an amplicon of ca. 800 bp. PCR amplification was carried out in a Mastercycler (Eppendorf, Hamburg, Germany) with the following conditions: initial denaturation at 94 °C for 5 min followed by 30 cycles of 94 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min. Final extension was carried out at 72 °C for 5 min. Each DNA amplification was performed in 200 µl microtubes using a 25 µl reaction mixture containing 50–100 ng DNA template, PCR Master Mix 2X (Fermentas, Inc., Burlington, Ontario, Canada), 10 µM of the primer pair and double-distilled water to achieve the final volume.
Since the 16S rRNA gene is known for its low resolution within *Pseudomonas* at the species or subspecies level (Yamamoto et al., 2000; Ercolini et al., 2007), partial sequence analysis of the *rpo*B gene was also performed. PCR amplification of *rpo*B was performed with the set of primers PSF (5′-AGT-TCA-TGG-ACC-AGA-ACA-ACC-3′) as forward/ rpoB-PTR (5′-CCT-TGA-CGG-TGA-ACT-CGT-TTC-3′) as reverse under the conditions described by Sajben et al. (2011). The amplification started with an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and elongation at 72°C for 1 min, and a final extension step at 72°C for 10 min. The PCR-amplified 16S rRNA and *rpo*B gene products were separated on a 1.5% agarose gel (GellyPhor, Euroclone) stained with SYBR Safe (Invitrogen, Minneapolis, MN, USA) and photographed using a UV transilluminator. Molecular size markers (100-bp DNA ladder, Euroclone) were included in each agarose gel.
Amplification products were sent to Macrogen Europe (Amsterdam, Netherlands) for sequencing. Concerning data analysis, partial *rpo*B and complete 16S rRNA gene sequences (approximately 310bp and 800bp, respectively) were analyzed with NCBI BLAST search (http://www.ncbi.nlm.nih.gov/BLAST, Altschul et al., 1990). Species names were assigned whenever the degree of homology was higher than 97%.

3.2.5 Randomly amplified polymorphic DNA (RAPD) analysis

Gram-negative psychrotrophic bacteria isolated from unprocessed milk samples were RAPD-typed to study their genetic variability and to distinguish closely related strains. RAPD-PCR reactions were performed with primers M13 (5′-GAGGGTGGCGGTTCT-3′) (Huey & Hall, 1989) and OPAA-10 (TGGTCGGGTG) (Martins et al., 2006). Briefly, the amplification with M13 started with an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 20 sec, and elongation at 72°C at

0,5°C/sec, and a final extension step at 72°C for 2 min (Andrighetto et al., 1998).While the amplification with OPAA-10 was carried out using the following protocol: 40 cycles consisting of denaturation at 94°C for 1 min, primer annealing at 37°C for 1 min, and extension at 72°C for 90 s. To complete the synthesis of all strands, the procedure was concluded with extension at 72°C for 7 min (Martins et al., 2006). Grouping of the RAPD-PCR profiles was obtained with the BioNumeric 5.1 software package (Applied Maths, Kortrjik, Belgium) using the UPGMA (unweighted pair group method using arithmetic averages) cluster analysis. The reproducibility value of the RAPD-PCR assay, calculated from two repetitions of independent amplification of psychrotrophic type strains, was higher than 90%.

3.2.6 Detection of the *apr*X gene in psychrotrophic strains (*apr*X –PCR)

The presence of the *apr*X gene, encoding for a known alkaline heat-resistant metalloprotease, was searched for in psychrotrophic bacteria, using an *apr*X-PCR test. PCR amplification of the *apr*X gene was performed with the set of primers SM2F (5′-AAA-TCG-ATA-GCT-TCA-GCC-AT-3′)/SM3R (5′-TTG-AGG-TTG-ATC-TTC-TGG-TT-3′) with an amplification product of approximately 850 bp, under the conditions described by Marchand et al. (2009). PCR amplification was carried out in a Mastercycler (Eppendorf), and the cycle parameters were 5 min at 95°C for initial denaturation followed by 30 cycles with denaturation for 30 s at 95°C, annealing for 30 s at 60°C, extension for 1 min at 72°C and a final elongation step of 72°C for 8 min.

3.3 Results and discussion

3.3.1 Psychrotrophic bacterial counts and isolation of gram-negative psychrotrophic (GNP) strains

The psychrotrophic bacterial content in the 56 raw milk samples analyzed in this study ranged between 2.43 and 6.46 log cfu ml^{-1}, representing from 49.1 to 99.99 % of Standard Plate Count (SPC). Total count of mesophilic bacteria in the whole set of bulk milk samples ranged from 3.78 to 6.89 log cfu ml^{-1}. SPC values are higher than those previously reported in Italy (Bava et al., 2011) as a consequence of the refrigeration temperature (> 8 °C) required by Grana Padano production rules. Eighteen milk samples contained more than 5 log-units total bacterial count, being the average content of psycrotrophs 91,63% ± 14,40 which not differed from that of milk samples characterized by lower bacterial count (93,63 % ± 13,33).
Psychrotrophs make a contribution to SPC similar to that reported by other authors in Brasil (Nörnberg et al., 2010) and Italy (Bava et al., 2011); differently, Cempírková (2002), in a study conducted on seven farms over an 18 month period, highlighted a high correlation ($p<0.001$) between psychrotrophic Gram-negative bacteria (PB) and the total count of mesophilic aerobic bacteria (TB), with a lower proportional PB/TB index (0.18). In the microbiological quality assessment of raw milk in Denmark, Holm and others (2004) confirmed that psychrotrophic bacteria were dominant in 28 % of the cases where TB exceeded 30,000 cfu mL^{-1}. Contrary to this, under conditions of unhygienic milking, the number of psychrotrophic Gram-negative bacteria in raw milk accounted for 75-99 % of the total microbial population, with the predominance of the *P. fluorescens* (Marshall, 1982; Cox, 1993; Muir, 1996).
The combination of longer storage times and lower temperatures of raw milk throughout the dairy chain prior to heat treatment creates selective conditions for growth of thermo-resistant enzyme-producing psychrotrophic bacteria, altering the structural and sensory properties of the finished dairy product (Chen et al., 2003). In 1980, Gehringer identified that high raw milk quality can only be achieved if the occurrence of psychrotrophic bacteria is less than 10 % of

the total bacterial count. However, cooling and holding refrigerated raw milk longer prior to processing causes changes in the microbial population. Under such conditions, the dominant Gram positive bacteria are replaced by Gram-negative and Gram-positive psychrotrophic bacteria.

A total of 99 colonies were isolated from 56 different milk samples originating from 28 separate dairy farms, in order to minimize the chances of them being the same strain even if the choice to isolate 2 colonies with different morphologies from each sample do not allow to draw conclusions about the prevalence of the species.

Of these, 80 were Gram-negative bacteria (80.8%), confirming the predominance of GNP in milk, which has already been reported in other studies (Jayarao & Wang, 1999), and were selected for further analysis. In this work bacterial isolates were identified at the species level by partial *rpo*B gene sequencing in the case of low 16S rRNA gene sequence heterogeneity. In particular the housekeeping *rpo*B gene, encoding the beta-subunit of RNA polymerase, provided improved phylogenetic resolution over the 16S rRNA gene for the *Pseudomonas* genus and can be used to complement the information gathered from the 16S rRNA gene, as reported by previous studies (Moore et al., 1996; Anzai et al., 2000; Yamamoto et al., 2000; Adékambi et al., 2009).

In total, 46 isolates were identified at the species level, based on 16S rRNA gene sequence, and 34 based on partial *rpo*B gene sequence since the sequencing of the 16S rRNA gene did not enable species identification. Eight strains could not be associated with any previously associated species, not even with the *rpo*B gene sequencing. Partial *rpo*B gene sequence analysis recognized them as *Pseudomonas* sp., sharing an identity by more than 97%.

Among the identified GNP, *Pseudomonas* was the predominant genus with a prevalence of 78.75 %, followed by Enterobacteriaceae (21,25%). These findings are in agreement with results of other researchers who stated that *Pseudomonas* represents a relevant part of raw milk microbiota (Ercolini et al., 2009; Giannino et al., 2009) and that the total psychrotrophic raw milk microbiota can comprise up to 90% *Pseudomonas* spp. (Champagne et al., 1994; Dogan & Boor, 2003). On the contrary, some studies reported Enterobacteriaceae to be predominant psychrotrophs in raw milk in Brasil and Spain (Suarez & Ferreiros, 1991; Nörnberg et al., 2010). Enterobacteriaceae are frequently encountered in milk and during cheese production (Sorhaug & Stepaniak, 1997; Morales et al., 2003; Mounier et al., 2005; Chaves-Lopez et al., 2006), although they have been considered negative flora (eg. markers of hygiene, cheese texture defects, blowing and off-flavours) and can be associated with raw milk samples from mastitic animals (Nam et al., 2010). Among the Pseudomonadaceae, ten different species were identified (Table 3.3.1). *P. fluorescens* and *P. aeruginosa* were the predominant species with a percentage of 30.16 % and 22.22 % respectively, followed by *P. putida* (11.11 %), *P. fulva* (6.34%), *P. fragi* and *P. mosselii* (4.76%), *P. rhodesiae* (3.17%). One strain of *P. libanensis*, *P. teatrolens* and *P. chlororaphis* subsp. *aurantiaca* were found in raw milk samples. Among the Enterobacteriaceae, *Serratia marcescens* was the most frequently isolated species, with a percentage of 52.94 %, followed by *Hafnia alvei* (29.41%), *Citrobacter freundii* (11.76%) and one strain of *Enterobacter cloacae* (Table 3.3.2). *S. marcescens* is the most frequently occurring species of the *Serratia* genus and is found in soil, water, and sometimes on starchy foods such as bread (Grimont & Grimont, 2006). *Serratia* strains have already been described in milk (Tornadijo et al., 1993) and cheese (Martin-Platero et al., 2009) and have been shown to affect milk and cheese sensory quality due to high proteolytic activity and dimethyl sulphide production (Morales et al., 2003; Chaves-Lopez et al., 2006). In recent years, *S. marcescens* has also been studied for its ability to produce lipase, which is responsible for flavour defects in milk and dairy products during cold storage (Abdou, 2003). *H. alvei* has been found in raw milk and raw milk cheeses (Tornadijo et al., 2001; Morales et al., 2005b; Kagkli et al., 2007), and

water was recently suggested as a possible contamination source for these species in milk (Kagkli et al., 2007).
All the psychrotrophic strains considered in this study were characterised by RAPD-PCR analysing amplification profiles obtained with two primers. A notable genotypic heterogeneity and a high degree of variability among *Pseudomonas* and Enterobacteriaceae strains was evident indeed a degree of homology higher than 90% was detected among strains belonging to the same species only for 2 *P. fluorescens* (PS16 and PS24 strains) (Figure 3.3.1).

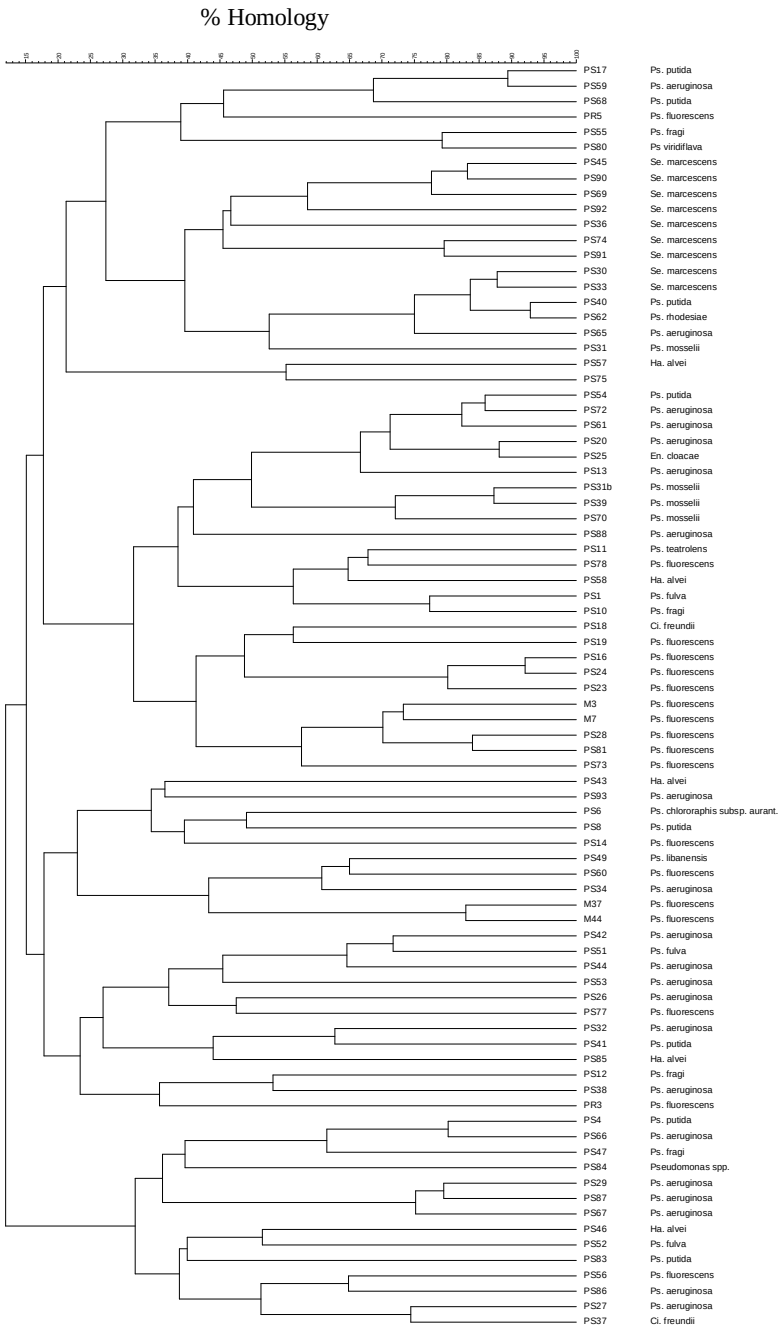

Figure 3.3.1 - Dendrogram derived from profiles by the Random Amplification of Polymorphic DNA (RAPD-PCR) generated with primers M13 and OPAA-10 of the 80 GNP strains isolated from different raw milk samples. The profile grouping was done with the BioNumeric 5.0 software package, using the unweighted pair group method with arithmetic averages (UPGMA) cluster analysis.

3.3.2 Phenotypic properties

According to their negative Gram reaction and the catalase and oxidase activities, the strains were considered to be *Pseudomonas* and Enterobacteriaceae, and were specified by molecular identification. Individual results of phenotypic characterization for *Pseudomonas* and Enterobacteriaceae strains are listed in Tables 3.3.1 and 3.3.2, respectively. All the *Pseudomonas* and Enterobacteriaceae isolates were catalase-oxidase positive and catalase positive-oxidase negative, respectively. Enterobacteriaceae gave gas production, except for the *S. marcescens* strains. Most psychrotrophic strains were actively motile after incubation, and produced a different and distinctive pigmentation varying from cream/yellow/fluorescent (pyoverdin pigment)/brown/blue (pyocyanin pigment) of *Pseudomonas* spp. to light pink/dark red (prodigiosin pigment) of *S. marcescens*. *P. fluorescens* strains mostly produced yellow/cream and blue pigments while *P. aeruginosa* strains showed yellow and brown/cream pigmentation. The other psycrotrophic strains showed a gradation of colors from cream/yellow to orange/brown.

Table 3.3.1 - Phenotypic properties of 63 *Pseudomonas* strains isolated from raw milk

***Pseudomonas* strains**		**Growth in BHI broth[a]**			**Gas production**	**Motility**	**Pigment production**
		7 °C	**22 °C**	**30 °C**			
PS34	*P. aeruginosa*	+ (3)	+ (1)	+ (1)	-	-	Brown/cream
PS20	*P. aeruginosa*	+ (3)	+ (1)	+ (1)	-	+	Yellow
PS86	*P. aeruginosa*	+ (4)	+ (1)	+ (1)	-	+	Beige
PS65	*P. aeruginosa*	+ (3)	+ (1)	+ (1)	-	-	Yellow/beige
PS26	*P. aeruginosa*	+ (3)	+ (1)	+ (1)	-	-	Brown
PS27	*P. aeruginosa*	+(23)	+ (1)	+ (1)	-	+	Yellow/cream
PS38	*P. aeruginosa*	+ (3)	+ (1)	+ (1)	-	+	Yellow
PS44	*P. aeruginosa*	+ (1)	+ (1)	+ (1)	-	+	Brown/cream
PS32	*P. aeruginosa*	+ (2)	+ (1)	+ (1)	-	+	Yellow
PS42	*P. aeruginosa*	+ (6)	+ (1)	+ (1)	-	+	Brown/yellow
PS53	*P. aeruginosa*	+ (8)	+ (1)	+ (1)	-	+	Yellow
PS87	*P. aeruginosa*	+ (3)	+ (1)	+ (1)	-	+	Cream
PS29	*P. aeruginosa*	+(23)	+ (1)	+ (1)	-	+	Brown/yellow
PS67	*P. aeruginosa*	+ (8)	+ (1)	+ (1)	-	+	Yellow
PS59	*P. aeruginosa*	+ (5)	+ (1)	+ (1)	-	+	Brown/cream
PS16	*P. fluorescens*	+ (5)	+ (1)	+ (1)	-	+	Yellow
PS19	*P. fluorescens*	+ (5)	+ (1)	+ (1)	-	±0,5cm	Yellow
PS23	*P. fluorescens*	+ (5)	+ (1)	+ (1)	-	+	Yellow/cream
PS24	*P. fluorescens*	+ (5)	+ (1)	+ (1)	-	+	Yellow/cream
PS14	*P. fluorescens*	+ (3)	+ (1)	+ (1)	-	+	Yellow
PS64	*P. fluorescens*	+ (2)	+ (1)	+ (1)	-	±0,5cm	Yellow/cream
PS60	*P. fluorescens*	+ (3)	+ (1)	+ (1)	-	±0,5cm	Yellow/cream

***Pseudomonas* strains**		**Growth in BHI broth[a]**			**Gas production**	**Motility**	**Pigment production**
		7 °C	**22 °C**	**30 °C**			
PS77	*P. fluorescens*	+ (5)	+ (1)	+ (1)	-	±0,5cm	Blue
PS78	*P. fluorescens*	+ (6)	+ (1)	+ (1)	-	±0,5cm	Blue/brown
PS28	*P. fluorescens*	+ (3)	+ (1)	+ (1)	-	+	Cream/orange
PS81	*P. fluorescens*	+ (5)	+ (1)	+ (1)	-	-	Yellow/cream
PS68	*P. fluorescens*	+ (9)	+ (1)	+ (1)	-	+	Yellow/cream
PS56	*P. fluorescens*	+ (6)	+ (1)	+ (1)	-	+	Beige
PS73	*P. fluorescens*	+ (2)	+ (1)	+ (1)	-	+	Cream
M3	*P. fluorescens*	+ (2)	+ (1)	+ (1)	-	+	Blue
M7	*P. fluorescens*	+ (5)	+ (1)	+ (1)	-	+	Blue
M37	*P. fluorescens*	+ (3)	+ (1)	+ (1)	-	+	Blue
M44	*P. fluorescens*	+ (1)	+ (1)	+ (1)	-	+	Blue
PR5	*P. fluorescens*	+ (2)	+ (1)	+ (1)	-	+	Blue
PS55	*P. fragi*	+ (3)	+ (1)	+ (1)	-	+	Colorless
PS12	*P. fragi*	+ (4)	+ (1)	+ (1)	-	-	Cream
PS47	*P. fragi*	+ (5)	+ (1)	+ (1)	-	-	Yellow/cream
PS52	*P. fulva*	+ (5)	+ (2)	+ (1)	-	+	Cream
PS10	*P. fulva*	+(30)	+ (1)	+ (1)	-	+	Cream
PS51	*P. fulva*	+ (6)	+ (1)	+ (1)	-	+	Yellow/cream
PS1	*P. fulva*	+ (3)	+ (1)	+ (1)	-	+	Dark yellow
PS49	*P. libanensis*	+ (6)	+ (1)	+ (1)	-	+	Fluorescent
PS70	*P. mosselii*	+ (5)	+ (1)	+ (1)	-	-	Yellow/cream
PS31	*P. mosselii*	+(23)	+ (1)	+ (1)	-	+	Brown/cream
PS39	*P. mosselii*	+ (1)	+ (1)	+ (1)	-	+	Yellow/cream
PS4	*P. putida*	+(20)	+ (1)	+ (1)	-	+	Cream
PS93	*P. putida*	+(12)	+ (1)	+ (1)	-	+	Cream
PS40	*P. putida*	+ (6)	+ (1)	+ (1)	-	+	Yellow
PS41	*P. putida*	+ (1)	+ (1)	+ (1)	-	+	Dark yellow
PS8	*P. putida*	+(12)	+ (1)	+ (1)	-	+	Beige/cream
PS83	*P. putida*	+ (5)	+ (1)	+ (1)	-	-	Yellow/cream
PS17	*P. putida*	+ (5)	+ (1)	+ (1)	-	+	Yellow/cream
PS62	*P. rhodesiae*	+ (2)	+ (1)	+ (1)	-	±0,5cm	Pink/cream
PS84	*P. rhodesiae*	+ (3)	+ (1)	+ (1)	-	+	Pink/cream
PS66	*Pseudomonas* sp.	+ (3)	+ (1)	+ (1)	-	-	Cream
PS88	*Pseudomonas* sp.	+ (2)	+ (1)	+ (1)	-	+	Yellow
PS72	*Pseudomonas* sp.	+ (6)	+ (1)	+ (1)	-	+	Yellow/cream
PS54	*Pseudomonas* sp.	+ (1)	+ (1)	+ (1)	-	+	Orange/yellow

***Pseudomonas* strains**		**Growth in BHI broth[a]**			**Gas production**	**Motility**	**Pigment production**
		7 °C	**22 °C**	**30 °C**			
PS61	*Pseudomonas* sp.	+ (1)	+ (1)	+ (1)	-	+	Fluorescent
PS13	*Pseudomonas* sp.	+ (3)	+ (1)	+ (1)	-	+	Yellow/brown
PS75	*Pseudomonas* sp.	+ (5)	+ (1)	+ (1)	-	±0,5cm	Cream
PR3	*Pseudomonas* sp.	+ (2)	+ (1)	+ (1)	-	-	Blue
PS11	*P. teatrolens*	+ (3)	+ (1)	+ (1)	-	-	Yellow
PS6	*P. chlororaphis* subsp. *aurantiaca*	+ (5)	+ (1)	+ (1)	-	-	Orange

[a] Numbers in brackets ( ) indicate the days of incubation until growth of *Pseudomonas* strains at different temperatures occurred

Table 3.3.2 - Phenotypic properties of 17 Enterobacteriaceae strains isolated from raw milk

Enterobacteriaceae strains		**Growth in BHI broth[a]**			**Gas production**	**Motility**	**Pigment production**
		7°C	**22°C**	**30°C**			
PS18	*Cit. freundii*	+ (4)	+ (1)	+ (1)	+	-	Brown/cream
PS37	*Cit. freundii*	+ (3)	+ (1)	+ (1)	+	+	Cream
PS25	*Ent. cloacae*	+ (5)	+ (1)	+ (1)	+	+	Colorless
PS57	*H. alvei*	+ (1)	+ (1)	+ (1)	+	+	Yellow/cream
PS85	*H. alvei*	+ (2)	+ (1)	+ (1)	+	+	Colorless
PS58	*H. alvei*	+ (1)	+ (1)	+ (1)	+	+	Yellow/cream
PS43	*H. alvei*	+ (1)	+ (1)	+ (1)	+	+	Brown/cream
PS46	*H. alvei*	+ (9)	+ (1)	+ (1)	+	+	Brown/cream
PS33	*S. marcescens*	+ (3)	+ (1)	+ (1)	-	+	Red/pink
PS69	*S. marcescens*	+ (5)	+ (1)	+ (1)	-	+	Red/pink
PS74	*S. marcescens*	+ (5)	+ (1)	+ (1)	-	+	Red/pink
PS90	*S. marcescens*	+ (5)	+ (1)	+ (1)	-	+	Red/pink
PS91	*S. marcescens*	+ (2)	+ (1)	+ (1)	-	+	Red/pink
PS92	*S. marcescens*	+ (3)	+ (1)	+ (1)	-	+	Red/pink
PS45	*S. marcescens*	+ (6)	+ (1)	+ (1)	-	+	Red/pink
PS36	*S. marcescens*	+ (2)	+ (1)	+ (1)	-	+	Red/pink
PS30	*S. marcescens*	+(18)	+ (1)	+ (1)	-	+	Red/pink

[a] Number in brackets ( ) indicate the incubation time required until growth of Enterobacteriaceae strains at different temperatures occurred

3.3.3 Growth and enzymatic activity at different temperatures

The growth ability of the 80 GNP strains was investigated at different temperatures (7, 22, 30 °C) over time to understand how storage conditions favoured or minimized their growth. For both *Pseudomonas* and Enterobacteriaceae, growth was visible for all the strains at the tested temperatures, as reported by other authors (Marchand et al., 2009), though growth rate differed during incubation. With the exception of one strain, *P. fulva* grew faster at 30 °C than at 22 °C, there were no detectable differences among the isolates in the growth test at 22 °C and 30 °C, and growth occurred already after 24 hours of incubation (Tables 3.3.1 and 3.3.2). Most of the strains were able to grow at a restricted temperature (7 °C) in the early days of incubation. Regarding *Pseudomonas* spp., growth was generally visible from 24h (6 strains) to 6 days, except for 10 strains showing lower growth rates for up to 30 days of incubation. Among the Enterobacteriaceae, fast growth was observed in almost all the strains between 24h (3 strains of *H. alvei*) and 6 days. Nine days were required to detect the growth of one of *H. alvei*, and one strain of *S. marcescens* showed the lowest growth (18 days).
All psychrotrophic strains were also tested for the production of extracellular proteases, lipases and lecithinases at 7, 22 and 30 °C up to 20 days. Individual results of enzymatic activities for *Pseudomonas* and Enterobacteriaceae strains are listed in Tables 3.3.3 and 3.3.4, respectively. Forty-one strains were found to be positive for all 3 enzymatic activities, and, *P. fluorescens* stood out as the species with the most number of positive strains for all enzymatic activities at the tested temperatures. Only a few data are available with regard to the influence of temperature on psychrotroph enzymatic activity, and these are limited to protease.
The highest number of positive strains at all incubation temperatures was observed for lipolytic activity (59/80), followed by proteolytic (31/80) and lechitinase activities (28/80). Enzymatic activities varied among the *Pseudomonas* and Enterobacteriaceae strains and were markedly influenced by incubation temperature, 30 °C being the optimal one.
With regard to protease production, *P. fragi*, *P. putida*, *P. fulva* and *P. teatrolens* species failed to produce proteases at all incubation temperatures, as did 3 strains of *P. aeruginosa* and 1 of *P. fluorescens*, *P. mosselii* and *Pseudomonas* sp.; among the Enterobacteriaceae, the production of proteases was restricted to *S. marcescens*. The protease production of *Pseudomonas* strains was studied by Marchand et al. (2009) who highlighted that *P. fragi* failed to produce proteases only at 30 °C, and observed the lowest number of protease producing strains at 30 °C. Conversely, in this study, the highest number of positive GNP for proteolytic activity was found at 30 and 22 °C 30 °C being the optimal temperature. Almost all the strains displayed marked proteolytic activity within 2 days at 30 °C, and within 3 days at 22 °C. Twenty-seven out of 63 of the *Pseudomonas* strains displayed protease production at 7 °C within at least 4 days, while four out of the 17 Enterobacteriaceae strains were protease positive, starting from the sixth day at 7 °C.
Lipase production on tributyrin agar plates was faster at 30 °C, nevertheless 68 strains displayed a noticeable lipolytic activity after incubation at 22 °C within 3 days; moreover 49 out of 63 *Pseudomonas* (77.77%) and 9 out of 17 Enterobacteriaceae (52.94%) strains showed lipase production at 7 °C within 10 days of incubation. Three strains belonging to *P. fluorescens* and one *Pseudomonas* sp. showed lipolytic activity after only 3 days at 7 °C, while it took at least 6 days for Enterobacteriaceae strains to produce lipolityc activity.
It was also observed that the number of *Pseudomonas* producing lecithinases increased with incubation temperatures, from a 39.68% of positive strains at 7 °C to a 55.55% of lecithinase-producing strains at 30 °C. On the other hand, almost half the Enterobacteriaceae (47%) were able to degrade lecithin at 30 °C and 22 °C, whereas only 10% displayed lecithinase activity at 7 °C.

Our data only partially agree with Dogan and Boor (2003) who found no evidence of lipolysis and lechitinase activity for *P. putida* strains, while the strains isolated in this study expressed lipolytic activity.

3.3.4 Assessment of *apr*X gene in psychrotrophic strains

Proteolytic psychrotrophic bacteria are the main microorganisms responsible for the spoilage of milk and milk products, due to their ability to produce thermostable proteases that hydrolyze casein and decrease the yields and sensory qualities of dairy products (Sørhaug & Stepaniak, 1997). *Pseudomonas* are known for their production of metalloproteases in the cold chain of raw milk (Craven & Macauley, 1992; Sorhaug & Stepaniak, 1997; Marchand et al., 2009). The production of monomeric extracellular proteases of molecular weight varying between 23 kDa and 56 kDa is a common feature of *P. fluorescens*. In this study the 80 GNP strains were screened for the presence of *apr*X gene by aprX-PCR test, even though they did not exhibit extracellular proteolytic activity when grown on skim milk agar. The *apr*X gene was detected in 19 out of 63 *Pseudomonas* strains, and was widespread among *P. fluorescens* (14/19), *P. rhodesiae* (2/2), *P. libanensis* and *P. chlororaphis* subsp. *aurantiaca* (Table 3.3.3). Among the Enterobacteriaceae, no amplification product was detected (Table 3.3.4), though all *S. marcescens* strains were proteolytic.
Martins and others (2005) already illustrated the presence of the *apr*X gene in proteolytic psychrotrophic bacteria isolated from raw milk. The *apr* gene, coding for heat-stable alkaline metalloproteases with a pH optimum of 6.5–8.0 (Fairbairn & Law, 1986; Woods et al., 2001), has been detected in proteolytic *P. fluorescens*, *P. tolaasii*, *P. aeruginosa*, *Pseudomonas* sp., *S. marcescens*, and *Flavobacterium-Cytophaga* isolated from soil (Bach et al., 2001).

Table 3.3.3 - An overview of *Pseudomonas* strains: enzymatic characterization at 7, 22 and 30 °C and detection of *apr*X gene

***Pseudomonas* strains**		**Proteolysis[a]**			***aprX***	**Lipolysis[a]**			**Degradation of lecithin[a]**		
		[b]7°C	**[c]22°C**	**[d]30°C**		**[b]7°C**	**[c]22°C**	**[d]30°C**	**[b]7°C**	**[c]22°C**	**[d]30°C**
PS34	*P. aeruginosa*	-	-	-	-	+ (10)	+ (2)	+ (1)	-	-	-
PS20	*P. aeruginosa*	-	+(2)	+ (1)	-	+ (9)	+ (3)	+ (2)	+ (7)	+ (2)	+ (2)
PS86	*P. aeruginosa*	-	+ (2)	+ (1)	-	-	+ (3)	+ (1)	-	-	-
PS65	*P. aeruginosa*	+ (5)	+ (3)	+ (1)	-	+ (7)	+ (3)	+ (3)	+ (6)	-	-
PS26	*P. aeruginosa*	-	-	-	-	-	-	-	-	+ (3)	+ (2)
PS27	*P. aeruginosa*	-	+ (1)	+ (1)	-	+ (6)	+ (1)	+ (1)	-	+ (2)	+ (1)
PS38	*P. aeruginosa*	-	+ (1)	+ (1)	-	+ (9)	+ (2)	+ (2)	-	+ (2)	+ (1)
PS44	*P. aeruginosa*	-	-	-	-	-	+ (6)	+ (3)	-	-	-
PS32	*P. aeruginosa*	-	+ (2)	+ (1)	-	+ (9)	+ (2)	+ (1)	-	+ (2)	+ (1)
PS42	*P. aeruginosa*	-	+ (2)	+ (1)	-	-	+ (2)	+ (1)	-	-	-
PS53	*P. aeruginosa*	-	+ (1)	+ (1)	-	+ (7)	+ (1)	+ (1)	-	+ (2)	+ (1)
PS87	*P. aeruginosa*	-	+ (2)	+ (1)	+	-	+ (4)	+ (1)	-	-	-
PS29	*P. aeruginosa*	-	+ (2)	+ (1)	-	+ (9)	+ (3)	+ (3)	-	-	-
PS67	*P. aeruginosa*	-	+ (2)	+ (1)	-	+ (8)	+ (2)	+ (1)	-	+ (2)	+ (2)

***Pseudomonas* strains**		**Proteolysis[a]**			***aprX***	**Lipolysis[a]**			**Degradation of lecithin[a]**		
		[b]7°C	**[c]22°C**	**[d]30°C**		**[b]7°C**	**[c]22°C**	**[d]30°C**	**[b]7°C**	**[c]22°C**	**[d]30°C**
PS59	*P. aeruginosa*	-	-	-	-	-	+ (4)	+ (2)	-	+ (3)	+ (2)
PS16	*P. fluorescens*	+ (7)	+ (2)	+ (1)	+	+ (7)	+ (2)	+ (2)	+ (6)	+ (3)	+ (1)
PS19	*P. fluorescens*	+ (6)	+ (3)	+ (1)	+	+ (6)	+ (1)	+ (1)	+(11)	+ (5)	+ (3)
PS23	*P. fluorescens*	-	+ (2)	+ (1)	+	+ (9)	+ (2)	+ (2)	+ (6)	+ (6)	+ (2)
PS24	*P. fluorescens*	+ (5)	+ (6)	+ (2)	+	+ (7)	+ (1)	+ (1)	+ (6)	+ (2)	+ (2)
PS14	*P. fluorescens*	+ (5)	+ (3)	+ (2)	+	+ (6)	+ (1)	+ (1)	+ (7)	+ (6)	+ (2)
PS64	*P. fluorescens*	+ (7)	+ (2)	+ (1)	+	+ (5)	+ (3)	+ (3)	+ (6)	+ (2)	+ (1)
PS60	*P. fluorescens*	+ (5)	+ (2)	+ (2)	-	+ (6)	+ (2)	+ (2)	+(10)	+ (2)	+ (2)
PS77	*P. fluorescens*	+(10)	+ (5)	+ (3)	-	+ (6)	+ (2)	+ (3)	+ (7)	+ (5)	+ (5)
PS78	*P. fluorescens*	+(10)	+ (3)	+ (2)	+	+ (6)	+ (2)	+ (3)	+(11)	+ (5)	+ (3)
PS28	*P. fluorescens*	+(10)	+ (3)	+ (2)	+	+ (7)	+ (2)	+ (1)	-	-	-
PS81	*P. fluorescens*	+(10)	+ (2)	+ (2)	+	+ (4)	+ (2)	+ (1)	-	-	-
PS68	*P. fluorescens*	-	-	-	-	+ (6)	+ (2)	+ (1)	-	-	-
PS56	*P. fluorescens*	+ (7)	+ (3)	+ (2)	-	+ (7)	+ (2)	+ (3)	+ (7)	+ (2)	+ (2)
PS73	*P. fluorescens*	+ (7)	+ (3)	+ (2)	+	+ (8)	+ (2)	+ (2)	-	-	-
M3	*P. fluorescens*	+ (8)	+ (2)	+ (2)	+	+ (3)	+ (1)	+ (2)	+(12)	+ (4)	+ (1)
M7	*P. fluorescens*	+ (8)	+ (2)	+ (1)	+	+ (3)	+ (1)	+ (1)	+ (8)	+ (4)	+ (2)
M37	*P. fluorescens*	+ (5)	+ (2)	+ (1)	+	+ (6)	+ (1)	+ (1)	+ (7)	+ (3)	+ (2)
M44	*P.fluorescens*	+ (5)	+ (2)	+ (2)	+	+ (3)	+ (1)	+ (1)	+ (7)	+ (3)	+ (1)
PR5	*P. fluorescens*	+ (6)	+ (2)	+ (1)	-	+ (6)	+ (2)	+ (3)	+(10)	+ (4)	+ (2)
PS55	*P. fragi*	-	-	-	-	+ (9)	+ (2)	+ (2)	-	+ (4)	+ (2)
PS12	*P. fragi*	-	-	-	-	+ (6)	+ (2)	+ (2)	-	-	-
PS47	*P. fragi*	-	-	-	-	+ (6)	+ (2)	+ (1)	-	-	-
PS52	*P. fulva*	-	-	-	-	+(10)	+ (3)	+ (3)	-	-	-
PS10	*P. fulva*	-	-	-	-	+(10)	+ (3)	+ (2)	-	-	-
PS51	*P. fulva*	-	-	-	-	+ (9)	+ (6)	+ (3)	-	-	-
PS1	*P. fulva*	-	-	-	-	+(10)	+ (6)	+ (3)	-	-	-
PS49	*P. libanensis*	+ (7)	+ (2)	+ (1)	+	+ (6)	+ (2)	+ (3)	+ (8)	+ (3)	+ (2)
PS70	*P. mosselii*	-	-	-	-	-	+ (1)	+ (1)	+(10)	+ (1)	+ (1)
PS31	*P. mosselii*	+(10)	+ (2)	+ (1)	-	+ (9)	+ (1)	+ (1)	-	+ (2)	+ (1)
PS39	*P. mosselii*	+(10)	+ (1)	+ (1)	-	-	+ (1)	+ (1)	+(13)	+ (1)	+ (1)
PS4	*P. putida*	-	-	-	-	-	+ (3)	+ (1)	-	-	-
PS93	*P. putida*	-	-	-	-	-	+ (5)	+ (3)	-	+ (3)	+ (1)
PS40	*P. putida*	-	-	-	-	+(10)	+ (2)	+ (3)	-	-	-
PS41	*P. putida*	-	-	-	-	+ (8)	+ (2)	+ (1)	-	-	-
PS8	*P. putida*	-	-	-	-	-	+ (3)	+ (1)	-	-	-

***Pseudomonas* strains**		**Proteolysis[a]**			***aprX***	**Lipolysis[a]**			**Degradation of lecithin[a]**		
		[b]7°C	**[c]22°C**	**[d]30°C**		**[b]7°C**	**[c]22°C**	**[d]30°C**	**[b]7°C**	**[c]22°C**	**[d]30°C**
PS83	*P. putida*	-	-	-	-	+ (8)	+ (3)	+ (3)	-	-	-
PS17	*P. putida*	-	-	-	-	+ (7)	+ (3)	+ (1)	-	-	-
PS62	*P. rhodesiae*	+ (5)	+ (2)	+ (1)	+	+ (9)	+ (2)	+ (3)	+ (7)	+ (2)	+ (2)
PS84	*P. rhodesiae*	+ (5)	+ (2)	+ (1)	+	+ (7)	+ (3)	+ (1)	+ (7)	+ (5)	+ (2)
PS66	*Pseudomonas* sp.	-	+ (2)	+ (1)	-	-	+ (3)	+ (2)	-	-	-
PS88	*Pseudomonas* sp.	-	+ (2)	+ (1)	-	+(10)	+ (4)	+ (1)	-	-	-
PS72	*Pseudomonas* sp.	-	-	-	-	-	+ (2)	+ (1)	-	-	-
PS54	*Pseudomonas* sp.	-	+ (2)	+ (2)	-	+ (8)	+ (2)	+ (2)	+(10)	+ (2)	+ (2)
PS61	*Pseudomonas* sp.	-	+ (5)	+ (2)	-	+ (8)	+ (5)	+ (2)	-	+ (5)	+ (3)
PS13	*Pseudomonas* sp.	+ (4)	+ (1)	+ (1)	-	+ (6)	+ (2)	+ (1)	+(10)	+ (1)	+ (1)
PS75	*Pseudomonas* sp.	+ (7)	+ (2)	+ (1)	-	+ (8)	+ (2)	+ (2)	-	-	-
PR3	*Pseudomonas* sp.	+ (5)	+ (2)	+ (4)	-	+ (3)	+ (2)	+ (3)	-	-	-
PS11	*P. teatrolens*	-	-	-	-	-	+ (6)	+ (2)	-	-	-
PS6	*P. chlororaphis* subsp. *aurantiaca*	+ (5)	+ (2)	+ (1)	+	+ (5)	+ (2)	+ (1)	+ (7)	+ (2)	+ (2)

[a] Numbers in brackets ( ) indicate the incubation time required to visualize enzymatic activities

[b] Proteolysis and lipolysis were visualized up to 10 days and degradation of lecithin up to 20 days

[c] Proteolysis , lipolysis and degradation of lecithin were visualized up to 7 days

[d] Proteolysis and lipolysis were visualized up to 3 days and degradation of lecithin up to 5 days

Table 3.3.4- An overview of Enterobacteriaceae strains: enzymatic characterization at 7, 22 and 30 °C and detection of *apr*X gene

Enterobacteriaceae strains		**Proteolysis[a]**			***apr*X**	**Lipolysis[a]**			**Degradation of lecithin[a]**		
		[b]7°C	**[c]22°C**	**[d]30°C**		**[b]7°C**	**[c]22°C**	**[d]30°C**	**[b]7°C**	**[c]22°C**	**[d]30°C**
PS18	*Cit. freundii*	+ (4)	+ (1)	+ (1)	+	-	-	-	-	-	-
PS37	*Cit. freundii*	+ (3)	+ (1)	+ (1)	+	-	+ (3)	+ (1)	-	-	-
PS25	*Ent .cloacae*	+ (5)	+ (1)	+ (1)	+	-	-	-	-	-	-
PS57	*H. alvei*	+ (1)	+ (1)	+ (1)	+	+ (6)	+ (2)	+ (3)	-	-	-
PS85	*H. alvei*	+ (2)	+ (1)	+ (1)	+	+ (7)	+ (3)	+ (1)	-	-	-
PS58	*H. alvei*	+ (1)	+ (1)	+ (1)	+	+ (6)	+ (1)	+ (2)	-	-	-
PS43	*H. alvei*	+ (1)	+ (1)	+ (1)	+	-	-	-	-	-	-
PS46	*H. alvei*	+ (9)	+ (1)	+ (1)	+	+ (6)	+ (2)	+ (3)	-	-	-
PS33	*S. marcescens*	+ (3)	+ (1)	+ (1)	-	+ (9)	+ (1)	+ (2)	-	+ (1)	+ (1)
PS69	*S. marcescens*	+ (5)	+ (1)	+ (1)	-	-	+ (2)	+ (2)	+(12)	+ (2)	+ (2)
PS74	*S. marcescens*	+ (5)	+ (1)	+ (1)	-	+(10)	+ (2)	+ (3)	+(12)	+ (1)	+ (1)
PS90	*S. marcescens*	+ (5)	+ (1)	+ (1)	-	-	+ (2)	+ (2)	+(10)	+ (1)	+ (1)
PS91	*S. marcescens*	+ (2)	+ (1)	+ (1)	-	-	+ (3)	+ (1)	-	+ (3)	+ (2)
PS92	*S. marcescens*	+ (3)	+ (1)	+ (1)	-	+ (6)	+ (3)	+ (1)	+ (7)	+ (5)	+ (2)
PS45	*S. marcescens*	+ (6)	+ (1)	+ (1)	-	+(10)	+ (2)	+ (2)	-	+ (1)	+ (1)
PS36	*S. marcescens*	+ (2)	+ (1)	+ (1)	-	-	+ (3)	+ (2)	-	+ (4)	+ (2)
PS30	*S. marcescens*	+(18)	+ (1)	+ (1)	-	+ (8)	+ (2)	+ (3)	-	-	-

[a] Numbers in brackets ( ) indicate the incubation time required to visualize enzymatic activities

[b] Proteolysis and lipolysis were visualized up to 10 days and degradation of lecithin up to 20 days

[c] Proteolysis , lipolysis and degradation of lecithin were visualized up to 7 days

[d] Proteolysis and lipolysis were visualized up to 3 days and degradation of lecithin up to 5 days

3.4 Conclusions

Psychrotrophic bacteria play a major role in the spoilage of cold milk and dairy products, and their proteolytic and lipolytic enzymes constitute one of the main limiting factors for maintaining the technological and sensory quality of milk. The presence of GNP bacteria in raw milk, bacteria harbouring spoilage features, and the predominance of *Pseudomonas* spp. and Enterobacteriaceae have been evidenced in Lombardy raw milk, where high biodiversity is highlighted. This study provides a better understanding of the outgrowth of GNP bacteria in milk, and the different enzymatic traits that are demonstrated to be more influenced by temperature conditions than growth rate. A strict control of storage temperature, along with the reduction of GNP contamination, can constitute an effective control measure against spoilage events. Indeed, the prevention of deterioration in processed milk and dairy products calls for a rapid detection of enzymes exhibiting high protein and lipid degrading effects in raw milk. Therefore more research is needed towards the early detection of spoilage phenomena.

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3.6 Characterization of volatile compounds in milk contaminated with psychrotrophic bacteria by SPME gas chromatography-mass spectrometry

Psychrotrophic Gram-negative bacteria, mostly *Pseudomonas* spp. and Enterobacteriaceae, play a leading role in spoilage of milk and dairy products (Walker, 1988; Sorhaug & Stepaniak, 1997; Morales et al., 2003; Chaves-Lopez et al., 2006). The main source of psychrotrophic bacteria that become predominant during the refrigerated storage of raw milk, is inadequately sanitized milking equipment (Cousin, 1982) and common sources of post-pasteurization contamination by psychrotrophs are improperly cleaned pasteurizers and filling machines (Gruetzmacher & Bradley, 1999). In milk and dairy products spoilage strongly depends on the production of heat-resistant extracellular enzymes by psychrotrophic bacteria. Mainly proteases and lipases are capable of releasing undesired metabolic compounds arising from nutrient degradation at low temperatures and responsible for off-flavours in milk. Proteases that degrade casein, are associated with textural changes (structural defects) in milk such as gelation and increased viscosity and with unclean and bitter flavours in cheese and other dairy products. On the other hand, rancid, soapy and fruity aromas (flavour defects) are caused by lipases due to the fat breakdown in cream, butter, cheese and UHT products. Literature references have reported VOCs profile of raw (Bassette et al., 1966; Vazquez-Landaverde et al., 2005; Vazquez-Landaverde et al., 2006; Hettinga et al., 2008) pasteurized and sterilized (Contarini et al., 1997; Valero et al., 1999; Valero et al., 2001; Contarini & Povolo, 2002) milk samples. Off-flavour development at a given storage time, usually at the end of shelf-life of the product, (Urbach & Milne, 1987; Jenq et al., 1988; Cormier et al., 1991; Leong et al., 1992; Vallejo-Cordoba & Nakai, 1994; Marsili, 1999b; Contarini & Povolo, 2002) and VOCs changes affected by packaging material (Karatapanis et al., 2006) in milk have also been studied. Recent studies reported the production of volatile compounds associated with bacteria isolated from different foodstuffs, such as beef (Ercolini et al., 2009) and shrimps (Jaffrès et al., 2011) and seven different species of *Pseudomonas* of dairy origin were screened for their VOC production in cheese (Morales et al., 2005) but very limited information exists in the literature on changes in flavour compounds of milk spoiled with different psychrotrophic bacteria. In addition, studies on the correlation between spoilage-related molecules release and the development of specific microbial species in milk are still not available. *Pseudomonas* is the psychrotrophic genus of greatest concern with respect to the spoilage of milk and dairy products (Ternström et al., 1993; Ralyea et al., 1998). Enterobacteriaceae have also been frequently encountered in milk and during cheese production (Sorhaug & Stepaniak, 1997; Morales et al., 2003; Mounier et al., 2005; Chaves-Lopez et al., 2006), and they are considered as a negative flora (i.e. marker for hygiene, cheese texture defects, blowing and off-flavours). The purpose of the second study of this PhD thesis was to investigate the spoilage potential of lipo-proteolytic psychrotrophic bacteria, previously isolated from raw milk (Decimo et al., 2014), assessing their volatile fraction using solid-phase microextraction (SPME) and gas chromatography coupled to mass spectrometry (GC-MS). A further aim of this work was to identify molecular markers for the early detection of milk spoilage.

3.7 Material and methods

3.7.1 Bacterial strains and milk contamination

The strains tested in this study had previously been isolated from raw milk samples, identified and characterized for their enzymatic activity at different temperatures (Decimo et al., 2014). Since the spoilage potential of bacteria is mainly related to their lipo-proteolytic activity, five

psychrotrophic strains, showing enzymatic activities both at 7 and 22°C, except for *P. mosselii* PS39 that was negative for lipase production at 7°C, were selected for the milk contamination and VOC monitoring experiments as follows.
Aliquots of 200 mL of whole UHT milk were spiked with 10^6 CFU mL $^{-1}$ of an overnight BHI cultures of each of the following psychrotrophic strains incubated at 30 °C: *Pseudomonas fluorescens* PS14, *Ps. fragi* PS55, *Ps. mosselii* PS39, *Ps. rhodesiae* PS62 and *Serratia marcescens* S92. This inoculum level was in chosen since bacteria need to reach a similar population before significant proteo-lipolytic activity occurs (Cogan, 1980; Outtara et al., 2004). The control sample of UHT milk was incubated along with the samples contaminated with psychrotrophic bacteria at 10°C for 5, 10, 20 days and at 5°C for 20 days.

3.7.2 VOC determination by SPME/GC-MS

Volatile compounds in UHT milk were analysed using a gas chromatography device, model GC 6850, Agilent (Agilent Technologies, Barcelona, Spain) coupled with a mass spectrometer 5975 C VL (Agilent) series GC/MSD with the Triple-Axis Detector (TAD) after solid-phase microextraction.
Briefly, spoiled UHT milk samples (7 ml) were heated in 15 ml vials sealed with polypropylene screw-on caps and PTFE/silicone septa (Supelco Bellefonte, PA, USA) for 15 min at 45°C to equilibrate the system.Volatiles were extracted from the headspace of the vial for 30 min with a 75 µm carboxen/polydimethylsiloxane (CAR/PDMS) SPME fiber (Supelco). Peak separation was carried out on a 60 m length × 0.25 mm internal diameter × 1 µm film thickness column (Quadrex 007-5MS-60-1.0, DB5, Supelco). Volatile compounds were analyzed following the gas chromatographic conditions described by Contarini and Povolo (2002), modifying MS settings and oven temperature. Briefly thermal desorption of volatile compounds was carried out by keeping the SPME fiber in the split/splitless injector at 270°C for 3 min in splitless mode; oven temperature was held at 40°C for 8 min, programmed to 150°C at a rate of 4°C/min, and held at 150°C for 10 min. Helium was used as a carrier gas at a flow rate of 1.0 ml/min. MS temperature adopted were as follows: interface, 270°C; source, 230°C; quadrupole, 150°C; acquisition was performed in electron impact (EI) mode (70 eV) and mass range used was *m/z* 33-300.
Peak identification of compounds was conducted by comparing their retention times and the collected ion (mass) spectra to the reference spectral library, NIST MS search 2.0 library (NIST; Perkin–Elmer; Waltham, MA). An 85-90% level of certainty in the spectral match criteria was used as a cut-off for spectral identification. The limit of detection (LOD) of a compound was considered to be the minimum concentration at which it could be identified by its mass spectrum, retention time and with a relative abundance of at least 3 times the signal-to-noise ratio. The peak areas of volatile compounds were taken to be their relative abundances and were estimated as mean value of duplicate analysis for each sample.

3.7.3 Sensory evaluation

Descriptive aroma analysis was performed on milk samples stored at 10°C after 10 day of incubation. Descriptive sensory test was performed to differentiate between the aroma of milk spoiled by the 5 different psychrotrophic strains. Samples was evaluated by a panel of 10 tasters (7 females and 3 males) selected from the members of the Department of Nutrition, Food Science and Technology of the Complutense University of Madrid. The panellists were previously trained in the sensory assessment of dairy products. During the training, reference models (rotten egg, cut grass, fruity, sweaty, burnt milk or pudding, sulphur, metallic, baby

vomit, rancid, pungent, alcohol or acetic acid, unpleasant buttery, cooked) were prepared in order to familiarise the testers with the expected flavours resulting from psychrotrophic contamination of milk. Before sensory analysis, the samples were kept to temperate for 15 min at room temperature. For evaluation, 100 ml of milk were placed into 250-ml Pirex sniff-top bottles wrapped in aluminum foil. The bottles were wrapped in aluminium foil to prevent panelists from visually assessing the samples. Panellists were also asked to give information about the rate of coagulation of milk after sniffing samples.

3.8 Results and discussion

3.8.1 VOC determination of spoiled UHT milk samples during storage

Qualitative data were obtained by calculating the area of peak relative to the quantifier ion of the mass spectrum for each of the VOC. If the relative abundance of VOCs ranged from 7 to 8 log peak Area, the corresponding compounds were considered highly produced (++), on the other hand if the peak measurements resulted between 6,5-5 log Area, these compounds showed a medium production (+) while if the VOC area values were less than 4 log Area, the corresponding compounds were flagged as detected in trace amount (t)
In Figure 3.8.1 is shown an example of an SPME-GC/MS metabolite profile obtained for the UHT milk spiked with *P. fluorescens* PS14 at 10°C after 10 days of storage.

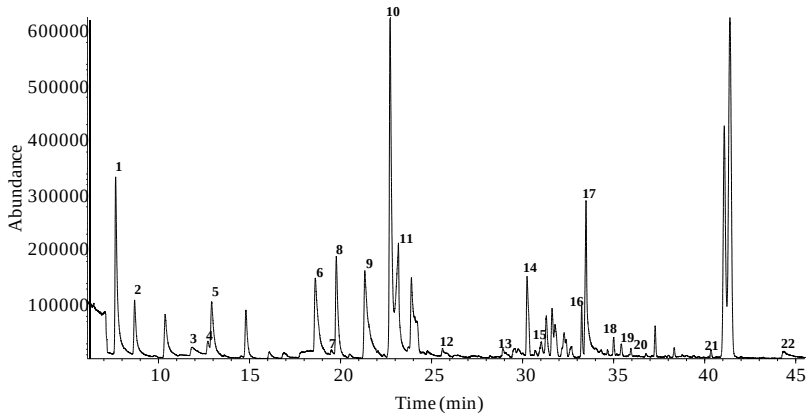

Figure 3.8.1 SPME-GC/MS chromatogram of UHT milk contaminated with *P. fluorescens* PS14. Peaks: (1) acetone; (2) dimethylsulphide; (3) hexane; (4) 2,3 butanedione; (5) 2-butanone; (6) 2-pentanone; (7) pentanal; (8) mercaptoacetone, (9) 3-methylbutanol;(10) disulphide dimethyl; (11) butanoic acid, (12) hexanal; (13) dimethyl sulfoxide; (14) 2-heptanone; (15) heptanal; (16) dimethyl sulfone; (17) hexanoic acid; (18) 5-hepten-2one-6-methyl; (19) benzaldehyde; (20) octanal;(21) 2-nonanone; (22) octanoic acid.

Fiftysix VOCs belonging to 7 chemical groups (aldehydes, ketones, fatty acids, esters, alcohols, sulphur compounds and hydrocarbons) were identified.

VOCs detected in control UHT milk before incubation (Table 3.8.1, T_0) were acetone, butan-2-one, pentan-2-one, pentanal, 2,3,3-trimethyl pentane, methyldisulfanylmethane, hexanal, heptan-2-one, methylsulfonylmethane, benzaldehyde. Methyl ketones with 3, 4, 5 and 7 carbon atoms, resulted the main group of volatile compounds as was reported for UHT milk at day 0 of storage by Valero et al. (2001) and for volatile fraction of direct UHT milk by Contarini and Povolo (2002). Whereas acetone and butan-2-one are supposed to be derived from bovine metabolism (Bassette et al., 1966; Gordon & Morgan, 1972; Urbacch & Milne, 1987), pentan-2-one and heptan-2-one are in part thermally-induced products, arising from β-ketoacid decarboxylation (Calvo & de la Hoz, 1992); they can also formed by β-oxidation of fatty acids, followed by decarboxylation (Grosch, 1982). As reported by Contarini and Povolo (2002) methyl ketones seemed to have the highest correlation to the severity of heat treatment on milk, being heptan-2-one a suitable marker for heat processing. Regarding aldehydes, the presence of pentanal and hexanal could be due to light exposure or Cu oxidation (Cadwallader & Howard, 1998; Marsili & Miller, 1998) or to oxidation of unsatured fatty acids whereas benzaldehyde could be generated from phenylalanine (Nursten, 1997). Differently from our data, Valero et al. (2001) found that the main aldehydes in UHT milk were acetaldehyde, butanal, hexanal, 2-methyl butanal, 3-methyl butanal and benzaldehyde. Methyldisulfanylmethane and methylsulfonylmethane probably derived from methionine (Dumont & Adda, 1979) and have a strong contribution (along with methyl ketones) to the aroma of UHT milk (Badings, 1984) and have also been related to the intensity of thermal treatment (Bosset et al., 1994). Vazquez-Landaverde et al. (2006) reported that whole UHT milk contained substantially high concentrations of hydrogen sulfide, methanethiol, carbon disulfide, dimethyl trisulfide, and dimethyl sulfoxide being methanethiol, dimethyl trisulfide and dimethyl sulfide the most important contributors to the sulfurous note in milk. The 2,3,3-trimethyl pentane hydrocarbon was identified in this study in the headspace of control UHT milk sample whereas 2,2,4-trimethyl pentane was found in whole pasteurized milk packaged in different material during storage time by Karatapanis et al. (2006).

As shown in Table 3.8.1, a general trend to VOCs production during the storage time at 10°C was observed in control UHT milk sample. The formation of carbon disulphide, heptanal, 6-methylhept-5-en-2-one was highlighted after 5 days of incubation while hexane (trace amount), 3-methylbutan-1-ol (t), pentan-1-ol (t), 2,2-dimethyl heptane, nonan-2-one, octanal (t) were formed after 10 days and methylsulfanylmethane, butane-2,3-dione were found only after 20 days. Whereas some compounds such as alcohols, aldehydes, sulphur compounds, and hydrocarbons detected at 10°C, was not noticed in same samples stored at 5°C after 20 days.

Table 3.8.1 VOCs detected in UHT milk at T_0 and during storage time (days) at 10°C and 5°C

VOCs	T_0	Storage time (d) at 10°C			Storage time (d) at 5°C
		5	10	20	20
Alcohols					
3-Methylbutan-1-ol	nd	nd	**t**	**t**	nd
Pentan-1-ol	nd	nd	**t**	+	nd
Aldehydes					
Pentanal	+	+	+	+	+
Hexanal	+	+	+	+	+
Heptanal	nd	+	+	+	nd
Octanal	nd	nd	nd	[b]**t**	**t**
Benzaldehyde	+	+	+	+	+
Ketones					
Acetone	+	+	+	+	+
Butane-2,3-dione	[a]nd	nd	nd	+	+
Butan-2-one	+	+	+	+	+
Pentan-2-one	+	+	+	+	+
Heptan-2-one	+	+	+	+	+
6-Methylhept-5-en-2-one	nd	+	+	+	+
Nonan-2-one	nd	nd	+	+	+
Sulphur compounds					
Methylsulfanylmethane	nd	nd	nd	+	nd
Carbon-disulphide	nd	+	+	+	nd
Methyldisulfanylmethane	+	+	+	+	+
Methylsulfonylmethane	+	+	+	+	+
Hydrocarbons					
Hexane	nd	nd	+	**t**	nd
2,3,3-Trimethylpentane	+	+	+	+	+
2,2-Dimethyl heptane	nd	nd	+	+	nd

[a]nd, not detected

[b]t, molecole occuring in trace amount

The results of the VOC analysis of the milk samples spoiled with five different psychrotrophic bacteria during the storage time at 10°C and 5°C are reported in Tables 3.8.2 and 3.8.3. Qualitative data indicated that the VOCs patterns of spoiled milk samples were partially identical and considerable differences were found concerning the evolution of VOCs production during the storage time.

Seven ketones (acetone, butan-2-one, pentan-2-one, heptan-2-one, 6-methylhept-5-en-2-one, nonan-2-one, butane-2,3-dione), 4 fatty acids (acetic, butanoic, hexanoic, octanoic acids), 4 sulphur compounds (methylsulfanylmethane, methyldisulfanylmethane, methylsulfonylmethane**,** methylsulfinylmethane), 4 aldehydes (exanal, heptanal, octanal, benzaldehyde) and 2 alcohols (2-methylpropan-1-ol**,** 3-methylbutan-1-ol) were detected in all

spoiled milk samples at both incubation temperatures (Tables 3.8.2 and 3.8.3), whereas others, distinctive of specific strains, were produced only at 10 °C.

With regard to VOC production during storage at 10 °C the progressive formation of new volatile compounds, mostly sulphur compounds, fatty acids, esters and alcohols was evident from 5 to 20 days. Among the predominant alcohols found in spoiled milk, 2-methylpropan-1-ol was produced by *P. fragi*, *P. rhodesiae* and *S. marcescens* at all sampling times and it was detectable in milk spiked with *P. mosselii* after 10 days of storage whereas it was always absent in the headspace of UHT control sample and of milk contaminated with *P. fluorescens*. 3-methylbutan-1-ol, occurred in trace amount in UHT control samples from the 10th day of storage, was produced in milk samples contaminated with *P. fragi*, *P. mosselii*, *P. rhodesiae* and *S. marcescens* already after 5 days, while the branched-chain alcohol was present only in minimal traces after 5 days of incubation in milk spoiled with *P. fluorescens* PS14 and increased during the storage time, being appreciable after 10 days. Morales et al. (2005) reported that 3-methylbutan-1-ol was mainly produced by *P. libanensis* and *P. rhodesiae* in laboratory-scale cheeses and ethanol, 3-methylbutan-1-ol and 2-methylpropan-1-ol were the predominant alcohols in cheeses inoculated with Enterobacteriaceae (Morales et al., 2004). Regarding aldehydes production, no significant differences were recorded between UHT control and milk samples contaminated with psychrotrophic strains. Octanal, only found in minimal traces at the end of storage of UHT control milk, showed a small peak in all chromatograms of the spoiled milk. Valero et al. (2001) reported the production of octanal not in whole but only in skimmed UHT milk in low concentrations (starting) from 45 days of storage at 25°C as a result of lipid oxidation. Concerning ketones, butane-2,3-dione (diacetyl) production was recorded in all spoiled milk samples at different storage times. *P. rhodesiae*, *S. marcescens* and *P. mosselii* were the strongest producing-species since the 5th day of incubation; diacetyl was detected in the headspaces of milk contaminated with *P. fluorescens* and *P. fragi* after 10 and 20 days of storage at 10 °C, respectively. Strangely, butan-2-one was found in minimal traces in milk spoiled with *S. marcescens* after 5 and 10 days of storage, despite its important presence in UHT control milk. The ketone butan-2-one could be derived from butane-2,3-dione, which is produced by fermentation of lactose and metabolism of citrate. The reduced amounts of butan-2-one by *Serratia marcescens* could be attributed to the variations of the enzyme activity in metabolic pathway. Nonan-2-one was almost absent only in UHT control sample and in milk spoiled with *P. fragi* and *P. mosselii* after 5 days of storage. Among sulphur compounds methylsulfanylmethane, showing a low detection level in UHT milk control, was the major sulphur compound found in the headspace of all spoiled milk samples at all incubation time. In particular it occurred in trace amount in *P. fluorescens* spoiled milk after 5 days of storage while it was generated in appreciable amount in all other contaminated samples during incubation time. *P. rhodesiae* and *S. marcescens* showed formation of the methylsulfinylmethane already after 5 days of storage while no production of this sulphur compound was found in unspoiled UHT milk during the whole storage time as well as in milk spoiled with *P. fluorescens* and *P. fragi* till 5 and 10 days of storage, respectively. No fatty acids were found in the headspaces of UHT control sample during storage time and in milk spoiled with *P. fragi* after 5 and 10 of storage at 10 °C. *P. fluorescens* released butanoic and hexanoic acids at all storage times while acetic and octanoic acids were detected after 20 and 5 days of storage, respectively. *P. mosselii* produced butanoic acid already after 5 day of storage, hexanoic and octanoic acids after 20 days and acetic acid was identified with a small peak only at the end of storage. *P. rhodesiae* and *S marcescens* seemed to be the strongest acid-producing species, showing an early appearance of all four fatty acids during storage.

Qualitative data indicated that each contamination resulted in complex and specific VOC profiles of the milk samples during the storage time at 10 °C (Table 3.8.2). Some branched-chain alcohols (3-methylbutan-2-ol and 3-methylhexan-2-ol) and pentan-1-ol occurred only in the headspace of milk spoiled with *P. fragi* at different times The only distinctive aldehyde was nonanal, found in the headspace of *P. fragi* and *P. rhodesiae* after 10 days and at the end of storage, respectively. Among distinctive ketones, 3-hydroxybutan-2-one (acetoin) was the most interesting ketone, due to its absence in UHT control sample and its early detection in milk spoiled with *S. marcescens* and *P. rhodesiae*. Hexan-2-one was detected only in the headspace of milk samples spiked with *P. fluorescens* and *P. mosselii* at the end of storage while *P. rhodesiae* was the only species releasing pentane-2,3-dione since 10th day of incubation. The samples spoiled by *P. mosselii* and *P. fluorescens* displayed the production of distinctive sulphur compounds (methylsulfanyldisulfanylmethane, methylsulfonylsulfanylmethane, 1-sulfanylpropan-2-one), being *P. mosselii* the faster producer (already after 5 days of storage). Milk contaminated with. *S. marcescens* was characterized by the presence of pentanoic and 3-methylbutanoic acids already after 5 days of storage and heptanoic acid from 10th day of incubation while it took *P. rhodesiae* more than 10 days to produce detectable amount of these compounds. *P. mosselii* was found to release the highest diversity of methyl esters during storage, producing methyl butanoate from the 5th day, and S-methyl butanethioate and S-methyl ethanethioate after 10 days. The milk contaminated with *S. marcescens* was the richest in ethyl esters displaying ethyl butanoate from the 5th day and ethyl hexanoate and ethyl acetate after 10 and 20 days of incubation respectively; in addition, 3-methylbutyl acetate was detected after 10 days of storage. The sample spoiled with *P. fragi* showed the presence of ethyl acetate, methyl butanoate and ethyl butanoate after 5, 10 and 20 days respectively. Among hydrocarbons, milk contaminated with *P. fragi* was characterized by the presence of 3,3-dimethylhexane during the entire storage time while heptane and 3-methylhexane were produced only by *P. rhodesiae* after 10 days of storage as well as hexane in milk spoiled with *P. fluorescens*.

Overall, among alcohols, 3-methylbutan-1-ol and 2-methylpropan-1-ol were the predominant alcohols in the headspace of spoiled milk samples during storage time at 10°C, being *P. fragi* the species that produced the greater diversity of alcohols. *P. rhodesiae* resulted as the species producing the highest number and diversity of ketones (9 different ketones) during storage at 10°C, whereas Morales et al. (2005) found that strains belonging to *P. rhodesiae* and *P. lundensis* produced seven different ketones in cheese samples, being *P. lundendis* the strongest producer. *P. mosselii* was by far the strongest sulphur compound-producing species (7 different sulphur compounds). Our results are in agreement with those of Morales et al. (2005) where methylsulfanylmethane was the major sulphur compound being *P. libanensis*, *P. brenneri* and *P. rhodesiae* the strongest producers. *P. rhodesiae* was the species producing the highest number of fatty acids, in agree with results reported for the headspace of cheese made from milk samples inoculated with six different *Pseudomonas* strains, where *P. rhodesiae* produced high amounts of butanoic and hexanoic acids and the highest levels of total acids (Morales et al., 2005). The highest production of fatty acids was noticed also in milk spoiled with *S. marcescens*, responsible of flavour defects and impairment in milk and dairy products during cold storage for its ability to produce lipase as reported by Abdou (2003). Moreover, *Serratia* genus has been already described in milk (Tornadijo et al., 1993) and cheese (Martin-Platero et al., 2009) and has been shown to affect milk and cheese sensory quality due to high proteolytic activities and dimethyl sulphide production (Morales et al. 2003; Chaves-Lopez et al., 2006;). *P. fragi* resulted the strongest producer of ethyl-acetate responsible for the fruity flavor defect, as previously reported in literature (Pereira & Morgan, 1958; Reddy et al., 1968; Cormier et al., 1991; Hayes et al., 2002). Other esters were detected in milk spoiled with *P. mosselii* and *S. marcescens*, differently from Morales et al.(2005) that found *P. rhodesiae* the strongest ester-producing species in cheese samples.

Table 3.8.2 VOCs detection in spoiled UHT milk during storage time (days) at 10°C

VOCs	UHT control			*P. fluorescens* PS14			*P. fragi* PS55			*P. rhodesiae* PS62			*P. mosselii* PS39			*S. marcescens* S92		
	5	10	20	5	10	20	5	10	20	5	10	20	5	10	20	5	10	20
Alcohols																		
2-Methylpropan-1-ol	[a]**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	+	+	+	+	+	+	**nd**	+	+	+	+	+
3-Methylbutan-1-ol	**nd**	[b]**t**	**t**	**t**	+	++	+	+	+	++	++	++	+	+	++	++	++	++
3-Methylbutan-2-ol	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	+	+	+	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**
3-Methylhexan-2-ol	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	+	+	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**
Pentan-1-ol	**nd**	**t**	+	**nd**	**nd**	**nd**	+	+	+	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**
Aldehydes																		
Exanal	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Heptanal	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Octanal	**nd**	**nd**	**t**	**t**	**t**	**t**	**t**	**t**	**t**	**t**	**t**	**t**	**t**	**t**	**t**	**t**	**t**	**t**
Nonanal	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	+	**t**	**nd**	**nd**	+	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**
Benzaldehyde	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ketones																		
Acetone	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Butan-2-one	++	++	++	+	+	++	+	+	+	++	++	++	+	++	++	**t**	**t**	+
3-Hydroxybutan-2-one	**nd**	**nd**	**nd**	**nd**	**nd**	+	**nd**	**nd**	**nd**	+	+	++	**nd**	**nd**	**nd**	+	++	++
Pentan-2-one	+	+	++	+	+	++	+	+	+	+	+	++	+	++	++	+	++	++
Hexan-2-one	**nd**	**nd**	**nd**	**nd**	**nd**	+	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	+	**nd**	**nd**	**nd**
Heptan-2-one	+	+	+	+	+	+	+	+	+	+	++	++	+	+	+	+	++	++
6-Methylhept-5-en-2-one	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nonan-2-one	**t**	+	+	+	+	+	**t**	+	+	+	+	+	**t**	+	+	+	+	+
Butane-2,3-dione	**nd**	**nd**	+	**nd**	+	+	**nd**	**nd**	+	++	++	++	+	+	+	++	++	+
Pentane-2,3-dione	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	+	+	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**

VOCs	UHT control			*P. fluorescens* PS14			*P. fragi* PS55			*P. rhodesiae* PS62			*P. mosselii* PS39			*S. marcescens* S92		
	5	10	20	5	10	20	5	10	20	5	10	20	5	10	20	5	10	20
Sulphur compounds																		
Methylsulfanylmethane	**t**	**t**	+	**t**	++	++	+	++	++	++	++	+	+	+	++	++	++	++
Methyldisulfanylmethane	+	+	++	+	+	+	+	+	+	+	+	+	++	++	++	+	+	+
Methylsulfanyldisulfanylmethane	**nd**	**nd**	**nd**	**nd**	**nd**	+	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	+	+	+	**nd**	**nd**	**nd**
Methylsulfonylmethane	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Methylsulfonylsulfanylmethane	**nd**	**nd**	**nd**	**nd**	**nd**	+	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	+	+	+	**nd**	**nd**	**nd**
Methylsulfinylmethane	**nd**	**nd**	**nd**	**nd**	+	+	**nd**	**nd**	+	+	+	+	**t**	+	+	+	+	+
1-Sulfanylpropan-2-one	**nd**	**nd**	**nd**	**nd**	+	+	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	+	+	+	**nd**	**nd**	**nd**
Fatty acids																		
Acetic acid	**nd**	**nd**	**nd**	**nd**	**nd**	+	**nd**	**nd**	+	+	++	++	**nd**	**nd**	**t**	++	++	++
Butanoic acid	**nd**	**nd**	**nd**	+	+	+	**nd**	**nd**	+	++	++	++	+	++	++	+	+	+
Pentanoic acid	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	+	+	**nd**	**nd**	**nd**	+	+	+
Hexanoic acid	**nd**	**nd**	**nd**	+	+	+	**nd**	**nd**	+	++	++	++	**nd**	**nd**	+	+	+	+
Heptanoic acid	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	+	**nd**	**nd**	**nd**	**nd**	+	+
Octanoic acid	**nd**	**nd**	**nd**	**nd**	+	+	**nd**	**nd**	+	+	++	++	**nd**	**nd**	+	**nd**	+	+
3-Methylbutanoic acid	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	+	**nd**	**nd**	**nd**	+	+	+
Esters																		
Ethyl hexanoate	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	+	+
Ethyl butanoate	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	+	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	+	+	+
3-Methylbutyl acetate	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	+	+
Ethyl acetate	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	+	+	+	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	+
S-Methyl ethanethioate	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	+	+	**nd**	**nd**	**nd**
S-Methyl butanethioate	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	+	+	**nd**	**nd**	**nd**
Methyl butanoate	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	+	+	**nd**	**nd**	**nd**	+	+	+	**nd**	**nd**	**nd**

(*Continued*)

VOCs	UHT control			*P. fluorescens* PS14			*P. fragi* PS55			*P. rhodesiae* PS62			*P. mosselii* PS39			*S. marcescens* S92		
	5	10	20	5	10	20	5	10	20	5	10	20	5	10	20	5	10	20
Hydrocarbons																		
Heptane	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	+	+	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**
3-Methylhexane	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	+	+	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**
3,3-Dimethylhexane	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	+	+	+	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**
Hexane	**nd**	**nd**	**nd**	**nd**	+	+	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**

[a]nd, not detected
[b]t, molecole occuring in trace amount, < 4 logArea
+, relative abundance between 6,5 – 5 logArea
++, relative abundance between 7 -8 logArea

Regarding to VOCs releasing after 20 days storage at 5 ˚C (Table 3.8.3) a common feature in GC profiles both of UHT control milk and all contaminated samples was the presence of the 2,3,3-trimethyl pentane hydrocarbon. It is worthwhile to note that this compound was not produced by psychrotrophic strains during storage at 10°C. Differently some ketones (nonan-2-one, butane-2,3-dione) and sulphur compounds (methyldisulfanylmethane and methylsulfinylmethane) were not observed in UHT control milk and in samples spoiled with *P. fluorescens, P. fragi* and *P. mosselii* at 5 °C differently from the same samples incubated at 10 ˚C. 2-methylpropan-1-ol alcohol was produced only by *P. rhodesiae.* At 5 ˚C no detection of fatty acids was recorded in milk samples spoiled with *P. fragi* and *P. mosselii*. *P. rhodesiae* produced only acetic acid while the highest number of fatty acids was found in milk spoiled with *P. fluorescens* and *S. marcescens*.

Table 3.8.3 VOCs detected in spoiled UHT milk at 5°C after 20 days of storage

VOCs	UHT control		*P. fluorescens*	*P. fragi*	*P. rhodesiae*	*P. mosselii*	*S. marcescens*
	T_0	T_{20}	PS14	PS 55	PS62	PS39	S92
Alcohols							
2-methylpropan-1-ol	[a]**nd**	**nd**	**nd**	**nd**	+	**nd**	**nd**
3-methylbutan-1-ol	**nd**	**nd**	+	+	+	+	+
Aldehydes							
Exanal	+	+	+	+	+	+	+
Heptanal	+	+	+	+	+	+	+
Octanal	[b]**t**	**t**	**t**	**t**	**t**	**t**	**t**
Benzaldehyde	+	+	+	+	+	+	+
ketones							
Acetone	++	++	++	++	++	++	++
Butan-2-one	++	++	+	+	+	+	+
Pentan-2-one	+	+	+	+	+	+	+
Heptan-2-one	+	+	+	+	+	+	+
6-methylhept-5-en-2-one	**t**	+	+	+	+	+	+
Nonan-2-one	**t**	**t**	**nd**	+	+	+	+
Butane-2,3-dione	**t**	**t**	+	**t**	+	+	+
Sulphur compounds							
Methylsulfanylmethane	**nd**	**t**	+	+	+	+	+
Methyldisulfanylmethane	+	+	**nd**	+	+	+	+
Methylsulfonylmethane	+	+	+	+	+	+	+
Methylsulfinylmethane	**nd**	**nd**	**t**	**t**	+	**nd**	+
Fatty acids							
Acetic acid	**nd**	**nd**	+	**nd**	+	**nd**	**nd**
Butanoic acid	**nd**	**nd**	+	**nd**	**nd**	**nd**	+
Hexanoic acid	**nd**	**nd**	+	**nd**	**nd**	**nd**	+
Octanoic acid	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	+
Hydrocarbons							
2,3,3-trimethyl pentane	+	+	+	+	+	+	+

[a]nd, not detected; [b]t, molecole occuring in trace amount, < 4 logArea
+, relative abundance between 6,5 – 5 logArea
++, relative abundance between 7 -8 logArea

By comparing the chromatographic VOC profiles of spoiled milk samples at different stages of storage (both at 10°C and 5°C), it was possible to identify compounds that could be regarded as potential markers of psychrotrophic contamination.
The 3-methylbutan-1-ol branched-chain alcohol could be considered as a possible marker of psychrotrophic contamination due to its absence in unspoiled UHT milk and its production in all spoiled milk samples already from 5th day of storage at 10° and after 20 days at 5°C. 3-methylbutan-1-ol could derive from the proteolytic activity of the strains and from leucine catabolism (Smit et al., 2005). Moreover, among the detected volatiles, some molecules, absent or found in minimal traces in UHT control milk, have already been detected in spoiled samples after 5 days of storage at 10°C, so they could be regarded as a markers of bacterial spoilage development: 2-methylpropan-1-ol (*P. fragi*, *P. rhodesiae*, *S. marcescens*), 3-hydroxybutan-2-one (acetoin) (*P. rhodesiae* and *S. marcescens*), butanoic (*P. fluorescens*, *P. rhodesiae*, *P. mosselii*, *S. marcescens*) and hexanoic acids (*P. fluorescens*, *P. rhodesiae*, *S marcescens*), butane-2,3-dione (*P. rhodesiae*, *P. mosselii* and *S. marcescens*).
Some metabolites were detectable only when the milk was contaminated by a specific psychrotrophic strain during storage at 10°C. 3-methylbutan-2-ol, 3-methylhexan-2-ol and pentan-1-ol and 3,3-dimethylhexane hydrocarbon were only produced by *P. fragi*; pentane-2,3-dione, heptane and 3-methylhexane were detectable only in milk contaminated with *P. rhodesiae* while hexane was produced only by *P. fluorescens.*

There are several possible origins for detected bacterial volatile metabolites and the range of organic compounds that can be used as carbon and energy source by psychrotrophic bacteria is wide. Although it is difficult, and in some cases not possible, to attribute them to a specific biosynthetic pathway, some hypotheses can be made. Most of the compounds identified in this work could be associated with the microbial and enzymatic activities occurring during the spoilage of milk. It is known that aldehydes, resulted from the catabolism of amino acid, (Marilley & Casey, 2012) can be reduced to alcohols by alcohol dehydrogenases (e.g. 3-methylbutanal to 3-methyl-1-butanol) or oxidized to carboxylic acids by an aldehyde dehydrogenase (e.g. 3-methylbutanal to isovaleric acid/3-methylbutanoic acid). Additionally, pyruvate or citrate are starting materials for the formation of short-chain flavor compounds such as 3 hydroxybutan-2-one (acetoin) and butane-2,3-dione through glycolytic and lactate converting fermentations (Michal, 1999) The catabolism of pyruvate and leucine seems to play an important role in case of *P. rhodesiae* and *S. marcescens* since the products of these metabolic pathway (3-methyl-1-butanol, 2,3-butanedione, 3 hydroxybutan-2-one) were significantly found in the headspace of milk spoiled with these bacteria. Sulfur compounds usually arise from sulforated amino acids catabolism (Montel, 1998; Ardo, 2006**)**. Some methyl ketones such as nonan-2-one can derive from a lypolytic process but also from several other possible pathways, such as alkane degradation (Stanyer et al., 1996; Padda et al., 2001) bacterial dehydrogenation of secondary alcohols (Lukins & Foster, 1963; Padda et al., 2001). Ethyl esters were formed through chemical esterification of alcohols and carboxylic acids following microbial esterase activity (Talon et al., 1998; Toldra, 1998). Carboxylic acids deriving from hydrolysis of triglycerides and phospholipids (Toldra, 1998) can also be an initial substrate for oxidation with the formation of final odorous products. Aldehydes, such as hexanal, nonanal and also alcohols can derive from hydrolysis of triglycerides or from amino acid degradation (Montel, 1998).

3.8.2 Sensory analysis of microbially spoiled milk samples

The individual ability of each of the five psychrotrophic strains to produce odor changes in UHT milk was tested after 10 days at 10°C, in order to determine which volatile organic

compounds are associated to undesirable odor characteristics. Different sensory evolution patterns were observed according to the strains. Sensory characteristics were slightly better in the sample spoiled with *P. fluorescens,* while *P. fragi* was classified as moderately spoiling micro-organism. The poorest sensory qualities were found in samples contaminated with *P. rhodesiae*, *P. mosselii* and *S. marcescens*, considered the strongest odour spoiling micro-organisms with different evolutions in their spoilage activity. In milk contaminated with *P. fluorescens* "fresh milk" and "dairy odor" off-flavours were detected by panelists. *P. fragi* exhibited mainly "cottage cheese", "sweet whipped cream", "slightly fruity" and "strong butter" off-odors while for *P. mosselii* sulphur and rotten off-odours were noted. For the milk spoiled with *P. rhodesiae* a sensory descriptor of "baby vomit" and acid odours were systematically suggested by panelists and adverse sensory effects have been also detected in milk spoiled with *S. marcescens*, producing metallic/oxidized and grassy odours. Our results showed that the spoilage potential of the five psychrotrophic strain was revealed by their ability to produce different off odours, most likely induced by volatile compounds. Volatile compounds detected in milk sample spoiled by *P. mosselii* could be responsible for characteristic flavors, such as sulphur compounds for rutten/putrid odors while 3-hydroxybutan-2-one, 2,3-butanedione, 3-methyl-1-butanol and an high number of fatty acids produced by *P. rhodesiae* and *S. marcescens* led to the formation of strong butter/buttermilk and sour, rancid and nauseous off-odors. *P. fragi* displayed a moderate spoilage potential by producing typical fruity off-odors due to the release of esters, as was already reported in several studies (Cormier et al., 1991) *P. fluorescens* milk samples were always described as adequate for consumption considering the off-flavour as a minor sensory change, probably due to a less production of distinctive VOCs during storage time. This finding is in accordance with the results of sensory analysis of the spoilage aromas of milk containing *Pseudomonas* species by Hayes et al. (2002).

Sensory analysis confirmed the differences in the bacteria-specific VOC profiles found by SPME/GC-MS analysis that contributed to the overall decrease in milk quality.

In the visual aspect, the descriptive analysis detected slight structural changes, such as coagulation, for milk samples inoculated with *P. fluorescens* and *P. fragi* whereas *P. rhodesiae, P. mosselii* and *S. marcescens* resulted the faster milk-coagulating strains.

3.9 Conclusions

It may be concluded from the results obtained in the present work that psychrotrophic strains belonging to different species of the genus *Pseudomonas* and *Serratia marcescens* produce a large variety of VOCs in milk during storage time and that these abilities are species-dependent, as reported by Morales et al. (2005) regarding the VOCs production in cheese by *Pseudomonas*. A general increase in the magnitude of the peaks of volatile compounds was observed with increasing temperature and storage time confirming that VOC profile of psychrotrophic strains is time-temperature dependent. In addition, the presence of these bacteria affect negatively the structural and sensory characteristics of milk, being *P. rhodesiae*, *P. mosselii* and *S. marcescens* the main spoilage species. Using SPME/GC-MS method, it was possible to identify potential markers for psychrotrophic milk spoilage (3-methylbutan-1-ol, 2-methylpropan-1-ol, 3-hydroxybutan-2-one, butane-2,3-dione, butanoic and hexanoic acids).
Determination of milk off-flavours has been the subject of numerous investigations (Badings & Neeter, 1980; Urbach, 1990; Kim & Morr, 1996; Contarini & Povolo, 2002; Toso et al., 2002; Marsili, 2003; Karatapanis et al., 2006) but SPME/GC-MS analysis of contaminated milk with psychrotrophic bacteria, mainly *Pseudomonas*, is a relatively new field of research and still requires extensive basic research to evaluate the candidate compounds, which may serve as biomarkers.
In seeking potential markers of milk spoilage, we feel that the emphasis should be placed on screening for headspace compounds or fragments produced by more strains and species of psychrotrophs in milk. In the future, it would be of utmost interest to study in deep the VOC presence in spoiled milk, confirming the possible markers detected in this work and finding new ones for prevention of quality loss and extension of milk shelf-life.

3.10 References

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3.11 Fatty acids released from cream by psychrotrophs isolated from bovine raw milk

Use of cooling, imperfect cleaning and inadequate disinfection of milking machines in primary milk production remain the main reasons for the occurrence of psychrotrophic bacteria in raw milk. On average, the psychrotrophic microbiota of bulk raw milk accounts between 10 and 50% of the total bacterial load (Chambers, 2005). Also psychrotrophic bacteria are mesophile organisms, they are able to grow at low temperatures and, moreover, they are endowed with proteolytic and lipolytic activities (Cousin, 1982). Munsch-Alatossava and Alatossava (2006) considered this group to be the most relevant spoiling microorganisms in the dairy industry because of their capacity to produce heat resistant enzymes (proteinasas and lipases) responsible of quality issues and sensory defects, resulting in a limited shelf-life of milk and dairy products.
The triglyceride fat fraction of milk accounted by more than 96% of total lipid, located in the core of fat globule, which are arranged from a conjugated ester of long chained fatty acids with glycerol molecules, enveloped by a thin layer composed phospholipids, glycolipids, and several proteins, (Evers et al., 2008). Obviously, to release the free fatty acids (FFAs) and cause changes and flavour defects (rancid or soap flavours if, respectively, short chain or medium or long chain fatty acid are released) in the milk, several consecutive cleavages are necessary, beginning with the breaking of the surrounding globule fat membrane followed by the lysis of the triglycerides into glycerol and fatty acids, which are accumulated in the milk gave rise to the above mentioned defects (Stepaniak 2004). The fat globule is susceptible to the action of many lipolytic agents, such as physiological, physiomechanical, and chemical factors (Deeth & Fitz-Gerald, 2006), the latter involving lipases (McPherson & Kitchen, 1983). Lipases are carboxylesterases that hydrolyse the bond ester of acylglycerols Those that hydrolyse acylglycerols of less than 10 carbon-chain fatty acids are named esterases, or carboxylases (EC 3.1.1.1) while those hydrolysing acylglycerols more than 10 carbon-chain fatty acids are the lipases, or triacylglycerol acylhydrolases (EC 3.1.1.3). Esterases are active in aqueous solutions, while true lipases work in lipid–water interfaces rather than in aqueous phase (the ‘‘interfacial activation phenomenon’’) (Brockerhoff & Jensen, 1974; Anthonsen et al., 1995) and most are also capable of hydrolysing esterase substrates (Jaeger et al., 1994; 1999). Lipolytic enzymes can be of endogen origin, in which case most of the lipases are associated with the casein micelle structure (Stepaniak, 2004) or of exogenous origin, secreted as a result of the bacterial metabolic action.
It is generally known that *Pseudomonas* spp. produce thermostable lipases (McPhee & Griffiths, 2011) active on milk fat and responsible for spoilage as a result of the production of short and medium chain fatty acids (Deeth, 2006). A high FFA accumulation causes the deterioration of technological properties (e.g. a worse whipping ability of cream) and sensory attributes of milk, which is reflected by a rancid or soap off-flavour that may negatively influence the quality of dairy products (Antonelli et al., 2002, Hanuš et al., 2008). According to Vyletělová et al. (2000) the medium-chain fatty acids (C12–C16) are primarily liberated by the action of *Pseudomonas fluorescens* lipolytic enzymes while short-chain fatty acids (C6–C10) are sporadically released or in very small quantities.
Despite many researches dealing with the ability of psychrotrophs, mainly *Pseudomonas* spp., to produce thermostable enzymes and their effect on the most important compounds of milk, fatty acids profiles of lipolytic psychrotrophs belonging to different genera and species has not been well fully explained. Due to the relevance of the aforementioned issues, the present work was envisaged in an attempt to clear up the ability of psychrotrophic bacteria, belonging to different bacterial species, to break lipids and to quantify the fatty acids released as a result of this action.

3.12 Material and methods

3.12.1 Microorganisms

The strains tested for the lipid breakdown in this study were previously isolated from raw milk samples, identified and characterized for their enzyme activity (Decimo et al., 2014). Fifteen psychrotrophic strains were selected showing different lipolytic activity at 7 and 22°C: *Pseudomonas fluorescens* (n=5) *P. fulva* (n=2), *P. putida* (n=1), *Ps. mosselii* (n=1), *P. fragi* (n=1), *P. rhodesiae* (n=1), *Hafnia alvei* (n=1) and *Serratia marcescens* (n=3).

3.12.2 Physico-chemical analysis of cream

For experiments, UHT cream purchased in the market was used. The pH of cream was determined using a Crison Digit-501 pH-meter (Crison Instruments LTD, Barcelona, Spain). Water activity (a_w) was measured using a Decagon CX1 hygrometer (Decagon Devices Inc., Pullman, WA, USA) at 25 °C. Moisture content (oven air-drying method) and ash (muffle furnace) were analysed following the AOAC (1995) method. The fat content was determined using the method of Bligh and Dyer described by Hanson and Olley (1963). All determinations were performed in triplicate and mean values of the replicates were considered.

3.12.3 Preparation of partially purified extracellular lipase

The selected strains was subcultured in Brain Heart Infusion (BHI) broth (Biolife, Milan, Italy) supplemented with 2% of skim milk and incubated at 30°C for 24 h. Then, one mL of each culture was inoculated in 500 mL erlenmeyer sterile flasks containing 250 mL of the above mentioned culture medium BHI. The flasks were incubated at 30°C with constant shaking at 125 rpm for 48 h. to stimulate enzyme production. After incubation, the culture was centrifuged at 12,000 *g* for 30 min at 4°C to separate cells and, then, the supernatant was precipitated with ammonium sulphate $(NH_4)_2SO_4$ to 50% saturation and stirred for 2 h on ice bath. After centrifugation at 12,000 *g* for 30 min at 4°C, the pellet was recovered by dissolving it in Tris-HCl 0,1 M pH 7.4 buffer and, afterwards, dialyzed overnight against the same buffer at 4°C and then collected in a sterile vial. The final solution was lyophilized (Telstar, Lioalfa-10, Madrid, Spain) and the dry extract stored by frizzing at - 20°C. For analysis, the lyophilizated was dissolved in a minimal volume of Tris-HCl 0,1 M pH 7.4.

3.12.4 Changes in the substrate specificity of crude enzyme

The specificity of the lipase activity of partially purified lipase was studied using four different triacylglycerides (TAGs), namely, glyceryl esters of tributyrate, tricaproate, trimyristirate and trioleate. The substrate media were prepared by sonicating 1%, 0.1%, 0.06% and 0.06% of the above mentioned triacylglycerides, respectively 100 mL of Agar Noble (Difco, BD, Sparks, Md.) for 2 min using an ultrasonic apparatus (model UP-400S, Dr. Hielscher GmbH, Stuttgart, Germany), which operates at 24 kHz, 400 W and 60 μm. The medium with triacylglycerols was plated in Petri dishes and 50 μl of each of the 15 crude enzymes was deposited into the wells (diameter 6 mm). The specific lipase activity was defined by a clear or turbid zone formation around the wells after incubation at 30°C. The halo performed was periodically recorded with a calliper from 24 h of incubation until its stabilization. The area was measuring using the following formula: $A = \pi [(r + r')^2 - r'^2]$ being r the radius of the halo and r' the radius of the well (3mm).

3.12.5 Preparation of spoiled cream samples

One mL of each partially purified lipase of the 15 psychrotrophic strains was used to inoculate 20 mL of UHT cream and the mixture incubated at 30°C for 24 h, according to Ouattara et al. (2004) who reported that a significant lipolysis is observed in the first 12-24 h. The incubation was extented for four additional days to verify the changes in the free fatty liberated with the incubation time. After that, the cream samples were lyophilized and frozen until gas chromatrography analysis.

3.12.6 Free fatty acid analysis

For quantitative lipid extraction the method proposed by Segura and Lopez-Bote (2014) was applied. Briefly, in a 2 mL safe-lock micro test tube, 170 mg of lyophilised spoiled cream sample were accurately weighed. Two steel balls (2 mm diameter) and 1.5 mL of chloroform/methanol (8/2) (v/v) mixture were added. After being tightly capped, the tubes were placed on the adapters and homogenized for 2 min at 20 Hz in a Mixer Mill MM400 (Retsch Technology, Haan, Germany). The final biphasic system was allowed to separate by centrifugation (8 min, 12,000 g). The solvent was decanted into a previously weighed 4 mL vial. The extraction was repeated three times. Solvent was evaporated under nitrogen stream at 25 °C. Samples were kept in a desiccator until constant weight (about 2 h). Lipid content was then gravimetrically determined.

The lipid fractionation into major lipid classes (apolar lipids , AL, free fatty acids, FFA and polar lipids, PL) was carried out according to the method of Ruiz et al. (2004). Briefly, a 0.2 g lipid extract was dissolved in a mixture of hexane/chloroform/methanol (95/3/2) (v/v/v) in the proportion 4/3 (w/v) and added to a 500 mg aminopropyl minicolumn (Varian, Harbor City, USA), which was previously activated with 7.5 mL of hexane. AL and FFA were eluted with 5 mL of chloroform and-diethyl ether/acetic acid (98/2) (v/v), respectively. PL were successively eluted with 2.5 mL methanol/chloroform (6/1) (v/v) and 2.5mL of 0.05 M sodium acetate in methanol/chloroform (6/1) (v/v). Both fractions were combined and analysed together.

Total lipid extracts were methylated by dissolving the sample in 1 mL of 5% sodium methylate methanolic solution in culture tubes (Olivares et al., 2009). After being tightly capped, the tubes were vortexed and heated for 1 h at 70 °C, shaking every 15 min. After tempering, 1 mL of 5% sulphuric acid methanolic solution was slowly added. The tubes were vortexed and shaking again in the previously conditions. After tempering, the fatty acids methyl esters (FAMEs) were extracted two times with 1.5 mL of petroleum ether and directly injected in the GC system.

Identification and quantification of FAMEs were performed by gas–liquid chromatography following the conditions described by Segura and Lopez-Bote (2014), modifying the oven temperature. The FAMEs were analyzed in a gas chromatograph (HP 6890 Series GC System) equipped with flame ionization detector and a J&W GC Column, HP-Innowax Polyethylene Glycol (30 m × 0.316 mm × 0.25 μm). Nitrogen was used as a carrier gas. The oven temperature was programmed for initial holding for 1.50 min at 80 °C, then heating to 210 °C at a rate of 4.5 °C/min , followed by a second heating to 250 °C at a rate of 7 °C/min followed by a final holding for 3 min. The flame ionization was held at 250 °C. FAMEs peaks were identified by comparing their retention times with those of the authentic internal standard, (tridecanoic acid methyl ester) from Dr. Ehrenstorfer GmbH, Augsburg, Germany.

3.13 Results and discussion

3.13.1 Physico-chemical characteristics of cream

Selected physico-chemical parameters (moisture, fat content, ash, a_w and pH) of UHT cream were determinated, achieving the following values (percentage on wet matter): moisture 59.44 ± 0.03; fat 11.34 ± 3.62; ash 0.41 ± 0.01; a_w 0.949 ±0.006 and pH 6.77 ± 0.05.

3.13.2 Lipolytic activity of partially purified lipases

Lipases from *Pseudomonas* spp. can actively hydrolyze a variety of natural vegetable oils and synthetic TAGs (emulsified triglyceride substrates) ranging from tributyrin to triolein (Stepaniak & Sorhaug, 1989; McPhee & Griffiths, 2011), and they show preference for triglycerides rather than monoglycerides, and for medium-chain glycerides, such as those containing 8 to 19 carbon fatty acids (Bozoglu et al., 1984). *P. fluorescens* lipase showed the highest lipolytic activity with regard to long chain TAGs, especially triolein. Most of the lipases have a preference for the sn-1 and sn-3 positions of TAGs (Rogalska et al., 1993). In this study the lipolytic activity from the fifteen selected psychrotrophic strains on the TAGs aforementioned in material and methods was investigated.

The changes in lipolytic activity on tributyrin, tricaproin, trimyristin and triolein of psychrotrophic strains are shown in figures 3.13.1, 2, 3 and 4, respectively. Generally, all strains presented lipolytic activity against the selected triacylglycerols and as expected, the activity of most strains increased with the incubation time. The lipolytic strains more active toward tributyrin were *P. fragi* PS55 and *P. fulva* PS1 while *P. rhodesiae* PS62 and *S. marcescens* S92 showed the lowest activity. The others psychrotrophic lipases showed a variable activity on this acylglycerol. The halo area at 216 h of incubation ranged between 6.16 cm^2 for *P. fragi* PS55 and 1.99 cm^2 for *P. rhodesiae* PS62 (Figure. 3.13.1). The lipolysis patterns observed on other substrates were quite similar to that of the tributyrin even the absolute levels achieved after 216 hours Briefly, in tricaproin (Figure. 3.13.2), the halos were comprised between 6.3 cm^2 of *P. fluorescens* PS14 and 2.09 cm^2 of *P. mosselii* PS39. In the case of trimyristin (Figure. 3.13.3) two strains (*P. rhodesiae* PS62 and *P. mosselii* PS39) gave rise to the lowest halos area (1.45 and 1.21 cm^2, respectively). On the contrast, the halos of five strains (*P. putida* PS17, *P. fragi* PS55, *P. fluorescens* PS73, *P. fluorescens* PS81 and *P. fluorescens* PS56) reached the highest final values, between 5.15 and 4.37 cm^2, i.e. 3.5 to 4-fold higher than those of *P. rhodesiae* PS62 and *P. mosselii* PS39. The remaining strains showed intermediate activities. Finally, for triolein (Figure 3.13.4), four strains (*P. fluorescens* PS14, *P. putida* PS17, *P. fluorescens* PS81 and *P. fluorescens* PS73) showed a clear higher lipolytic power (halos area between 5.2 and 3.9 cm^2), than the remaining ones, which gave rise to halos lower than 3.0 cm^2. It is noteworthy that *P. fluorescens* PS81strain showed the fastest activity since evident at 24 h of incubation while the time to detect the lipase activity elapsed, in general, 48 – 72 hours when tricaproin and trimyristin were used as substrate and more than 72 hours in the case of triolein. These results partially agree with those of Schuepp et al. (1997) that reported that the FII from *P. fragi* CRDA037 presented the highest activity on trimyristin followed on triacetin and then on tributyrin. Nishio et al. (1987) reported that the purified extracellular lipase from *P. fragi* 22.39B exhibited the highest affinity toward tributyrin, in agreement with the data here obtained. *P. fluorescens* PS14 displayed high activity on both glycerol triocaproate and trioleate, which is in partial agreement with Chakraborty and Paulraj (2009) that indicated a preferential specificity of the lipase from *P. fluorescens* MTCC 2421 toward cleaving shorter short-chain length fatty acid TAGs but a scarce hydrolytic action against toward monounsaturated TAGs (i.e. triolein) with longer fatty acyl chain length. *P.*

putida PS17 showed high specificity toward the fatty acid triglycerides having medium to long carbon chain lengths (C14:0- C18:1n9) while *P. fulva* PS1 exhibited higher affinity on tributyrin *P. rhodesiae* PS62 was the strain with the lowest lipolytic activity against all tasted TAGs.

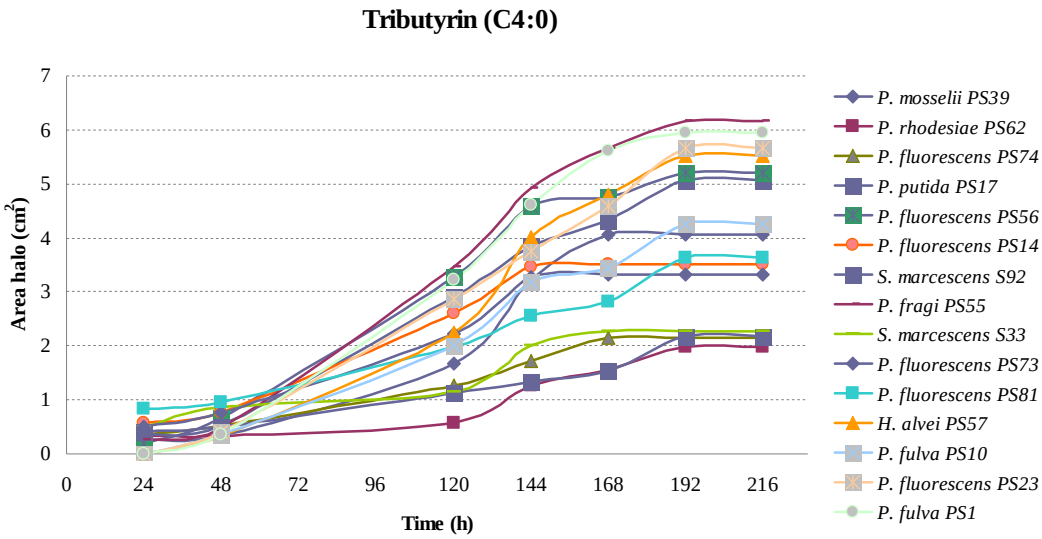

Figure 3.13.1 Specificity of lipases from psychrotrophic strains toward tributyrin TAG

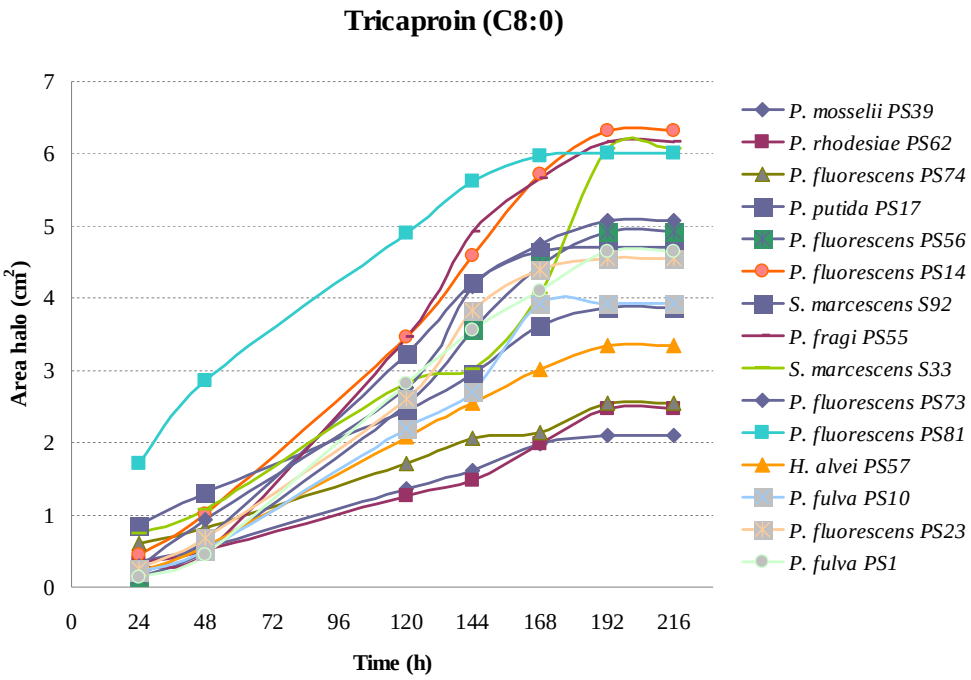

Figure 3.13.2 Specificity of lipases from psychrotrophic strains toward tricaproin TAG

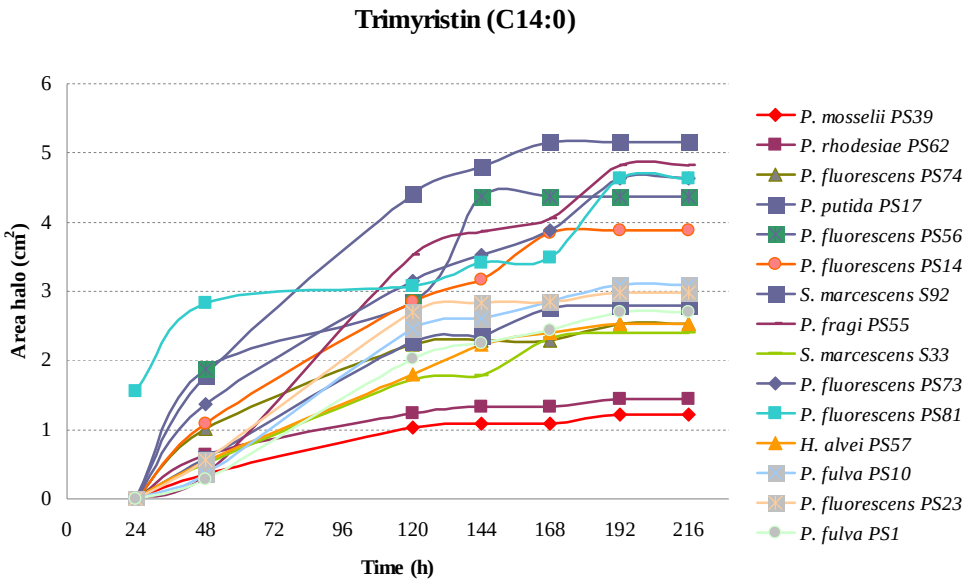

Figure 3.13.3 Specificity of lipases from psychrotrophic strains toward trimyristin TAG

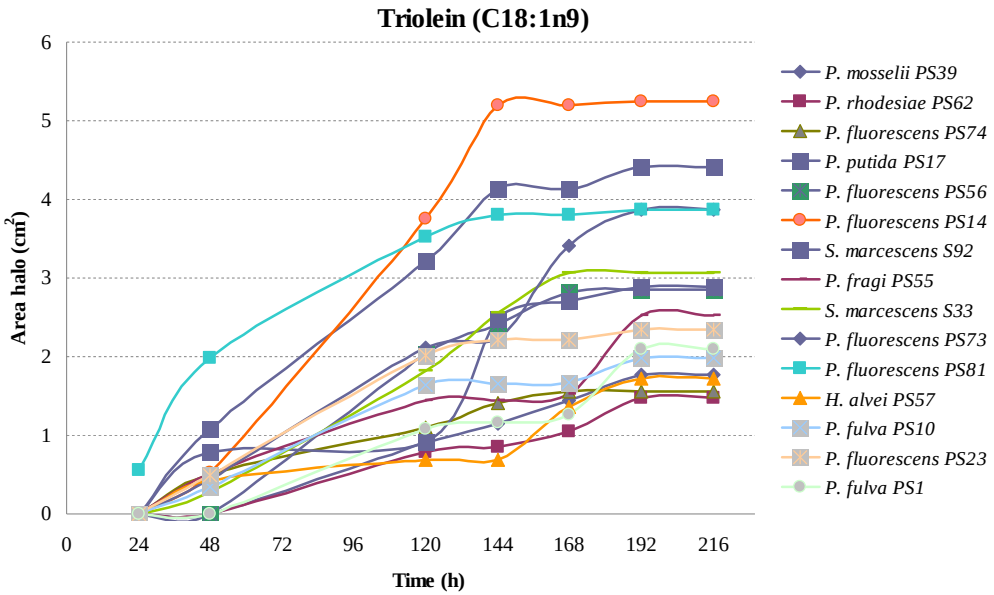

Figure 3.13.4 Specificity of lipases from psychrotrophic strains toward triolein TAG

3.13.3 Release of FFAs by psychrotrophs

To confirm the qualitative analyses of lipolytic activity, a study on the release of fatty acids from cream was performed by use of gas liquid chromatography. The results are presented in figure 3.13.5, in which the free fatty acids values reached after 1 and 4 days are shown. In control samples was observed a minor increase of FFAs, which has been attributed to the effect of the both the natural lipoprotein lipase (LPL) and the growth of the background microbiota (Taylor & MacGibbon, 2003). In inoculated samples, it may be observed that the lipolytic activity of the strains was variable. Briefly, among the fifteen tested lipolytic psychrotrophic strains, the species producing the highest concentrations in total FFAs at 24 h of incubation were *P. fluorescens* PS73 and *S. marcescens* S33 but the former was one of the most active after four days while *S. marcescens* S33 hardly increased the FFAs concentration in this period. The strongest total fatty acids liberated after 4 days of incubation were due to the *P. fluorescens*

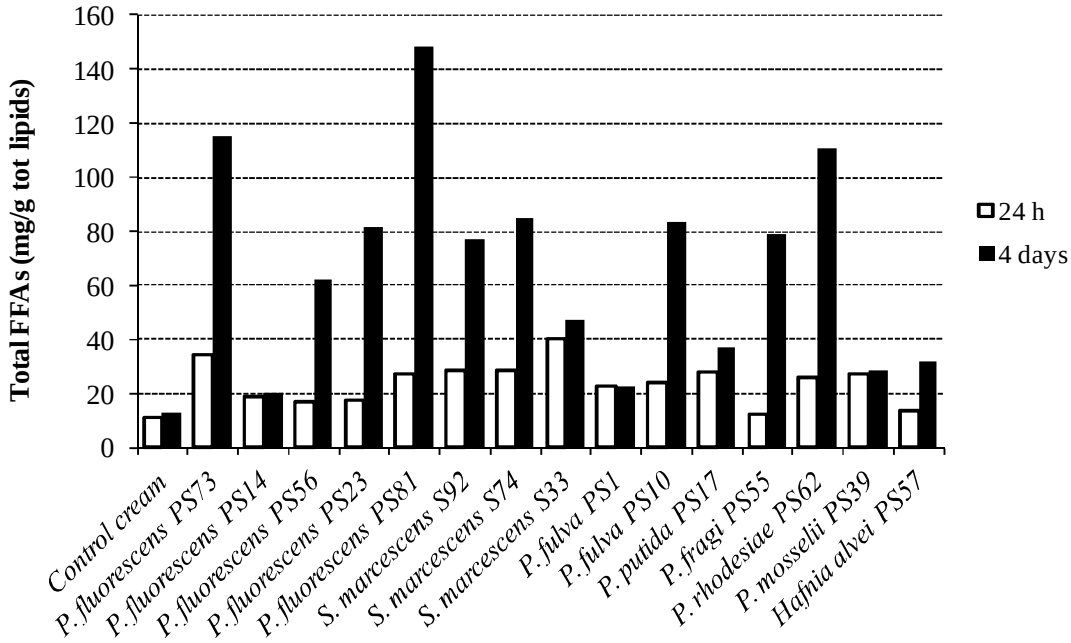

Figure 3.13.5 Total FFAs concentration (mg/g total lipid) in control and inoculated samples after incubation at 30 °C for 24 h and 4 days

PS81 activity. On the opposite side, in spite of *P. putida* PS17 was one of the major FFAs producer at 24 h, it did not show a significant increase in total FFAs concentration after 4 days of incubation, which could be related with the metabolic adaptation or reduction in the availability to break the milk fat fraction during the time. Other strains had an intermediate behavior, which may be clear deduced in figure 3.13.5.

In order to analyze the individual fatty acids liberated, four strains were selected, as model of high (*P. fluorescens* PS73), intermediated (*S. marcescens* S92), odd behavior (initially low activity but it accelerates during storage) (*P. fragi* PS55) and low (*P. mosselii* PS39) activity. Eighteen FFAs (C-8:0 to C-20:1) were identified in both the control cream lipid extract and inoculated samples, the FFAs detected in the highest quantities were, in general, those of the intermediate par carbon chain (C-12:0 to C-18:0) and unsaturated C-18:1 and C-18:2. This fact coincided with the most abundant triacylglycerols in the milk fat (Jensen, 1992). Other authors have reported similar phenomenon, e.g. Outtara et al. (2004) found that milk sample inoculated with *P. fragi* CBS-15-5260 the C-16:0 was the fatty acid detected in the largest amount (16.3 mg/kg) followed by C-18:1 (12.8 mg/kg), C-18 (9.8 mg/kg) and C-14 (7.3 mg/kg) although in this work the dominant FFA was C-18:1 (see table 1). The increased of the mentioned FFAs during cream storage was quite regular for *P. fluorescens* PS73 and *S. marcescens* S92 although the percentage of increased were variable (table 1) between 200 to 350% and 100 to 200%, respectively. Both strains roughly represent the action of the majority strains studied in the present research. On the other hand, the trend observed for *P. fragi* PS55 was different. This was the one of the lowest lipolytic strain after 24 hours, but very high increases of FFAs were observed during storage since the values were 4 to 7-fold higher after four days. This means that the average increase percentage was about 350% although in many fatty acid the percentage was higher than 450% (table 3.13.1) such as C-10:0, C-12:0, C-16:1 and others. The *P. mosselii* PS39 was also a strain with an initial low activity and the increase in the FFAs during storage was negligible.

These results indicated that the ability of lipolytic psychrotrophic strains to release fatty acids from milk fat varies according to the bacterial genus, among species and even with the strain of given specie. The results of the present work are in total agreement with those of Ouattara et al. (2004), who stated that lipolytic activity of psychrotrophic bacteria common in raw milk was species-specific. Therefore, no general prognosis of the concentration of FFA released could be made although it may be predicted that, in a greater or lesser degree, a lipid breakdown will occur if the raw milk storage is prolonged.

The proteolytic phenomenon are probably the major event of concern occurred in milk and dairy products but the fatty acid release by hydrolysis of milk triacylglycerols through the activity of psychrotrophic lipases are the main source primary cause for the undesirable changes in the flavour of these products. The normal content of FFA in milk is very low, accounting for a 0.1% of total fat (Walstra and Jenness, 1984) but if this concentration increases in about a 1%, it may be result in a rancid flavor in milk, which is caused by the accumulation of short-chain FFAs (butyric, caproic and caprylic acids). However, a higher amount of medium-chain FFAs (capric, lauric and myristic acids) leads to a soapy taint while long-chain saturated fatty acids, namely palmitic and stearic acids contribute little to off-flavour (Al-Shabibi et al., 1964; Vulfson, 1994). As psychrotrophic bacteria are able to develop under refrigeration, it is convenient to minimize its growth on raw milk and cream in order to avoid a high bacteria number during storage and, along with, to diminish the lipolytic activity. As is well known, these bacteria do not survive even mild heat treatment. It should be sufficient a thermization process (about 65 ºC for few seconds) to reduce *Pseudomonas* spp (and other Gram negative psychrotrophs) to a large extent. This reduction should be enough to produce negligible changes in both milk nutrients and sensory properties.

Table 3.13.1 Concentrations of free fatty acids (mg/100 g total lipid) in control and inoculated samples after incubation at 30°C for 1 and 4 days by four selected psychrotrophic strains

FFA	*P. fragi* PS55			*P. fluorescens* PS73			*S. marcescens* S92			*P. mosselii* PS39		
	1 day	4 days	[a]Δ	1 day	4 days	[a]Δ	1 day	4 days	[a]Δ	1 day	4 days	[a]Δ
C8:0	1.29	1.78	+	0.44	1.27	++	0.53	0.84	+	1.01	1.12	+
C10:0	0.40	2.15	++++	0.89	3.16	+++	0.72	1.75	++	0.69	0.93	+
C12:0	0.46	3.06	++++	1.24	4.71	+++	0.99	2.82	++	0.85	0.95	+
C14:0	1.40	7.59	++++	3.05	11.58	+++	2.60	7.05	++	2.75	2.75	-
C14:1	0.14	1.06	++++	0.42	1.63	+++	0.33	1.03	+++	0.24	0.25	+
C15:0	0.17	0.72	+++	0.31	1.08	+++	0.27	0.67	++	0.29	0.29	-
C15:1	0.04	0.15	+++	0.07	0.22	+++	0.06	0.14	++	0.07	0.07	-
C16:0	4.81	18.97	+++	8.72	28.11	+++	7.79	18.01	++	8.97	8.98	-
C16:1	0.25	1.64	++++	0.66	2.31	+++	0.62	1.64	++	0.56	0.56	-
C17:0	0.17	0.35	++	0.16	0.50	+++	0.14	0.33	++	0.17	0.22	+
C17:1	0.06	0.30	++++	0.13	0.45	+++	0.11	0.31	++	0.09	0.11	+
C18:0	2.06	8.12	+++	4.10	11.65	++	3.65	7.84	++	3.81	3.82	-
C18:1	5.20	28.11	++++	12.11	41.05	+++	9.26	29.15	+++	6.37	7.23	+
C18:2	0.47	3.22	++++	1.49	4.21	++	1.16	3.00	++	0.72	0.97	+
C18:3	0.04	0.46	++++	0.19	0.74	+++	0.14	0.51	+++	0.08	0.11	+
C18:4	0.17	0.40	++	0.20	0.81	+++	0.15	0.53	+++	0.10	0.11	+
C20:0	ND	0.18	-	0.06	0.16	++	0.05	0.17	+++	0.05	0.05	-
C20:1	0.33	0.54	+	0.22	0.69	+++	0.17	0.50	++	0.15	0.16	-
Total	17.46	78.80	++++	34.44	114.33	+++	28.73	76.30	++	26.98	28.30	+

[a]Δ (1→4) - Percentage of the lipolytic activity increase after four days of the cream storage: + (< 100 %); ++ (100 - 200 %); +++ (200 - 350 %); ++++ (> 350 %)

3.14 Conclusions

Lipolysis in milk and dairy products derived from psychrotrophic bacteria is a constant concern in the dairy industry due to the production of off-flavours and off-odours. In this study, the lipolytic activity of fifteen psychrotrophic strains isolated from bovine raw milk has been assayed. Although it seems that the lipolytic activity of psychrotrophs is a general characteristic, no general pattern for acylglycerols breakdown was found since the behavior of selected strains was variable, being *S. marcescens* S33 and *P. fluorescens* PS81 the major FFAs producers, at 24 h and 4 days, respectively.

Moreover lipases from psychrotrophic strains showed a variable range of specificity toward fatty acid esters with different fatty acid chain lengths, being *P. fragi* PS55, *P. putida* PS17, *P. fluorescens* PS14 and *P. fulva* PS 10 the more active to cleave fatty acid triglycerides.

A thorough understanding of the mechanism of lipolysis and of the ability to control FFAs release by psychrotrophic bacteria during storage of milk may be required to minimize lipase-related problems, to avoid the deterioration of sensory properties and maintain the overall quality of dairy products.

3.15 References

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3.16 Proteomic characterization of extracellular proteases of *P. fluorescens* strains isolated from raw milk

The microbiota of raw milk depends on the health status of the cattle, the type of feed and the storage conditions. It has been reported that intensive cleaning of milking equipment together with prolonged refrigerated storage of raw milk at farm and processing plants favour the growth of psychrotrophic bacteria, particularly *Pseudomonas* spp. (MacPhee & Griffiths, 2002; Lafarge et al., 2004). The *Pseudomonas* genus corresponds to a diverse and ecologically significant group of bacteria which are found in a very large number of natural environments (terrestrial, freshwater or marine). Some of them are also plant, animal and human pathogen or responsible for food spoilage (Palleroni, 1992; Gonzalez et al., 2000; Park et al., 2000). Psychrotrophic bacteria are the key microorganisms responsible for spoilage of milk and dairy products, due to their ability to produce heat-resistant proteases that hydrolyse casein, leading to formation of bitter off-flavours and gelation of UHT milk (Mitchell & Marshall, 1989; Sorhaug & Stepaniak, 1997; Chen et al., 2003; Datta & Deeth, 2003;). Production of extracellular protease by *Pseudomonas* strains is a common feature during the late log phase and in the stationary phase of bacterial growth (Hellio et al., 1993; Rajmohan et al., 2002). The production of at least one monomeric extracellular protease of molecular weight varying between 23 kDa and 56 kDa, appears to be a common feature among strains of *P. fluorescens*, which has been reported as the predominant species among psychrotrophic bacteria (Ahn et al., 1999; Dogan & Boor, 2003; Dufour et al., 2008). Most research has focused on expression, production and biochemical properties of proteases produced by *P. fluorescens* strains associated with spoilage of milk dairy products (Barach et al., 1976; McKellar, 1989; Mitchell & Marshall, 1989; Kohlmann et al., 1991; Ching-hsing & McCallus, 1998; Liao & McCallus, 1998; Koka & Weimer, 2000; Nicodème et al., 2005; Liu et al., 2007; Dufour et al., 2008). Most of these proteases are metalloproteases which are rich in alanine and glycine residues and poor in cysteine and methionine residues. Calcium is essential for the activity and stability of these proteases (Mitchell et al., 1986). In milk, they preferentially hydrolyze the caseins $\kappa > \beta > \alpha_{S1}$. One metalloprotease called AprX which belongs to the serralysin family, has been characterized in *P. fluorescens* and its gene (*apr*X) was identified in several strains (Martins et al., 2005; Marchand et al., 2009). The *apr*X gene products of two *P. fluorescens* strains revealed estimated molecular weights of 50 kDa (Ching-hsing & McCallus, 1998) and 48.9 kDa (Kumura et al., 1999). Similar molecular weights were reported by other researchers, investigating food spoiling proteases (Koka & Weimer, 2000; Rajmohan et al., 2002; Nicodème et al., 2005, Marchand et al., 2009).

The aim of this work was to evaluate the extacellular caseinolytic potential of three *P. fluorescens* strains (PS19, PS60 and PS24), which have previously been isolated from bovine raw milk (Decimo et al., 2014). Moreover, proteolytic enzyme of PS19 *Pseudomonas fluorescens* strain, which showed the highest caseinolytic activity, was subjected to proteomic analysis.

3.17 Material and methods

3.17.1 Bacterial strains and culture conditions

In a previous work (Decimo et al., 2014), a large set of proteolytic psychrotrophic strains was isolated from bovine raw milk samples. Strains were further identified by a polyphasic (phenotypic and molecular) approach and tested for their proteolytic activity by agar diffusion assays. From this collection, three *P. fluorescens* strains (PS19, PS60 and PS24), which showed the highest proteolytic enzyme production on milk agar, were selected and further investigated.

The production of proteases by these *P. fluorescens* strains was examined in minimal salt medium (MSM) containing K_2HPO_4 (0.7%); KH_2PO_4 (0.2%), $MgSO_4 \cdot 7H_2O$ (0.02%), $(NH_4)_2SO_4$ (0.1%), glycerol (0.4%) supplemented with $CaCl_2$ at a final concentration of 1 mM (MS medium; Liao & McCallus, 1998). Growth was at 22 °C under stirring (180 rev min^{-1}) and aerobic conditions.

3.17.2 Preparation of crude enzyme extract

Cryopreserved strains were firstly recovered in Brain Heart Infusion broth (BHI) (Biolife, Milan, Italy) + 1 mM $CaCl_2$, before inoculation in MSM medium and incubated at 22 °C until growth was visually present (approx. 6 hours). Next, 300 µl (3%) of incubated BHI + 1 mM $CaCl_2$ broth was inoculated in 10 ml of MSM medium + 1 mM $CaCl_2$ and incubated overnight at 22 °C. This was repeated once again to stimulate the adaptation of the strains to the minimal salt medium with 1 mM $CaCl_2$ (Marchand et. al, 2009). Then, 30 ml of incubated MSM + 1 mM $CaCl_2$ was inoculated 2 l Erlenmeyer flasks containing 1L of MSM +1 mM $CaCl_2$. After 24 h of incubation at 22 °C under stirring (125 rev min^{-1}) and aeration, cells were harvested by centrifugation (6 000 g for 20 min at 4 °C) The filter-sterilized (0.22 µm) supernatant was heated at 95 °C for 8.45 min (Marchand et al., 2008, 2009) to select for heat-resistant proteases, and used as crude enzyme extract after storing at -20 °C before casein zymography.

3.17.3 Casein zymography and detection of extracellular caseinolytic activity

To detect the extracellular caseinolytic activity of the three *P. fluorescens* strains and to estimate the molecular mass of their bacterial proteases, a casein zymography was performed. After heating, 16 µl of each cell free supernatants (CFS), obtained from growth of the *P. fluorescens* strains in MSM, was dissolved in a 156.25 mM Tris-HCl buffer, pH 6.8 in the presence of 10% (w/v) SDS, 62.5% glycerol and 0.5% (w/v) bromophenol blue (5x Loading Buffer). Twenty µl of CFS solution were loaded for electrophoresis on a 12% polyacrylamide gel polymerized with 0.1% (w/v) casein (Sigma–Aldrich, Milan, Italy) (sodium caseinate) under non-reducing conditions, i.e. without β-mercaptoethanol. The samples were run initially at 60 V for 30 min and at 100 V for 100 min. The molecular mass standard ranged from 10kDa to 250 kDa (PageRuler Plus Prestained protein Ladder, Thermo Scientific, Milan, Italy). After electrophoresis migration, the gel was washed in 2.5% (v/v) Triton X-100 (renaturing solution) for 30 min and then incubated overnight at 37 °C in development buffer (50 mM Tris, 200 mM NaCl, 5 mM $CaCl_2 \times 2H_2O$ and 0.02% (v/v) Brij 35 (Sigma), pH 7.5). After incubation the gel was stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 dissolved in a mixture of 50% methanol (v/v) and 10% acetic acid (v/v) (staining solution), followed by discoloration in a solution containing 50% methanol (v/v) and 10% acetic acid (v/v) (destaining solution) until proteolytic activity appeared as clear bands on a blue background.
P. fluorescens PS19 crude enzyme extract was concentrated in a stirred cell ultrafiltration unit (Centricon-30; Millipore (UK) Ltd, Watford, UK) with a 10 kDa membrane and further tested on zymogram gel with 0.1% (w/v) of single casein fractions (κ, β, α_{S1}, Sigma).

3.17.4 Proteomic analysis of crude enzyme extract from PS19 *P. fluorescens* strain

P. fluorescens PS19 proteolytic bands of approximately 15, 25 and 45 kDa (as determined by zymogram analysis) was subjected to proteomic characterization. Proteomic analysis was performed using a LTQ-Orbitrap system (working in nanoLC–ESI-MS/MS mode), using a bottom-up approach, which involved denaturation, reduction, alkylation of Cys residues prior to enzymatic digestion of the protein and ESI-MS analysis of digestate. In detail, proteases were

digested *in-gel* solution and *in-solution* solution (FASP, filter aided sample preparation), using trypsin (Roche Diagnostics, Monza, Italy) as digestion enzyme.

3.17.5 *In gel* digestion

After destaining, gel lanes were excised and cut into three slices each corresponding to migration regions of different molecular weights (15, 25 and 45 kDa). Gel slices were subsequently digested with trypsin according to a protocol previously described by Lamond Lab (2007).
Briefly, gel pieces were incubated for 60 min at 56 °C with 10 mM dithiothreitol. (DTT) in 50 mM NH_4HCO_3 for cysteine reduction, immediately followed by incubation with iodoacetamide (IAA) 55 mM in 50 Mm NH_4HCO_3 for 45 min at room temperature and in the dark, for cysteines alkylation. After discarding iodoacetamide, gel pieces were rinsed several times in 50 mM NH_4HCO_3 (digestion buffer) and finally incubated in 100% acetonitrile (ACN) for dehydration. About one µg of trypsin (Roche) dissolved in 50 mM NH_4HCO_3 was incubated with each gel slice. After overnight digestion at 37 °C, the tryptic mixture was acidified with 2 µl of 50% trifluoroacetic acid solution and collected. Gel pieces were incubated for 10 minutes with 30% acetonitrile/3% trifluoroacetic acid (TFA) (extraction solution); the solution was collected and pooled with the initial peptide mixture. Gel pieces were incubated for additional 10 minutes with 100% acetonitrile; the solution was pooled with the initial one. The digestates were evaporated on a vacuum concentrator to about 5 µl. The peptides present in the concentrates were solubilized in 100 µl of 0.1% formic acid, desalted and concentrated using reverse-phase C18 handmade nano-columns (StageTips) (Rappsilber et al., 2007). Samples loaded on C18 StageTips were eluted with 80% acetonitrile, lyophilized and re-suspended in 10 µl of 0.1% formic acid for LC–MS/MS analysis.

3.17.6 *In solution* digestion

The digestion protocol reported by Manza et al. (2005) and Wisniewski et al. (2009) was also applied. This protein *in-solution* digestion was carried out using spin filter devices (filter-aided sample preparation (FASP) protocol).
The concentrated crude enzyme extract was transferred to a Microcon spin filter device (Millipore Amicon Ultra MWCO 3000 Da) (max 500 µl) and mixed with 250 µl of Urea buffer (UA) containing 8 M urea in 0.1 M Tris-HCl, pH 8.5. Filter units are used to remove interfering chemicals and small molecules and mass spectrometry-incompatible substances, before digestion, without substantial sample loss. The device was centrifuged at 14,000 × g for 20 min and the eluate was thrown away. All subsequent centrifugation steps were performed under the same conditions, allowing maximum concentration. Subsequently, 250 µl of DTT 10 Mm in UA (reducing solution) was added to the concentrated protein mixture followed by centrifugation. Then incubation with 250 µl of IAA 55mM in UA (alkylating solution) was performed for 30 min at room temperature in the dark. The device was centrifuged to remove excess iodoacetamide solution. Two additional wash steps were performed by adding 400 µl of UA buffer followed by centrifugation. About 2 µg of LysC (Roche) dissolved in 50 mM NH_4HCO_3 was incubated with protein mixture for 3 h.
The concentrated protein mixture was also subjected to trypsin digestion by adding 1 µg in 50 mM NH_4HCO_3 before diluiting urea concentration (< 1M) with 200 µl of 50 mM ammonium bicarbonate. Trypsin digestion was performed by incubation overnight at room temperature.
Afterward, peptides were collected in a low-binding tube using centrifugation, and the filter device was rinsed with 100 µl of ammonium bicarbonate and again centrifugated at 14,000 g for 10 min to recover all peptides. Eluted peptides were desalted and concentrated using reverse-

phase C18 handmade nano-columns (StageTips) (Rappsilber et al., 2007) and evaporated on a vacuum concentrator to about 5 µl. Samples loaded on C18 StageTips (The concentrated peptides) were stocked at -20 °C and re-suspended in 15 µl of 0.1% formic acid for nanoLC–MS/MS analysis.

3.17.7 NanoLC–ESI-MS/MS peptide analysis

Chromatographic separation was performed by injecting 5 µl of digested peptides resuspended in 0.1% formic acid on a C18 HALO PicoFrit column (75 µm × 10 cm, 2.7 µm particle size, 100Å, New Objective, USA). The sample was injected by an Ultimate3000 RSLCnano system and electrosprayed using a nanoESI source (Thermo). Peptide separation was performed by a reverse phase linear gradient from 1% acetonitrile, 0.1% formic acid to 40% acetonitrile, 0.1% formic acid over 60 min, at flow rate of 300 nl/min. The instrument operated in data-dependent mode to acquire both full MS and MS/MS spectra. Full MS spectra were acquired in profile mode by the FT analyzer in a scan range equal to 300–1500 m/z, using capillary temperature 220 °C, AGC scan 5×10^5 and resolution 60,000 FWHM at m/z 400. Tandem mass spectra were acquired by the linear ion trap (LTQ) for the 2 most intense ions exceeding 1×10^4 counts. MS/MS spectra acquisition was set as follows: centroid mode, resolution 15,000, precursor ions isolation width of 2 m/z, AGC target 1×10^4 and normalized collision energy 30 eV. Dynamic exclusion was enabled to reduce redundant spectra acquisition as follows: 3 repeat counts, 30 s repeat duration, 45 s of exclusion duration. The mass spectrometer and spectra analysis were fully automated and controlled by the Xcalibur software (version 2.0.7,Thermo Scientific) and operated in data-dependent acquisition (DDA) mode, to automatically switch between MS and MS/MS scans.

3.17.8 Mass spectral data elaboration and database searching

The software Proteome Discoverer 1.4 (Thermo) was used to extract peaks from spectra and to match them against a database dealing with *Pseudomonas* microorganisms (updated 08.07.2014.)
The selected protease was trypsin (cleaving at the C-terminus of Lys and Arg, unless followed by Pro), with maximum 2 allowed missed cleavages. One static (carbamidomethylation on Cys) and 2 dynamic modifications (deamidation on Asn and oxidation on Met) were selected. The precursor mass tolerance was set to 5 ppm, and fragment mass tolerance was set to 0.5 Da. Low-confidence peptide matches were filtered out by selecting peptide confidence = high. For each protein identified, a minimum of at least two unique matching peptides was considered as a prerequisite for protein validation with a high degree of confidence.

3.18 Results and discussion

3.18.1 Extracellular caseinolytic potential of *P. fluorescens* PS19, PS60 and PS24

Among the psychrotrophic strains isolated from bovine raw milk in a previous work (Decimo et al, 2014), three strains (PS19, PS60 and PS24) exhibited different proteolytic activity at 7 °C and 22 °C. These *P. fluorescens* strains, after growth in MSM supplemented with 1 mM $CaCl_2$, showed proteolytic activity on milk agar-well plates at 22 °C. Growth and enzyme production were examined in MSM containing 1 mM $CaCl_2$. because this medium was reported to increase extracellular protease production in *Ps. fluorescens* (Amrute & Corpe, 1978). Moreover, Liao and McCallus (1998) indicated that the divalent cation Ca^{2+} is essential for the activity of the *apr*X enzyme and they used this medium for the biochemical and genetic characterization of an

extracellular protease from *Ps. fluorescens* CY091. Rajmohan et al. (2002) and Nicodeme et al. (2005) also found that the addition of $CaCl_2$ to the growth medium significantly enhanced the specific-proteolytic activity of *Pseudomonas* spp.

The heat-resistant caseinolytic activity of the cell free supernatant of each *P. fluorescens* strains was further evaluated by casein zymography and one heat-stable protease of approximately 45 kDa was detected for all the three strains (Figure 3.18.1).This result is in agreement with data reported by other researchers (Noreau & Drapeau, 1979; Kohlmann et al., 1991; Ching-hsing & McCallus, 1998; Koka and Weimer, 2000; Nicodème et al., 2005; Dufour et al., 2008, Marchand et al., 2009). All tested strains produced protease at 22 °C, but the intensity of the protease patterns on zymogram varied; *P. fluorescens* PS19 exhibited the highest extracellular caseinolytic activity while strains PS60 and PS24 displayed a weak extracellular proteolytic potential. This might be indicative for a variable expression and/or activity of the proteases. Besides, this variability in regulation might be temperature dependent (McKellar & Cholette, 1987; Burger et al., 2000; Nicodème et al., 2005, Marchand et al., 2009). *P. fluorescens* PS19, being the more proteolytic strain, was further investigated and the extracellular caseinolytic activity of its concentrated (ultrafiltered at 10 kDa) protease extract was examined on zymogram with sodium caseinate and single casein fractions (α_s, β and κ). Surprisingly, two additional proteolytic bands with molecular masses of approximately 15 and 25 kDa was visualized on zymography gels (Figure 3.18.2). The presence of proteolytic activity due to low molecular weight bands was also reported by Rajmohan et al. (2002) after ultrafiltration of the culture supernatans of a *P. fluorescens* strain. The appearance of these bands was attributed to the concentrating effect of the applied ultrafiltration process before zymogram analysis (Rajmohan et al., 2002). Nevertheless, Marchand et al. (2009) reported that same *Pseudomonas* strains, after 8 days of incubation in milk, displayed 2 or 3 clearance zones of lower molecular weight on casein zymography. They assumed the appearance to be likely attributable to substrate depletion, by which the protease, being the only protein source left, is degraded into smaller active fragments. Although the production of low molecular mass proteases by a single strain of *P. fluorescens* has been reported (Rajmohan et al., 2002), the identification of the putative 15 and 25 kDa proteases has never been described. For this reason, the crude proteolytic extract of *P. fluorescens* PS19 was subjected to proteomic characterization.

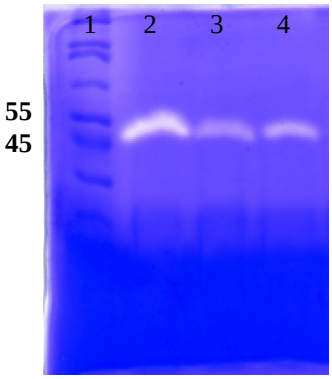

Figure 3.18.1 Extracellular caseinolytic activity of *P. fluorescens* strains: casein zymography under non reducing conditions.
1. Protein molecular weight ladder, 6.5–200 kDa
2. PS19 proteolytic enzyme extract
3. PS24 proteolytic enzyme extract
4. PS60 proteolytic enzyme extract

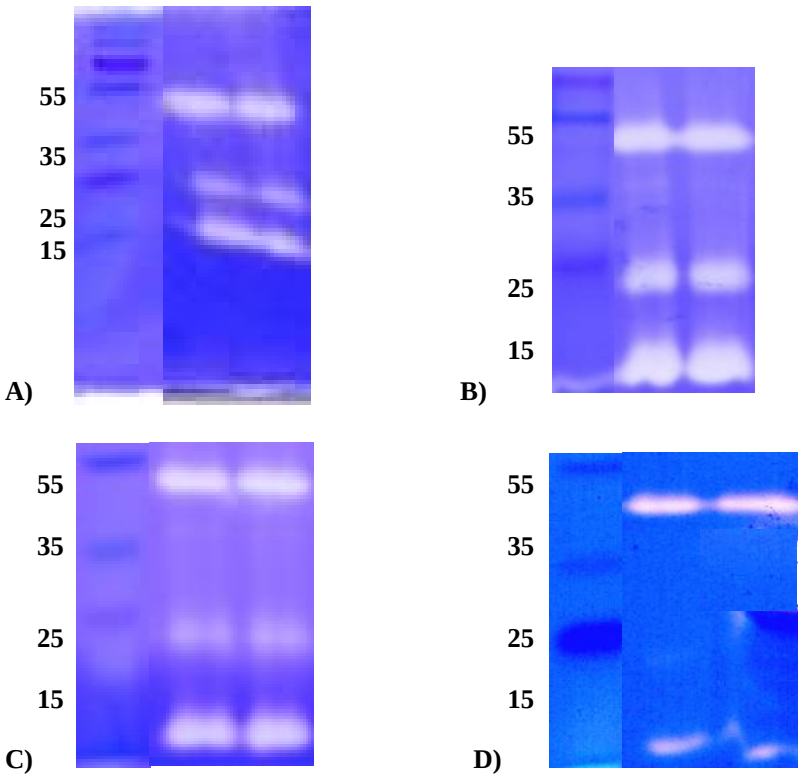

Figure 3.18.2. Extracellular caseinolytic activity of *P. fluorescens* strain PS19: casein zymography under non reducing conditions of concentrated proteolytic enzyme extract with A) 0,1% of sodium caseinate; B) 0,1% of α_s – casein; C) 0,1% of β – casein and D) 0,1% of κ – casein.
1. Protein molecular weight ladder, 10–250 kDa
2 and 3. PS19 concentrated proteolytic enzyme extract

3.18.2 NanoLC–ESI-MS/MS and identification of *P. fluorescens* PS19 extracellular proteases

The proteases of *P. fluorescens* PS19, as visualized at 45, 25, and 15 kDa on the zymogram (Fig. 2), were analyzed by nLC/MS/MS analysis after both *in gel* and *in solution* trypsinolysis. The aim was to verify if they correspond to any known protein of *Pseudomonas* spp. Nanoscale liquid chromatography coupled to tandem mass spectrometry (nanoLC–MS/MS) has become an essential tool in the field of proteomics for the analysis of peptides derived by enzymatic digestion of either purified proteins or moderately complex protein mixtures. In fact, its sensitivity has advantages over conventional LC–MS/MS that allow the analysis of peptide mixtures in sample-limited situations (Gaspari & Cuda, 2011).
Although MS strategies for the protein identification may vary (depending on the type of modification and on the available MS instrumentation), the analysis of either intact proteins (top–down approach) and/or peptides generated from enzymatic digestion (bottom–up or peptide mapping approach) is the most widely used approach (Reid & McLuckey, 2002).
For our purpose, we focused on bottom-up approach by digesting proteases the *P. fluorescens* PS19 strain both *in gel* and *in solution.*
The nLC/MS/MS analysis of the *in gel* trypsin-digested band at 45 kDa excised from the Coomassie-stained gel showed that it was similar to the sequence of *P. fluorescens* AprX metalloprotease C9WKP6 (SwissProt, accessed 20.02.2014) with a high coverage (55.14%). (Figure 3.18.3).
The nLC/MS/MS analysis of the *in gel* trypsin-digested protein band with molecular mass of 15 kDa excised from the SDS-PAA gel showed similarity to both the sequences of another AprX metalloprotease (accession number: E6Z7L2; coverage: 8.36%) and Protein F (accession number: Q51777; coverage: 10.73%) from *P. fluorescens.*
Only one matching peptide (MEPSSGLELIRK) was found for the *in gel* trypsin digestion of the 25 kDa band excised from the gel. It showed an homology to the sequence of hypothetical protein of *P. fluorescens* (accession number: CAA72727) and response regulator of *Pseudomonas* sp. TKP (accession number: WP_024077763).

```
                          MNETHASSPKAEPLSAARAVAAGIDFAELQVGPHGLFWNEYRPEDAACRV
tr|C9WKP6|C9WKP6_PSEFL    -------MSKVKDKAIVSAAQASTAYSQIDSFSHLYDR------------
                                 .*.:  : . *. *.  :::::  .*

                          WQWRDGQAHCLTPPTFSVRSRVYEYGGGAFCLTDDGLVFVNEADQQLYRQ
tr|C9WKP6|C9WKP6_PSEFL    -------------------------GGNLTVNGKPSYTVDQAATQLLRD
                                                  ** : :...    *::*  ** *:

                          SLSDETPEVLTSSECRYGDLQFANGQVLAVEENRDRHRLVAINLAGGQRH
tr|C9WKP6|C9WKP6_PSEFL    GAAYRDFDGNGKIDLTY------------------TFLTSATQSTMNKH
                          . : .  :   . :  *                    *.: . :  ::*

                          LLAEGADFYAAPTLSPDGRRLAWIEWNRPDQPWTATRLMVAERQRDHTFT
tr|C9WKP6|C9WKP6_PSEFL    GISGFSQFNTQQKAQAALAMQSWADVAN------VTFTEKASGGDGHMTF
                           ::  ::* :  . ..     :* :  .      .*    *.   .*

                          QPRCVAGDGAQESLQQPRFDDSGRLYCLTDRGGFWQPWVESTEGLSPLPS
tr|C9WKP6|C9WKP6_PSEFL    GNYSSGQDGAAAFAYLP----------GTGAGYDGTSWYLTNNSYTPNKT
                              . . ***      *           *. *    .*  :.:. :*  :

                          AAADHGPAPWQLGGCTWLPLSEDTYLASWTEEGFGRLGLCHGDGSREDFT
tr|C9WKP6|C9WKP6_PSEFL    PDLNN------------YGRQTLTHEIGHTLGLAHPGDYNAGNGNPTYND
                          .  ::               .  *:  . *       .   *:*.
```

```
                                  GDYSRFRHLAQDDQFIYCIAASPVSSASVIAIERKSRQVKTLAGGVVPLP
tr|C9WKP6|C9WKP6_PSEFL            ATYGQDTRGYSLMSYWSESNTNQNFSKGGVEAYASGPLIDDIAAIQKLYG
                                  . *.:  :  .  .:     :.   * . :    ..  :. :*.

                                  ADQISRPQTLRYPSGSGEAHGFFYPAMNDDTKPPLVVFIHGGPTSACYPM
tr|C9WKP6|C9WKP6_PSEFL            ANFNTRATDTTYGFNSNTGRDFLSATSNAD-KLVFSVWDGGGNDTLDFSG
                                  *:  :*.    *  .*. .:.*: .: * * *  : *:  **  :  :.

                                  FDPRIQYWAQRGFAVADLNYRGSSGYGRAYRQALHLSWGDVDVEDACAVV
tr|C9WKP6|C9WKP6_PSEFL            FTQ------NQKINLTATSFSDVGGLVGNVSIAKGVTIENAFGGAGNDLI
                                  *        :: : ::  .: . .*       *  ::  :.    .  ::

                                  GYLAERGLIDGDKAFIRGGSAGGYTALCALAFHKIFRAGASLYGVSDPVA
tr|C9WKP6|C9WKP6_PSEFL            IGNQVANTIKGGAGNDLIYGGGGADQLWGGAGSDTFVYGASSD--SKPGA
                                        . *.*. .     ..**   * . *  . *  ***    *.* *

                                  LGRVTHKFEGDYLDWLIGNPVEDAERYAARTPLLHANNISVPMIFFQGEL
tr|C9WKP6|C9WKP6_PSEFL            ADKIFDFTSGSDKIDLSG-------------ITKGAGVTFVNAFTGHAG
                                   .:: .  .*.    * *              : :. .::.   *

                                  DAVVVPQQTRDMVKALQDNGILVEAHYYADERHGFRKAGNQAHALEQEWL
tr|C9WKP6|C9WKP6_PSEFL            DAVLSYASG----TNLGTLAVDFSGHGVADFLVTTVGQAAASDIVA----
                                  ***:   .     . *   .: ...*  **        .  :. :

                                  FYRRVME
tr|C9WKP6|C9WKP6_PSEFL            -------
```

Figure 3.18.3 ClustalW sequence alignment of protein identified by proteomic analysis of proteolytic 45 kDa band from *P. fluorescens* PS19. Peptides as retrivied by mass spectrometry are shown in bold. Symbols (.), (:) and (*) represent the degree of conservation within the compared sequences.

The nLC/MS/MS analysis of the *in solution* trypsin-digest of the concentrated (UF 10 kDa) proteolytic extract showed that it was similar to the sequences of extracellular metalloprotease AprX from *P. fluorescens* (acc. no. C9WKP6; coverage 15.72%), of 60 kDa chaperonin GroEL from *P. fluorescens* (acc. no. C0J914; coverage 30.05%; 3) and of 50S ribosomal protein L25 from *P. fluorescens* (acc. no. F1BCI0; coverage 11.06%).
Combing the results from nLC/MS/MS analysis of both *in gel* and *in solution* digestion, the correspondence of the 45 kDa protease to metalloprotease C9WKP6 was demonstrated. In addition, it can be likely hypothesized that the 15 kDa protease is a fragment of this AprX metalloprotease (Fig. 3.18.4). On the contrary, the 25 kDa protease showed no homology to any known protein of *Pseudomonas* spp.

```
. :**************:*****
1   MSKVKDKAIVSAAQASTAYSQIDSFSHLYDRGGNLTVNGKPSYTVDQAATQLLRDGAAYR   60  C9WKP6
C9WKP6_PSEFL
1   -----------------------------------SWQPSYTVDQAATQLLREGAAYR   23  E6Z7L2
E6Z7L2_PSEFL

** ************************************************************
61   DFDGNGKIDLTYTFLTSATQSTMNK**HGISGFSQFNTQQK**AQAALAMQSWADVANVTFTEK  120  C9WKP6
C9WKP6_PSEFL
24   DFBGNGKIDLTYTFLTSATQSTMNK**HGISGFSQFNTQQK**AQAALAMQSWADVANVTFTEK   83  E6Z7L2
E6Z7L2_PSEFL

:************************************************************
121  ASGGDGHMTFGNYSSGQDGAAAFAYLPGTGAGYDGTSWYLTNNSYTPNK**TPDLNNYGR**QT  180  C9WKP6
C9WKP6_PSEFL
84   TSGGDGHMTFGNYSSGQDGAAAFAYLPGTGAGYDGTSWYLTNNSYTPNKTPDLNNYGRQT  143  E6Z7L2
E6Z7L2_PSEFL

************************************************************
181  LTHEIGHTLGLAHPGDYNAGNGNPTYNDATYGQDTRGYSLMSYWSESNTNQNFSKGGVEA  240  C9WKP6
C9WKP6_PSEFL
144  LTHEIGHTLGLAHPGDYNAGNGNPTYNDATYGQDTRGYSLMSYWSESNTNQNFSKGGVEA  203  E6Z7L2
E6Z7L2_PSEFL

************************************************************
241  YASGPLIDDIAAIQK**LYGANFNTRATDTTYGFNSNTGRDFLSATSNADK**LVFSVWDGGGN  300  C9WKP6
C9WKP6_PSEFL
204  YASGPLIDDIAAIQK**LYGANFNTR**ATDTTYGFNSNTGRDFLSATSNADKLVFSVWDGGGN  263  E6Z7L2
E6Z7L2_PSEFL

************
301  DTLDFSGFTQNQKINLTATSFSDVGGLVGNVSIAKGVTIENAFGGAGNDLIIGNQVANTI  360  C9WKP6
C9WKP6_PSEFL
264  DTLDFSGFTQNQ-----------------------------------------------  275  E6Z7L2
E6Z7L2_PSEFL


361  KGGAGNDLIYGGGGADQLWGGAGSDTFVYGASSDSKPGAADK**IFDFTSGSDKIDLSGITK**  420  C9WKP6
C9WKP6_PSEFL
276  ------------------------------------------------------------  275  E6Z7L2
E6Z7L2_PSEFL


421  GAGVTFVNAFTGHAGDAVLSYASGTNLGTLAVDFSGHGVADFLVTTVGQAAASDIVA  477  C9WKP6
C9WKP6_PSEFL
276  ----------------------------------------------------------  275  E6Z7L2
E6Z7L2_PSEFL
```

Figure 3.18.4. ClustalW sequence alignment of proteins identified by proteomic analysis of *P. fluorescens* PS19 concentrated (UF 10 kDa) proteolytic extract (*in solution* digest) and proteolytic band of 15 kDa. Peptide coverage is indicated by highlighted characters: PS19-Trypsin retentate (shown in bold), PS19-Trypsin 15 kDa (underlined and shown in bold).

3.19 Conclusions

Three strains (PS19, PS24 and PS60) of *Pseudomonas fluorescens* previously isolated from bovine raw milk, have been further investigated in this study.
All strains secreted one thermostable protease of about 45 kDa, which was characterized in *P. fluorescens* PS19 by nLC/MS-MS and assigned to *P. fluorescens* AprX metalloprotease C9WKP6. The proteomic characterization of 15 kDa protease of *P. fluorescens* PS19 showed a high similarity with AprX metalloprotease C9WKP6 whereas the 25 kDa protease showed no homology to any known protein of *Pseudomonas* spp.

3.20 References

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3.21 Identification of peptides from hydrolysis of casein fractions by *P. fluorescens* PS19 enzyme extract

Breakdown of proteins (proteolysis) is important in a wide variety of food products. Proteolysis may have beneficial effects and may be essential for desirable qualities in food products, such as flavour development and texture changes during the ripening of cheese. However, uncontrolled or unwanted proteolysis can adversely affect the quality of foods.
Proteolyis in milk is caused by both native proteases and proteases produced by psychrotrophic microorganisms during refrigerated storage of the milk (Fairbairn & Law, 1986; Grufferty and Fox, 1988). Psychrotrophic microorganisms are those that grow at 7 °C, although their optimal growth temperature may be higher. During cold storage after milk collection, they dominate the microbiota and are responsible for many quality problems in dairy products. Under sanitary conditions, <10% of the total microflora are psychrotrophs, compared to >75% under unsanitary conditions (Suhren, 1989). The numbers of psychrotrophs that develop after milk collection depend on the storage temperature and time. Among the psychrotrophs, *Pseudomonas* spp. are most frequently reported in raw milk. Psychrotrophs produce extracellular proteases (metalloproteases, that require the presence of a metal ion such as calcium for optimal activity) (Mitchell and Ewings, 1985) that contribute to sensory defects in milk and dairy products, releasing (with formation of) peptides, responsible of undesiderable bitter off-flavour. Optimal enzyme synthesis occurs in the majority of psychrotrophs at 20–30 °C, but considerable synthesis occurs at lower temperatures. For example, production of extracellular protease by *Pseudomonas fluorescens* at 5 °C was 55% of that produced at 20 °C (McKellar, 1982). Heat-stable proteases from psychrotrophs attack all forms of casein, with preferential hydrolysis for κ-casein, then β-casein, and finally α_s-casein (Fairbairn and Law, 1986; Cox, 1993). There is controversy in the literature as to whether the major whey proteins are susceptible to bacterial proteases (Fairbairn and Law, 1986; Sorhaug and Stepaniak, 1997). An extracellular protease isolated from *P. fluorescens* M3/6, produced after incubation in reconstituted nonfat dry milk stored at 7 °C, was characterized and shown to have activity on α-, β-, and κ-caseins (Kohlmann et al., 1991). Hydrolysis of κ-casein can result in destabilization of the casein micelle and the production of small peptides that contribute to bitter flavours (Cromie, 1992).
The aim of this work was to characterize by LC/MS the peptidic profiles generated by the action of thermostable protease from *P. fluorescens* PS19 strain on milk casein fractions and to identify casein peptides useful as markers to early recognize the activity of psychrotrophic strains in (un)processed milk.

3.22 Material and methods

3.22.1 Hydrolysis of casein fractions by enzyme extract from *P. fluorescens* PS19

The crude enzyme extract was obtained from the *P. fluorescens* PS19 strain isolated from bovine raw milk (Decimo et al., 2014), which was selected for its high proteolytic potential on milk agar plates and extracellular caseinolytic activity on caseinate zymogram gel. The proteolytic extract from the cell free supernatant of *P. fluorescens* PS19, grown in minimal salt (MS) medium supplemented with 1 mM $CaCl_2$ (Liao & McCallus, 1998) was concentrated by ultrafiltration (UF) through 10 kDa molecular weight cut-off (MWCO) ultrafiltration membrane. The UF retentate was used for hydrolytic activity experiments.
Hydrolytic activity on α_s, β and κ caseins was tested by incubating 5 mg of single protein in 5 mL of Tris-HCl (20 mM, pH 6.7) with 16 µL of concentrated crude proteolytic enzyme extract of *P. fluorescens* PS19. The reaction mixtures were incubated for 24, 48, 96, 120 and 144 h at 7

°C and for 4, 8, 24, 48 and 96 h at 22 °C and aliquots taken from the mixtures, were submitted to RP-HPLC analysis. Two controls, consisting of the peptide and the enzyme only, respectively, were used to determine spontaneous degradation of the casein fraction or the enzyme extract.

3.22.2 Reversed-phase – High performance liquid chromatography (RP-HPLC)

Separation of peptides in hydrolysates from *P. fluorescens* PS19 crude enzyme extract digesta was performed on an RP-HPLC apparatus. This consisted of an Alliance 2695 HPLC system (Waters, Vimodrone, Italy) equipped with a Waters 2996 diode array detector (DAD). Hydrolysates (5–50 µL) were separated on a PLRP-S column (2.1 mm i.d. × 250 mm, 5 µm, 300 Å; Polymer Laboratories Ltd, Church Stretton, Shropshire, UK) kept at 40°C. The eluents used for the separation were: solvent A, 0.1% (v/v) trifluoroacetic acid (TFA) in MilliQ-treated water, and solvent B, 0.1% (v/v) TFA in acetonitrile. Trifluoroacetic acid and acetonitrile were from Thermo Scientific (Rockford, IL, USA) and Sigma–Aldrich (Milan, Italy), respectively. The elution gradient, expressed as the solvent B proportion, was as follows: 0–5 min, 5%; 5–65 min, 55%; 65–70 min, 95%; 70–72 min, 95%, 72–76 min, 5%. The flow rate was 0.2 mL min^{-1}, and run-to-run time, 90 min. Absorbance was recorded at 210 nm and data were processed using the Empower software package (Waters).

3.23 Results and discussion

In order to determine whether *P. fluorescens* PS19 concentrated proteolytic enzyme extract hydrolyses α_s, β and κ caseins, the substrates were incubated with enzyme in 20 mM Tris-HCl (pH 6.7) buffer at 22 °C and 7 °C for different times. Subsequently, the digestion products were separated by RP-HPLC.

Previous studies have been performed to understand the proteolytic activities on milk proteins and on the casein molecules, in particular Koka and Weimer (2000) indicated that a protease isolated from *P. fluorescens* RO98 preferentially hydrolysed κ-casein in artificial casein micelles. With the same objective, Costa et al. (2002) showed that an extract of *P. fluorescens* RV10 culture proteolysed κ-casein and β-casein. More recently, Nicodème (2006) characterized an extracellular protease from *Pseudomonas aureofaciens* LBSA1 and identified some peptidic bonds which had been cleaved by this enzyme on different purified casein molecules. Mu et al. (2009) studied the proteolytic activity of Ht13 purified enzyme from raw milk-associated *P. fluorescens* Rm12, and reported that the enzyme can cleave α_s casein, β casein and κ casein, obtaining after hydrolysis at least three, two and one fragments, respectively. It was generally accepted that κ casein resides at the surface of the casein micelle as it exists naturally (Creamer, 1991; Walstra, 1990). The stability of the casein micellar system can be attributed directly to the unique properties of κ casein. Baglinière et al. (2013), studying the destabilisation of UHT milk due to the activity of *P. fluorescens* F *apr*X protease, found that the enzyme hydrolysed all casein fractions with a preference for β-casein. It has been reported that metalloproteases, as *Apr*X, preferentially hydrolyse the κ-casein then β-casein and finally α_{s1} casein. Generally, this enzyme can hydrolyse all casein molecules (Cousin, 1982; Fairbairn and Law, 1986; Koka and Weimer, 2000).

In our study, a progressive hydrolysis of α_s casein fraction [which was previously found by casein zymography to be the most extensively hydrolysed by PS19 (data not shown)] by the *P. fluorescens* PS19 concentrated enzyme extract was highlighted by HPLC. As expected, casein degradation was higher at 22 °C and no new peptidic fragments formed when hydrolysis was

longer than 96 h at 22 °C or 6 d at 7 °C, as reported in Fig. 3.23.1 and Fig. 3.23.2 for α_s casein fraction.
The different peptides are under identification by HPLC/MS.

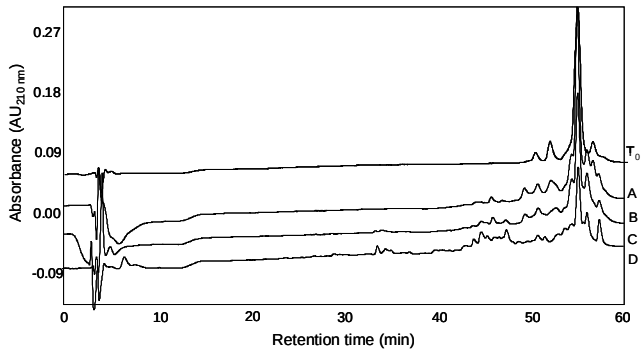

Figure 3.23.1 Chromatographic profiles of peptides generated from α_s-CN hydrolysis by enzymatic extract of *P. fluorescens* PS19 after 4 (A), 8 (B), 24 (C) and 96 (D) h of hydrolysis at 22 °C.

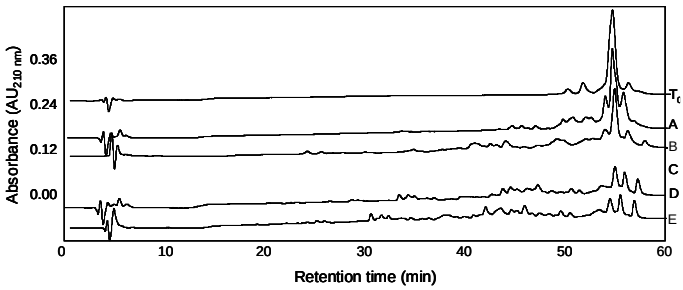

Figure 3.23.2 Chromatographic profiles of peptides generated from α_s-CN hydrolysis by enzymatic extract of *P. fluorescens* PS19 after 24 (A), 48 (B), 96 (C), 120 (D) and 144 (E) h of hydrolysis at 7 °C.

3.24 References

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Milan, December 2014

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

1. Decimo M, Morandi S, Silvetti T, Brasca M. 2014. Characterization of Gram-negative psychrotrophic bacteria isolated from bulk tank milk. Journal of Food Science **doi**: 10.1111/1750-3841.12645.

Characterization of Gram-Negative Psychrotrophic Bacteria isolated from Italian Bulk Tank Milk

Marilù Decimo, Stefano Morandi, Tiziana Silvetti, and Milena Brasca

Abstract: Eighty psychrotrophic bacterial strains, isolated from different northwest Italian bulk tank milks destined for Grana Padano cheese production, were identified by 16S rRNA gene amplification and partial sequence analysis of the *rpo*B gene. *Pseudomonas* spp. were the most commonly occurring contaminants, *P. fluorescens* being the predominant isolated species, along with *Enterobacteriaceae*, primarily *Serratia marcescens*. RAPD-PCR was used to study genetic variability and distinguish closely related strains; a high degree of genetic heterogeneity among the strains was highlighted. All the strains were characterized for their ability to produce proteases, lipases and lecithinases at different temperatures (7, 22, and 30 °C). Forty-one of the psychrotrophic strains were positive for all the enzymatic activities. The highest number of positive strains for all the incubation temperatures was found for lipolytic activity (59), followed by proteolytic (31) and lecithinase (28) activities, and the enzymatic traits varied among the *Pseudomonas* and Enterobacteriaceae strains. The proteolytic psychrotrophic strains were screened for the presence of the *apr*X gene, coding for a heat-resistant metalloprotease in *Pseudomonas* spp. The *apr*X gene was detected in 19 of 63 *Pseudomonas* strains, and was widespread in the *P. fluorescens* strains (14/19).

2. Zucali M, Bava L, Colombini S, Brasca M, **Decimo M**, Morandi S, Tamburini A, Crovetto GM. 2014. Management practices and forage quality affecting the contamination of milk with anaerobic spore-forming bacteria. Journal of the Science of Food and Agriculture **doi**: 10.1002/jsfa.6822.

Management practices and forage quality affecting the contamination of milk with anaerobic spore-forming bacteria

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Abstract

BACKGROUND: Anaerobic spore-forming bacteria (ASFB) in milk derive from the farm environment, and the use of silages and management practices are the main responsible of milk ASFB contamination. The aim of this study was to evaluate the relationships between feeding, milking routine and cow hygiene and milk and Grana Padano cheese (produced with and without lysozyme) ASFB contamination.

RESULTS: The study involved 23 dairy farms. ASFB in corn silage were on average 2.34 ± 0.87 $\log_{10}$ MPN g^{-1}. For grass, Italian ryegrass and alfalfa, ASFB ($\log_{10}$ MPN g^{-1}) were numerically higher for silages (3.22) than hays (2.85). The use of corn silages of high quality (high lactic and acetic acids concentrations) decreased the milk ASFB contamination, whilst the use of herbage silages did not affect it. The presence (>40%) of cows with dirty udders increased the ASFB contamination of milk, while forestripping had a positive effect (−9% ASFB). Ripened Grana Padano had an ASFB count below the analytical limit; *Clostridium tyrobutyricum* DNA was found only in wheels produced without lysozyme, which also showed late blowing.

CONCLUSION: The factors increasing milk spore contamination were corn silage quality, cow udder hygiene and inadequate milking routine. Late blowing was present only in cheeses without lysozyme.

3. Battelli G, Silvetti T, **Decimo M**, Brasca M. Volatile organic compounds produced in milk by *Enterococcus faecalis*. In Proceedings of the 10th International Meeting on Mountain cheese, Dronero (CN), Italy, 14-15 September 2011, pp. 75-76 ISBN: 978-88-902754-5-6.

Abstract
Enterococci can contribute positively to the development of flavour during cheese ripening as their presence is consistently reported in many raw milk cheeses. They can influence flavour taste and texture of cheeses as they produce several enzymes that interact with milk components, thus promoting important biochemical transformations. For this survey, *40 Enterococcus faecalis* strains, collected in different valleys in northwest Italy, were inoculated in milk and submitted to head-space solid-phase-micro- extraction gas chromatography-mass spectrometry analysis. The major volatile compounds detected were: ethanol, diacetyl, acetoin, acetic and benzoic acids. The variability was huge, demonstrating a very different enzymatic activity among strains. Apart from other important phenological parameters for characterizing strains that can be used as starter cultures, it seems useful to also give some information about their capability of enhancing flavour characteristics in the cheese.

4. Decimo M, Bacterial enzymatic activities as potential markers for assessing the technological properties of (un)processed milk. In Proceedings of the 17th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, University of Bologna, Cesena, 19-21 September, 2012, pp. 299-300.

Abstract
Psychrotrophic bacteria are responsible for the highest spoilage of unprocessed or heated milk during storage, because of their capacity to synthesize thermostable extracellular lipases and proteinases which can hydrolyze milk fat and proteins, raising many quality and technological problems. To date, still little is known about the presence and the hydrolytic activity of these enzymes in milk. On these bases, this PhD thesis research project aims to acquire further knowledge on the enzymatic activity of psychrotrophs, isolated from unprocessed milk, in order to assess presence and type of activity of lipases and proteinases and hence to predict the overall quality of (un)processed milk.

5. Battelli G, Bava L, **Decimo M**, Mattiello S, Povolo M, Sanna M, Zanini L, Sandrucci A, Brasca M. Individuazione di modelli di aziende zootecniche per produzioni di eccellenza di latte e derivati. In abstracts of the 3rd Dairy Meeting – AITeL, Milk and Dairy Products: Research and Innovation. Milano, 28 September 2012.

Abstract
Il latte rappresenta uno dei principali prodotti del settore agro-alimentare lombardo. La Lombardia detiene il primato in Italia per il latte bovino, con una produzione pari a circa il 37% di quella nazionale, essendo anche il primo produttore di formaggi, tra i quali figurano formaggi DOP di grande pregio. Una produzione così importante per l'economia del territorio deve avere carattere di eccellenza, un'eccellenza "globale" (microbiologica, nutraceutica e organolettica), che non sia a scapito del benessere animale e che sia eco-sostenibile. Le aziende che si distinguono per questi parametri di eccellenza potrebbero avvalersi di un adeguato riconoscimento commerciale e, in prospettiva, aderire ad un Marchio di Filiera che garantisca al consumatore non solo la qualità superiore del prodotto finito, ma anche una gestione aziendale rispettosa dell'ambiente e degli animali. Grazie alla collaborazione di competenze che comprendono tutta la filiera produttiva, è in corso una sperimentazione che, coinvolgendo 30 aziende zootecniche selezionate sulla base di parametri gestionali (criteri:

autosufficienza alimentare; quantità di insilati e/o foraggio), raggruppate in base alla collocazione geografica (LO-MN-CO/LC), vuole identificare modelli aziendali virtuosi che comprendono tutti i processi della filiera produttiva: la coltivazione delle essenze foraggere, la composizione della razione, la gestione della mandria in stalla e in sala di mungitura, lo spandimento dei liquami, la gestione del territorio, le modalità di stoccaggio del latte e la sua trasformazione, parametri che in diversa misura differenziano le aziende nelle tre zone considerate. Il latte prodotto da queste aziende, raggruppato nelle tre diverse zone, viene trasformato a Grana Padano da una unico caseificio. La sperimentazione prevede sul latte di ogni singola azienda, nella stagione estiva ed in quella invernale, analisi atte a valutarne la qualità microbiologica, compositiva e nutrizionale. La sperimentazione prevede inoltre l'analisi della qualità nutrizionale ed organolettica del formaggio a 9, 16 e 20 mesi.

6. Zucali M, Battelli G, **Decimo M**, Mattiello S, Povolo M, Sanna M, Zanini L, Guerci M, Tamburini A, Brasca M. Qualità del latte e sostenibilità di aziende zootecniche di tre differenti realtà lombarde. In abstracts of the 3rd Dairy Meeting – AITeL, Milk and Dairy Products: Research and Innovation. Milano, 28 September 2012.

Abstract
Il presente lavoro si propone di studiare 3 modelli di aziende zootecniche rappresentative della realtà lombarda (province di Como-Lecco, Lodi e Mantova) definiti in base al carico di animali per ettaro e livello di autosufficienza alimentare, percentuale di utilizzo di alimenti insilati nella razione, qualità e quantità di foraggio nella razione. In 30 aziende è stato valutato l'impatto ambientale attraverso la metodologia Life Cycle Assessment, monitorato il benessere animale, determinate le caratteristiche chimiche e microbiologiche del latte. Le aziende di Lodi, rispetto a quelle delle altre province, sono le più grandi sia in termini di capi allevati (128 bovine) che di ettari coltivati (55 ha), hanno acquistato più alimenti concentrati ($P<0,05$) e questo ha determinato un maggior surplus di azoto a livello aziendale ($P<0,005$). Sempre in queste aziende sono state rilevate maggiori percentuali di zoppie e diarree, sintomo quest'ultimo forse dovuto all'elevata quantità di silomais in razione (34,5%). I parametri di impatto ambientale non hanno presentato differenze significative nei tre gruppi di aziende, anche se è possibile notare un maggior impatto delle aziende del lodigiano. Simili nelle tre zone sono anche i contenuti di PUFA, MUFA, acidi grassi n-6 e n-3 del latte. La presenza di elevate percentuali di fieno nella razione sembra invece aver determinato una quantità più elevata ($P<0,05$) di alcuni idrocarburi quali 2-fitene, fitano, nonacosano, entriacontano e gli esteri del fitolo nel latte delle stalle di Mantova che sono caratterizzate da una minor presenza di silomais in razione. La carica batterica del latte di massa è rappresentata in gran parte da batteri lattici mesofili. Ciò indica la buona qualità microbiologica del latte data dalla corretta igiene in mungitura ed adeguata temperatura di conservazione. Nel latte delle aziende di Mantova i batteri coliformi erano presenti in quantità più elevate rispetto alle altre zone. I risultati ottenuti mostrano che il fattore che ha maggiormente influenzato alcuni parametri di sostenibilità ambientale, di benessere e le caratteristiche del latte è la percentuale di inclusione dei silomais nella razione delle bovine da latte.

7. Bava L, Zucali M, Sandrucci A, Guerci M, Battelli G, Brasca M, Povolo M, **Decimo M**, Tamburini A. How different farming systems can affect nutraceutical and traceable components of cow milk? XX Congresso Aspa – Animal Science and Production Association, Bologna, 11-13 June, 2013.

Abstract
The aim was to analyze different dairy farming systems to clarify if the management can influence the nutraceutical composition and traceable components of cow milk. All of the 29 farms studied were located in Northern Italy and they were members of a cheese factory which produced Grana Padano cheese P.D.O. Information about herd composition and milk production, feeding system and land utilization were obtained through personal interviews to the farmers. In each farm samples of bulk milk were collected for chemical and microbiological analyses. The farms had characteristics of high intensity: lactating cows was on average 78.4±46.6, stocking density 4.09±2.56 LU/ha, milk production (FPCM) 27.0±4.21 kg/d cow, milk fat 3.94±0.15% and milk protein 3.43±0.15%. Most of t! he farms included corn silage in the cow ration, forage intake was 59.5% and feed self-sufficiency was 63.6%. A cluster analysis was performed on farm characteristics (ha of land, number of cows, milk production level, dairy efficiency, feed self-sufficiency) and identified three different groups of farms. Cluster 2 included 11 farms characterized by low intensity level: few lactating cows (32.4), low stocking density (2.97 LU/ha), low corn silage intake (22.8% DMI) and high forage intake (64.1%). Milk microbial contamination (SPC, coliform count and clostridium spores count) was not statistically different among the groups while the lactic bacteria were higher in milk of cluster 2 compared to the others. Nutraceutical components (CLA, MUFA, omega 3 and omega 6 fatty acids) were similar among the clusters. Some non-volatile hydrocarbons present in the neutral lipid fraction of milk fat (phytane, phytene, phytyl C16, phytyl C18 unsaturated e phytyl C18 saturated) were significantly h! igher in the farms of Cluster 2 than in Cluster 1(10 farms) e 3 (7 farms). We supposed that these molecules were linked with the high forage content and low maize silage percentage of cows' rations in Cluster 2 farms. The results showed that the different level of intensity of farms involved had a low influence on nutraceutical composition of milk and that some non-volatile hydrocarbons in milk could be used as markers of forage composition of cow's diet.

8. Decimo M, Bacterial enzymatic activities as potential markers for assessing the technological properties of (un)processed milk. In Proceedings of the 18th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Universities of Padova and Udine, Conegliano, 25–27 September, 2013, pp. 239-240.

Abstract
Psychrotrophic bacteria are responsible for the highest spoilage of unprocessed or heated milk during storage because of their capacity to synthesize thermostable extracellular proteases and lipases. The diversity and enzymatic traits of 70 psychrotrophic strains, isolated from samples of unprocessed milk, were investigated, and the presence of the *apr*X gene was ascertained. Extracellular caseinolytic activity of three selected strains of *P. fluorescens* (two *apr*X+ and one *apr*X-) was evaluated and the approximate molecular mass of their proteases was estimated by casein zymography. Finally, the production of volatile organic compounds in UHT milk inoculated with 5 different psychrotrophic species was evaluated by SPME-GC/MS to investigate the relationship between the presence of these bacterial strains and the onset of certain off flavours in milk.

9. Decimo M, Cabeza MC, Ordonez JA, Silvetti T, Morandi S, Brasca M. Caratterizzazione di composti volatili in latte a seguito di sviluppo di batteri psicrotrofi. In abstracts of the 4th Dairy Meeting – AITeL, Milk and Dairy Products: Research and Innovation. Padova, 12 September 2014.

Abstract
I batteri psicrotrofi rappresentano una delle principali cause di deterioramento dei prodotti lattiero-caseari. Tali microrganismi infatti, seppure siano facilmente distrutti dai trattamenti termici, rilasciano enzimi termoresistenti (proteasi, lipasi e lecitinasi) in grado di influenzare le caratteristiche di *flavour*, colore e *texture* dei prodotti e conseguentemente la loro possibilità di conservazione.
Lo studio ha valutato i composti organici volatili (VOCs) rilasciati in latte a seguito dello sviluppo di batteri psicrotrofi con attività lipo-proteolitica mediante microestrazione in fase solida (SPME) accoppiata alla gas cromatografia/spettrometria di massa (GC-MS). *P. fluorescens* PS14, *P. fragi* PS 55, *P. mosselii* PS39, *P. rhodesiae* PS62 e *Serratia marcescens* S92, appartenenti alla collezione CNR-ISPA, sono stati inoculati in latte UHT, conservati a 5°C e 10°C per 20 giorni e analizzati a tempi stabiliti.
L'analisi dei dati indica che allo sviluppo di ciascun microrganismo durante la conservazione del latte sono associabili profili cromatografici complessi e specifici. Il latte UHT non contaminato mostra una ridotta quantità e diversità di composti volatili rispetto al latte inoculato; il numero di composti volatili.

10. Decimo M, Bacterial enzymatic activities as potential markers for assessing the technological properties of (un)processed milk. In Proceedings of the 19th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, University of Bari, Bari, 24-26 September, 2014, pp. 278-282.

Abstract
Psychrotrophic bacteria are responsible for the highest spoilage of unprocessed or heated milk during storage because of their capacity to synthesize thermostable extracellular proteases and lipases leading to gelation, off-odours/flavours, loss of sensory quality and shelf life. This PhD thesis dealt with the assessment of the enzymatic traits of 80 raw milk-associated psychrotrophic bacteria and the detection of specific molecules and metabolites derived from bacterial enzymatic degradation of milk components that could be used as a good markers to early recognize the activity for psychrotrophic strains in unprocessed milk.

Quaderni della Ricerca

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2 Brasca M, Bava L, Zucali M, Tamburini A, Guerci M, Sandrucci A, Mattiello S, Andreoli E, Battini M, **Decimo M**, Morandi S, Battelli G, Povolo M, Pellizzola V, Passolungo L, Sanna M, Zanini L. (2014) Modelli di azienda zootecnica per latte e formaggi d'eccellenza. – Regione Lombardia – Quaderni della ricerca n. 163.

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APPENDIX 3. INDEX OF FIGURES