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Effetto della temperatura di conservazione del latte, dalla raccolta all'affioramento, sulla microflora del latte, del sieroinnesto e del formaggio *Trentingrana*

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1. INTRODUCTION

1.1. FERMENTED DAIRY FOODS

Fermented dairy products as cheeses have been discovered by accident, but their history has also been documented for many centuries. The primary objective of cheese manufacture originally was to extend the shelf life and conserve the nutritious components of milk. This is achieved either by acid production and/or dehydration. Production of lactic acid by the starter microflora during cheese manufacture results in a decrease in the pH of the milk and this, in combination with cooking and stirring, promotes syneresis of the curd and expulsion of whey (Walstra, 1993). So the manufacture of most cheese varieties involves four ingredients: **milk**, rennet, **micro-organisms** and salt, which are processed through a number of common steps such as gel formation, whey expulsion, acid production and salt addition, followed by a period of ripening.

1.1. MILK PROPERTIES INVOLVED IN CHEESE MANUFACTURE

Milk is a very complex food in which over 100.000 different molecular species are found. Many factors affect milk composition such as breed, cow, herd and geographical variations.

The approximate composition of milk is

- 87.4 % of water
- 3.7 % of fat
- 4.8 % of lactose
- 3.4 % of casein
- 0.7 % of minerals

Depending upon the component, milk may be classified as:

- Oil in water emulsion (the fat globules are dispersed in the continuous serum phase)
- A colloid suspension (casein micelles, globular proteins and lipoproteins)
- A solution (lactose, soluble proteins, minerals, vitamins and other components)

1.2.1. Milk Lipids

The main milk lipids are triglycerides, which may represent up to 98% of the total lipids. Triglycerides have molecular weights ranging from 470 to 890 Da, corresponding to 24–54 acyl carbons. Triglycerides are esters of glycerol composed of a glycerol backbone with three fatty acids attached. The major (about 75 %) fatty acids found in milk triglycerides have a long chain (myristic, palmitic, stearic and oleic acids). Capric, caprylic, caproic, and butyric acids are the main fatty acids with short chain present in milk (Alais, 2000a). The final melting point of milk fat is about 37 °C and not higher because the fatty acids with higher melting point

dissolve in water. This allows the milk to be liquid at the temperature of the cow's body (Collins et al. 2003a).

The fat is predominantly present in spherical globules (Milk Fat Globules: MFG) varying in diameter from 0.1 to 15 μm. The diameter of MFG depends on cow breed, feed and season (Michalski et al. 2001). The core of the MFG mainly consists of triglycerides and is formed in the secretory cells of the mammary gland. Precursors of MFGs are formed at the endoplasmic reticulum and are transported through the cytosol as small lipid droplets covered by a non-bilayer coat material of polar phospholipids and proteins. During transport the lipid droplets grow in size, apparently due to droplet–droplet fusion. At the apical plasma membrane, the lipid droplets are secreted from the epithelial cell (Dhantine et al., 2000).

MFGs are surrounded by a native stabilizing membrane, allowing them to be compatible with the aqueous environment: the milk fat globule membrane (MFGM). The MFGM protects the triglycerides of the core from lipolysis and oxidation (Wiking et al. 2003). This complex MFGM, around 15 nm thick, originates from a primary membrane in the secretory cell, additioned with the membrane of the apical cell after secretion. During the secretion process, the droplets are covered by the plasma membrane and finally pinched off into the milk channel (Danthine et al. 2000). The MFGM is composed mainly of phospholipids, glycolipids, proteins (25-60% of MFGM mass), lipoproteins and enzymes such as butyrophilin and xanthine oxidase (Danthine et al. 2000).

The structure and texture of dairy gels and cheeses are affected by the interactions between the surface of milk fat globules and the casein matrix. In this respect, native milk fat globules do not interact with the protein network in dairy gels and act mainly as inert fillers or structure breakers, depending on their size and number (Michalski et al. 2002). Milk fat is recognized to contribute greatly to the texture, flavor and physicochemical properties of cheese. Particularly, depending on its solid fat content, it acts more or less as a plasticizer (Jaros et al. 2001). Furthermore, the **size** of MFGs affects the visco-elasticity of acid and rennet gels: for a given fat content, if native MFG were smaller, they would represent a greater number of weak points in the matrix and a larger surface area of MFGM, with a higher water-binding ability and enzymatic content. Conversely, large fat globules provide larger weak points, although less numerous and have been found to be more susceptible to coalescence and lipolysis during pumping. The presence of small or large native MFG results in different cheese flavour and aroma (Goudédranche et al. 2000; Saint-Gelais et al. 1997). Even the cheese texture is affected by the MFG size: cheeses produced with native small MFGs (~3 μm in diameter) contained more moisture, were less firm and had a more elastic texture than cheeses made of milk containing large MFGs (~6 μm); Michalski et al. (2003) explained these results by the larger surface area of small MFGs having a higher water binding capacity and the fact that thinner casein strands are formed in cheeses with small MFGs. The MFGs size may be affected by breeding practice and dairy technology: Wiking et al. (2004) showed that MFG grow larger when the fat production by the dairy cow increases the fat synthesis probably because of changing in fat synthesis regulated trough cow feeding. Ma and Barbano (2000) indicated that gravity

separation during creaming can also influence the MFG size in partially skim milk after creaming because gravity separation should produce a partial skim milk that has a larger proportion of smaller size MFG than when it is produced with a cream separator.

1.2.1.1. Damages to MFG

If the fat globule membrane is damaged or weakened, milk fat can be hydrolysed giving rancid flavour by indigenous or bacterial lipoproteins. **Mechanical treatments** increase the risk of inducing damage to the membrane. This may be due to imposed physical stress. Disruption of the membrane results in coalescence of MFGs. In the milk are present Lipoprotein lipase (LPL) which play a physiological role in the metabolism of plasma triglycerides and enter into the milk from the blood (Olivecrona et al. 2003). This enzyme may induce lipolysis of MFG triglycerides if MFGM is damaged. The levels of LPL in milk are enough to cause perceptible rancidity in milk within about 10 s (Collins et al. 2003a), but the LPL activity is not allowed under normal circumstances, because milk fat is protected from the action of LPL by the milk fat globule membrane (MFGM) and more than 80% of LPL is associated with the casein micelles (Stepaniak L.; 2004). However, if the MFGM suffers mechanical damage, for example by agitation or fast temperature change, significant lipolysis may occur quickly, resulting in the development of off-flavours (Collins et al. 2003a). LPL exhibits a preference for hydrolysis of triglycerides containing medium-chain fatty acids (C6–C12) and acts preferentially at the *sn*-1 and *sn*-3 positions of triglycerides (Collins et al. 2003b). LPL activity is of most significance in raw milk cheeses as the enzyme is completely inactivated after a 10 s treatment at 78 °C (Driessen 1989). The lipases and consequent lipolysis cause fat globules breakdown in cream (Craven et al. 1992), higher amount of free fatty acids (FFA) in milk resulting in the appearance of rancid flavours in dairy products, and low yield in hard cheeses (Chen et al. 2003, Sorough et al 1997). Another factor contributing to the damage of MFGs is air and foaming. Uptake of air in milk will further increase the damage of MFGs imposed by mechanical treatments. An inconsiderate milk treatment such as frequent drawing and icing may induce MFGM damages and consequent lipolysis. The majority of FFA formation occurs before milk reaches the dairy factory maybe because the implementation of automatic milking-systems in dairy production can cause an increase in lipolysis of bulk milk The mechanical stress that affects milk in the milking systems is a crucial factor in relation to milk quality. The supplied thermal or mechanical energy into the multi-component milk system can damage the fat globule membranes and liberates fatty acids from the triglyceride ester link (Klungel et al. 2000). Some of the deterioration of milk can be prevented by technological improvements, cooling strategies, and even by low fat content feeding diets, resulting in higher de novo synthesis and thereby creating stable MFGs (Wiking et al. 2003).

The fat destruction is a phenomenon caused in milk not only by natural enzymes present in milk but even by extra-cellular lipases and phospholipases (active on MFGM) deriving by **milk bacterial contamination** and in particular from psycrotrophic microorganisms, mainly *Pseudomands* which are able to develop during storage at low

temperatures in milk. Lipolysis is therefore spontaneous or induced. At the same time lipases can be remarkably heat stable and decompose dairy products even after a milk heat treatment. Due to a number of changes in the milking system, like cooling storage, the portion of psychrotrophic microflora grows, which is a significant lipase and protease carrier (Koka et al. 1991; Lopez-Fandino et al. 1993, Wiedmann et al. 2000). Insufficient cow housing and milking hygiene just as bad raw milk storage and treatment can lead to the growth of undesirable psychrotrophic milk microflora which can increase lipolysis intensity. There are also other factors increasing the FFA content in milk such as mastitis disorder with an increased somatic cell count in milk, late lactation stage, shorter intervals between milkings and the bad quality of particularly bulky fodders such as silage and hay (Hanus et al. 2008).

1.2.1.2. Creaming

The natural creaming process, or gravity separation, is used as a critical processing step for removing milk fat before manufacturing. The gravity separation technique employed may seem primitive; however, it has been shown that gravity separation is an essential part in the cheese-making so that in the traditional Italian dairy industry, milk is processed for typical products such as for long-ripening cheeses (e.g., Grana Padano, Grana Trentino and Parmigiano Reggiano).

The cheese-making processes involve different traditional stages that give specific features to the final product quality. Natural creaming is the traditional way to achieve the two main results that affect the quality of milk: a significant reduction in microorganisms and spore-forming bacteria present in raw milk (Dellaglio et al. 1969), which could determine cheese loss during the ripening stage through the late blowing defect, and a standardization of milk fat-to-casein ratio. Natural creaming is according to Stokes' law: separation velocity of a fat globule from the skim phase during creaming, can be calculated using Stoke's Law: fat has a lower density than the aqueous phase of milk; therefore, fat globules tend to rise (Ma and Barbano, 2000):

$$V = \frac{2\, r^2 (dm - dl)\, g}{9\, \eta}$$

r is the fat globule ray
dm is the density of the medium and dl is the density of the fat
g is the force applied (the gravity one)
η is the viscosity

According to this law, higher is the temperatures and lower is the viscosity resulting in a faster rate of fat separation than at lower temperatures but actually the optimal temperature is about 10-15 °C because at these low temperatures the fat globules agglutinate in bigger clusters than at higher temperature, so the ray (r in the law above) is much bigger. Ma and Barbano (2000) saw that in two hours at 15 °C, large fat globule clusters have already moved to the top of the cream layer and even if fat

content in the top cream layer continued to increase with separation time, the MFG mean diameter doesn't' increase.

Raw whole milk is stored in shallow separation basins at 14 to 20 °C (15 °C for Grana Trentino cheese) for 10 to 12 h to gravity separate milk (Panari et al. 2007). When the desired extent of fat separation is achieved, the partially skimmed raw milk with about 2% fat is drained from the bottom of the basins and transferred to cheese vats.

1.2.2. Milk Proteins

Approximately 3.0-3.5% of normal bovine milk is made up of protein; the concentration and composition of which can change during lactation. Originally, milk proteins were believed to be a simple homogeneous protein, but about a century or more ago, milk proteins were divided into two broad classes. The first fraction, which is about 80% of the protein in bovine milk, is precipitated at pH 4.6 (isoelectric pH) at 30 ºC, and is now called **casein**. The second minor fraction, makes up about 20% of protein, is soluble under those conditions, and is now referred to as whey protein or serum protein or non-casein nitrogen. The rest are trace fractions of glycoproteins (Alais, 2000b).

1.2.2.1. Caseins and their structure

The unique characteristic of caseins is their post-translational modifications, resulting in the phosphorylation at seryl and infrequently threonyl residues; hence, caseins are phosphoproteins. Casein is made up of many components, and the main types are α_{S1}-casein, α_{S2}-casein, β-casein, and κ-casein (Alais, 2000b) as defined and validated by analysis of DNA sequences. There are trace amounts of γ-casein occurring naturally on account of limited proteolysis of β-casein by plasmin (Alais, 2000b). The main casein components have several genetic variants and contain variable numbers of phosphoseryl residues, especially α_{S2}-casein exhibiting a large variability in phosphorylation. Another unique feature of caseins is the large amount of proline residues, especially in β-casein, which greatly affect the structure of caseins, because the proline residues disrupt the formation of αα-helical and β-sheet (Alais, 2000b). In addition, all casein proteins have different hydrophobic and hydrophilic regions along the protein chain. α_S-caseins are the major casein proteins containing 8-13 seryl phosphate groups, while β-casein contains about 5 phosphoserine residues, and it is more hydrophobic than α_S -caseins and κ-casein. Cause α_S -caseins and β-casein are highly phosphorylated, they are very sensitive to the concentration of calcium salts, that is, they will precipitate with excess Ca^{2+} ions. Unlike other caseins, κ-caseins are glycoproteins, and they have only one phosphoserine group. Hence, they are stable in the presence of calcium ions, and they play an important role in protecting other caseins from precipitation and make casein micelles stable. Casein is not heat sensitive; only temperatures up to or above 120 ºC cause the gradually insolubilisation of casein, whereas it is sensitive to pH and will precipitate at its isoelectric pH (Alais, 2000b).

Caseins in normal milk are in the form of colloidally dispersed particles, known as micelles containing on a dry basis of 92-93% of protein and 7-8 % colloidal calcium phosphate (CCP), which is comprised of

calcium, phosphate, and citrate. The shape of casein micelles as observed by electron microscopy is spherical, ranging in size from 20-300 nm in diameter (average about 100 nm) and a molecular mass ranging from 0.5-1.0 x 10^9 Da (Alais, 2000b). Due to the importance of casein and casein micelles for the functional behaviour of dairy products, the nature and structure of casein micelles have been studied extensively, but the exact structure of casein micelles is still under debate. Various models for casein micelle structure have been proposed and fall into three general categories, which are: coat-core, subunit (sub-micelles), and internal structure models. We are going to talk about the Walstra model (1990) which falls in the sub-micelle category. This model suggests that casein micelles are built of roughly spherical subunits or sub-micelles. The composition of sub-micelles is variable and the size is in range 12-15 nm in diameter, and each sub-micelle has 20-25 casein molecules. The sub-micelles are kept together by hydrophobic interactions between proteins, and by calcium phosphate linkages. There are two main types of sub-micelles; one mainly consisting of α_S- and β-caseins, hydrophobic regions buried in the centre of the sub-micelle, another type consisting of α_S- and κ-caseins, which is more hydrophilic because of the sugar residues on κ -caseins. The κ -caseins are located near the outside of the micelle with the hydrophilic part of the C-terminal end protruding from the micelle surface to form a 'hairy' layer that will avoid further aggregation of sub-micelles by steric and electrostatic repulsion. Consequently, micelles are stable, and they do not usually flocculate.

Two alternative models, which fall into internal structure category, have been proposed by Holt in 1996 and by Horne in 1998.

1.2.2.2. Caseins micelles and coagulation

There are several ways in which caseins can aggregate: by **proteolytic enzymes**, by acidification and by heat. The first way is the one used in cheese manufacturing and evolve in three reactions:

1 Rennet cleaves the Phe (105)-Met (106) linkage of κ –caseins resulting in a Casein Macro Peptide (CMP) diffusing away from micelles while the para-κ–casein (hydrophobic peptide) remain on the micelles. When k-casein is hydrolyzed, the casein micelle becomes unstable and susceptible to precipitation by calcium. With an increase in temperature up to 40 °C the rate of the rennet reaction increases.

2 Aggregation of the casein micelles occurs in a non-enzymatic phase because of hydrophobic interactions between casein molecules. Calcium assist coagulation by creating isoelectric conditions and by acting as a bridge between micelles. The temperature at the time of coagulation is very important: at temperatures lower than 15 °C the aggregation reaction becomes very slow.

3 After the gel forming, the micelles rearrange: there is a loss of paracasein as the milk curd firms and sineresys begins (Alais, 2000b).

Coagulation time (CT) is the point at which coagulation is first notable; the time when casein micelles have aggregated sufficiently to form visible flocs. Coagulation rate (CR) is a measure of how quickly the curd firms once coagulation has begun. Ideally, cheese-makers would like to minimize CT and maximize CR since both influence the processing time

required for cheese production. Most importantly, cheese-makers want to maximize curd firmness (CF) since it is the primary coagulation property that influences cheese quality, yield, and economic returns. A firm curd improves cheese yield by encouraging retention of milk components (Aleandri et al., 1990). Extensive research has been conducted to study the relationships between cow milk composition and coagulation properties (Pagnacco and Caroli, 1987; Politis and Ng-Kwai-Hang, 1988). Cow milk researchers agree that high fat and protein, casein in particular, contribute to better coagulation properties and cheese yield. In addition, research has shown that when protein or casein levels are high in cow milk, CT tends to be short, CR fast, and CF high (Marziali et al. 1986; Politis and Ng-Kwai-Hang, 1988).

1.2.2.3. Serum proteins

Traditionally, serum proteins or whey proteins is the term describing the milk proteins remaining in the serum after precipitation of caseins or after casein is removed. Whey proteins contain about 20% of the total milk protein. Most serum proteins are globular, soluble proteins with densely folded peptide chains. They are heat-sensitive and will denature and become insoluble once milk is heated till 100 °C. The two main components of serum protein in bovine milk are α-lactalbumin and β-lactoglobulin, and the rest are (blood) serum albumin, immunoglobulins, proteose-peptones, and trace amount of enzymes and proteins with specific metabolic functions, such as lysozyme and lactoferrin (Alais, 2000b).

1.3 MILK MICROBIOTA: STORAGE AND PROCESSING

Milk is sterile at secretion but is contaminated by bacteria before it leaves the udder. Except cases of mastitis, the bacteria at this point are few in number. The health of the dairy herd, milking and pre-storage conditions are basic determinants of milk quality (Aumaitre, 1999). Microbial counts in raw milk depend on the temperature at which milk is stored and on the time that elapses between milk production and collection (Soler et al.1995). Others sources of contamination by micro-organisms are unclean teats, storage equipment, milking room floor, feed trough, atmosphere of the cow's housing and transport equipment (Michel et al. 2001). Bacterial counts on teats and milk may be easy reduced by cleaning with a paper towel (Rasmussen et al. 1991). Michel et al. (2001) have shown that the microbial count and the balance between spoilage and useful cheese making micro-organisms can be influenced by a combination of milking practices (equipment, pre-milking and post-milking udder preparation). Information on the microbial content of milk can be used to judge its sanitary quality and the conditions of production. Before to be processed at the cheese factory, just after transport from farm to cheese factory, raw milk contains an indigenous microbial pool consisting of lactic acid bacteria (LAB) along with micro-organisms such as psychrotrophic species (e.g. *Escherichia coli* and *Pseudomonas sp.*) and spore forming bacteria such as *Bacillus sp.* or *Clostridiaceae* which are organisms from soil and can contaminate milk at various steps along the processing line including during storage in tankers

(Rasmussen et al. 1991; Michel et al. 1991; Sorhaug and Stepaniak 1997; Ercolini et al 2009; Verdier-Metz et al. 2009). LAB from raw cow milk comprise mainly of species lactococci, enterococci and streptococci, including *Lc. lactis* ssp. *lactis and cremoris, L. garviae, St. thermophilus, St. macedonicus, E. faecalis* and *E. durans.* Lactobacilli, leuconostocs and pediococci can be also found at low levels (Franciosi et al. 2009).

1.3.1 Milk storage

Refrigeration is undoubtedly the most widespread milk storage system used to avoid microbial proliferation between the milking and cheese making steps. We will consider the merits and disadvantages of this system. The main reasons for the spreading of refrigeration, and the consequent collection and transport of the milk in tanks, may be summed up as follows (Castagnetti et al. 1982):

- Technical reasons: it is well known that the application of low temperature avoids microbial proliferation keeping high the milk bacteriological quality to be maintained until processing. Keeping milk in clean containers at refrigerated temperatures immediately after milking process may delay the increase of initial microbial load and prevent the multiplication of micro-organisms in milk between milking at the farm and transportation to the processing plant (Bonfoh et al., 2003).
- Economic reasons: refrigeration makes it possible to rationalize the collection routes, and hence to reduce transport costs from the farm to the processing plants.
- Manpower reasons: refrigeration permits, within certain limits, a greater flexibility in working hours both for the farm workers and those employed in the processing factories.
- Other advantages offered by refrigeration system: it provides a storage tank which eliminates the difficulties arising from the use of cans and it reduces to a minimum any violent shaking of the milk or contact with the air.

The European Community has recently (EC 854/2004) set limits for microbial counts in raw milk at 3×10^5 just before cheese-transformation, with the aim to improve the overall conditions of hygiene on the farm and especially those of milking through a rapid cooling of milk after milking.

It should, however, be clearly stated that refrigeration is not a technique for improving milk quality, but only a physical process to conserve the original quality of the milk until it is processed. Milk storage at low temperatures, above all if prolonged, may have negative effects, especially on the organoleptic characteristics of the milk and dairy products, as well as on cheese's yield Milk stored in this way undergoes considerable changes as regards its physical, chemical, biochemical and microbiological characteristics involving practically all its components. From a microbiological point of view, refrigeration leads to growth and proliferation of psychrotrophic bacteria, which are known to produce very active (and thermostable) proteolytic and lipolytic enzymes (Cousin 1982; Koka et al. 1991; Lopez-Fandino et al. 1993, Wiedmann et al. 2000). Proteolitic enzymes can partially solubilize β-caseins. This causes a

decreased diameter and an increased hydration degree of casein micelles, both of which promote a greater stability. As a result, a less compact and more fragile final coagulum is attained (Manfredini and Massari 1989). Lipolytic enzymes can increase the levels of free fatty acids causing potential off-flavours. The lipases of certain psychrotrophic bacteria are resistant to heat treatment such as the curdle cooking during cheese making and thus, even though they do not give apparent lipolysis in raw milk, they can negatively influence the quality of ripened products giving rise to a rancid or soapy flavour in the cheese. Lipolysis can be induced in refrigerated cow milk not only by microbial but also natural lipases (secreted from the udder) which can be active on the milk fat globules once these have been damaged by strong stresses such as cooling and mechanical shocks for example agitation in tankers at the farms and shaking during transport to cheese factory (Chillard et al. 1984). So the high initial bacterial load of the milk together with a refrigerated storage and excessive agitation during transport can enhance lipolysis in raw cow milk (see 1.2.1.1. Damages to MFG).

Low-temperature storage also has considerable effects on the colloidal phase of the milk. As regards casein, there is a partial solubilization of the components, especially β-casein which tends to separate from the micelles, (not only for the proteolytic microbial system). If one considers that β-casein accounts for a third of the total casein in cow milk, this fact obviously leads to reduced cheese yield. As far as the mineral compounds are concerned, the temperature influences the balance between the soluble and colloidal forms. An increase in the content of soluble inorganic calcium and phosphorus is observed in refrigerated milk. These changes give rise to a decrease in micelle diameter. At the same time a rise in the hydratation degree of the casein micelles occurs. This double phenomenon leads to greater stability of the micelles which is clearly evident during cheese-making from the way the milk reacts to the rennet. The coagulation time increases and the coagulum is less compact, more fragile and consequently more difficult to process mechanically (Alais, 2000b).

It has been shown for cow milk that β- and α_{S2}-casein are the most sensitive to plasmin (Snoeren et al. 1979). The action of plasmin continues throughout storage. The proteolysis of the milk due to both the plasmin and the proteinases of the psychrotrophic bacteria causes losses of soluble nitrogen in the whey and reduced cheese yield.

An immediately thermization treatment of the milk would be the fastest and most efficient method of limiting microbial load and biochemical activities as it reduces both psychrotrophic multiplication and the activity of the natural lipase of milk without all the negative effects of refrigeration, but is not possible for the further processing of milk to hard cheese.

Milk storage can also be assured by freezing, and this technique has been experimented for cow milk. It is however obvious that freezing of fluid milk is not of interest as cheese accounts for only a small portion of the weight of milk, and thus the storage of large quantities of milk is not always justifiable technically nor economically.

1.3.2. Milk processing

The milk stored at the farm must be transported to the processing dairy plant and apart from the hygiene during milking (Alais, 2000d), even transport has an enormous influence to maintain the bacteriological quality of the milk. Studies carried out to date have indicated that the process of milk transportation may produce a number of undesired effects (Czerniewicz et al. 2006 and 2008), including :

- the possible lowering of the milk microbiological quality as a result of secondary contamination or elevated temperature of the transported raw material;
- aeration of milk during its flowing, pumping and transportation:
- deterioration of the quality and technological aptitudes of raw milk as a result of mechanical impact, like shakes and vibrations
- Intensification of lipolytic and proteolytic processes, especially in refrigerated storage of raw milk, as well as an increases acidity which, in turn, negatively affects its technological employ

In order to reduce negative effects of vibrations occurring during transportation the milk containers might be fill till the top to reduce the empty space above in the tank (Czerniewicz et al., 2008).

1.4 MICROBIOTA INVOLVED IN CHEESE MANUFACTURE

Certain micro-organisms produce chemical changes that are desirable in the production of dairy products as cheese: **Lactic acid bacteria** (LAB), able to ferment lactose to lactic acid, are normally present in the milk and are also used as starter cultures. Being linked to the lactic acid production, the definition of LAB is biological rather than taxonomical, i.e., the LABs are not a monophyletic group of bacteria. Most of the LAB belong to the genera *Lactobacillus, Pediococcus, Lactococcus, Streptococcus, Leuconostoc, Oenococcus, Weissella, Enterococcus, Carnobacterium, Tetragenococcus* and *Vagococcus,* are defined as gram-positive, non-sporulating, lacking cythocromes and catalase, aero tolerant, acid tolerant and nutritionally fastidious (Mucchetti and Neviani, 2006a). This definition excludes the dairy associated genera *Propionibacterium* and *Bifidobacterium*. Undoubtedly, the most important application of LAB, as before mentioned, is their use as starter strains in dairy products. In particular, *Streptococcus thermophilus, Lactococcus lactis, Lactobacillus helveticus,* and *Lactobacillus delbrueckii* ssp. *bulgaricus* are widely used dairy starters and are of major economic importance. In the milk fermentation processes, the proteolytic system of LAB plays the key role because it enables these bacteria to grow in milk, thereby ensuring successful fermentations. LAB require an exogenous source of amino acids, or peptides, which are provided by the proteolysis of casein, the most abundant protein in milk and the main source of amino acids (Savijoki et al., 2006).

Usually we can find two kind of LAB during the cheese ripening: Starter lactic acid bacteria (SLAB) and Non starter lactic acid bacteria (NSLAB). SLAB are added to milk at the beginning of cheese manufacture in order to obtain a fast acidification. By converting sugar (lactose) to lactic

acid (lactate), these bacteria are able to lower the pH from 6.6 (the usual pH before milk dairy processing) down even to 4.2. They also contribute to cheese ripening where their enzymes are involved in proteolysis and conversion of amino acids into flavour compounds (see Introduction 1.5). Starter bacteria are either added deliberately at the beginning of manufacture or may be natural contaminants of the milk. Either mesophilic or thermophilic starter cultures are used, depending on the cheese being manufactured; mesophilic cultures are used in the production of Cheddar, Gouda, Edam, Blue and Camembert, while thermophilic cultures are used for high temperature (50–55 °C) cooked hard cheeses such as Emmental, Parmesan and Grana. Starter bacteria encountered most often are members of the genera *Lactococcus, Lactobacillus, Streptococcus, Leuconostoc* and *Enterococcus*. Both mesophilic and thermophilic cultures can be subdivided into mixed (undefined) cultures in which the number of strains is unknown, and defined cultures, which are composed of a known number of strains. Daly (1983) reviewed the use of mesophilic cultures in the dairy industry. The whey used in the hard-cheese dairy factories is composed of single or multiple strains of thermophilic starters both lactobacilli such as *Lb. helveticus, Lb. fermentum, Lb. acidophilus, Lb. delbrueckii* ssp. *delbrueckii, Lb. delbrueckii* ssp. *bulgaricus, Lb. delbrueckii* ssp. *lactis* and cocci such as *Sc. thermophilus*. These cultures are generally produced, by incubating whey from the previous day's production, overnight at 40–45 °C. (Andrighetto et al. 2004; Lazzi et al. 2004). A similar natural whey starter containing thermophilic LAB is used to make Parmigiano Reggiano. This starter consists primarily of undefined strains of *Lb. helveticus* (75%) and *Lb. delbrueckii* ssp. *bulgaricus* (25%); however, their precise composition is uncontrolled and significant variations at strain level are likely to occur (Coppola et al., 1997; Gatti et al. 2003). A significant proportion of starter LAB are distributed within the curd during coagulation, and in cheese become entrapped around the periphery of fat globules at the interface between protein and aqueous phases (Laloy, et al. 1996). Fenelon et al. (2000) studied the effect of fat content on the microbiology and proteolysis in Cheddar cheese of fat contents ranging from 6 to 33% (w/w). In agreement with Laloy et al. (1996), starter populations on day 1 were highest in 33% fat cheese. However, over much of the 225 day ripening period, starter viability decreased with increasing fat content in the cheese. Additionally, decreased water activity in lower fat cheeses may have inhibited NSLAB growth. These authors also determined lactate dehydrogenase (LDH) activity, an indicator of cell autolysis, in cheese juice during ripening and they found out that, in agreement with what occurred to starter viability, the LDH activity increased with increasing of fat content in the cheese. This study suggests that fat content in cheese may exert an important influence on the growth, survival and autolysis of starters in cheese. Laloy et al. (1996) showed that, along ripening, lactococci became more intimately associated with the fat globule and appeared to be integrate into the fat globule membrane and alter its structure. These authors also suggested an interaction between proteolysis during ripening and the degree of association between starter cells and fat globule membrane. The influence of LAB cell surface characteristics on the adsorption or retention of LAB in cheese or cream was studied by Crow et al. (1995), Kiely et al.

(1997) and Law (2001) but is still mainly unknown. They grow during manufacture typically attains densities of 10^8 cfu / g within few hours of the beginning of manufacture (Crow et al. 1995). Starters give the only significant contribution to the microbial biomass of LAB in the young curd and they represents a considerable potentiality for cheese ripening reactions, which could be modulated through autolysis of the starter cells. Autolysis of starter cells may be influenced by the NaCl concentration and manufacturing conditions, such as elevated cook temperatures (Crow et al., 1995). Crow et al. (1995) reported that intracellular peptidases released into cheese from starter (auto) lysis accelerate the rate-limiting peptidolytic steps and contribute to the higher levels of amino acids in the cheese which accumulate faster following starter autolysis, and are major precursors of the compounds required for cheese flavour production (Urbach, 1995). These reactions provide not only important flavour compounds, but also precursors for further influence on growth rates and types of NSLAB in cheese which, in turn, could produce cheese flavour (Crow et al., 1995). Autolysis of *Lb. helveticus* has been reported in Grana cheese (Bottazzi et al.1992) and in experimental Swiss type cheese (Valence et al. 1998).

NSLAB populations consist mainly of mesophilic lactobacilli and reach 10^5-10^8 cfu / g up to the kind of cheese (Beresford et al., 2001). NSLAB are present in the cheese matrix as a result of contamination during the manufacturing procedure or come from milk or may also enter the curd through downstream contamination at salting, pressing or packaging during cheese ripening. The mechanism of entrapment of NSLAB within cheese curd is not clearly known but it is likely that their entrapment occurs during coagulation more or less in the same way as starter LAB do. Growth of NSLAB appeared to increase with increasing fat content in the cheese. Fenelon et al. (2000) ascribed the trends noted for NSLAB to an increasing level of glycoprotein and MFGM in higher fat cheeses that could potentially provide a substrate for the growth of mesophilic lactobacilli. All microorganisms present in the cheese product will convert milk compounds like sugar, fat and protein into various metabolites. Since the product is an environment where various micro-organisms are present, the growth of certain micro-organisms is influenced by the changes occurring, which results in differences in populations over the time (microbial population dynamics). In general, the NSLAB amount begins to increase in the cheese matrix because, during the first two weeks after manufacture, most of the residual lactose in cheese has been utilized by SLAB (Diaz-Muniz et al., 2006) and NSLAB are the main actor of cheese ripening cause their enzymatic activity (e.g. peptidases and lipases) very useful for the cheese flavour formation and definition. It has to be stressed that anyway most peptidase, aminotransferase, and lipase/esterase enzymes appear to be intracellular and would therefore require contact with their substrates in cheese through permeabilisation or complete autolysis of the cell during ripening.

A number of physical parameters control the growth of micro-organisms in cheese during ripening, including moisture, salt concentration, pH and ripening temperature. The extent of variation in these parameters is influenced by the cheese making process (Beresford et al. 2001).

Salt, moisture and water activity (Aw) are strictly interrelated and the inhibition of starter and spoilage bacteria by salt mainly reflects the effect of salt in reducing Aw. All micro-organisms require water for growth and one of the most effective ways of controlling their growth is to reduce the available water either through dehydration or addition of some water soluble component such as sugar or salt. An increase in the moisture content of cheese leads to increased susceptibility to spoilage. Aw is a thermodynamic concept defined as the relationship between the vapour pressure of the food (p) and that of pure water (p0) at the same temperature: Aw = p/ p0. The vapour pressure of a given food is always lower than of pure water due to the presence of dissolved substances, so that Aw is always lower than one. In cheese Aw is essentially dependent on the moisture content, pH, NaCl and other low molecular weight compounds as ash and non-protein nitrogen (NPN) (Esteban and Marcos 1990). During the first stages of cheese manufacture, Aw is 0.99, which supports the growth and activity of the starter culture. However, after whey drainage, salting and during ripening the common Aw values of hard cheese are known to be within the range $0.9 < Aw < 1.0$ (Saurel et al. 2004).

The optimum pH for the growth of most common bacteria is around neutral and growth is often poor at pH values < 5.0. Due to the accumulation of organic acids, hard cheese curd post-manufacture has a pH ranging between 5.3 and 5.7; such low pH values will not allow the survival of acid-sensitive species. The real inhibitor is thought to be the undissociated form of the organic acid. The principal organic acids found in cheese are lactic, acetic, and propionic acids which have pKas of 3.08, 4.75 and 4.87, respectively, such that lactic acid is the least and propionic acid the most effective inhibitor at the same concentration at the pH of cheese. However, lactate in cheese curd is invariably present at much greater concentrations than each of the other two acids, except in the case of Swiss cheese where the concentration of propionic acid may be higher than lactic acid in the ripened cheese (Beresford et al. 2001).

1.5 MICROBIAL METABOLISMS INVOLVED IN CHEESE RIPENING

The ripening of cheese is a concatenation of physico-chemical, biochemical and biologic events strictly connected among themselves. During ripening, the curd, characterised by light taste and flavour, undergoes deeper changes until it assumes the typical rheological and sensory characteristics of the final product. The whole process of ripening consists essentially in an enzymatic “digestion” of the curd. The substrate is represented by the casein, but the process involves also the soluble components (sugar, lactic acid, citric acid, *etc*.) and, according to cheese variety, lipids. The result is a deep changes consisting in the loss of moisture, the fermentation of lactose, the metabolisation of the lactic and citric acid, the more or less intense solubilisation of the casein and intermediate products, the hydrolysis of fat and the formation of the cheese rind. The main agents responsible for transformation (glicolysis, proteolysis,

lipolysis) are represented by the milk enzymes, the SLAB and NSLAB, rennet and polluting microflora enzymes.

The ripening of Grana Trentino as Grana Padano and Parmigiano Reggiano cheese take e *minimum* period of 12 months, but, in most cases, it lasts until 18 months. During this phase the cheese develops its typical flavour and aroma properties. This is related to the enzymatic activities present in the cheese which are related to the peculiarities of the cheese making process, such as the use of raw milk, the addition of natural whey starter culture and the use of calf rennet. The intensity and the specificity of the enzymatic action seems to be strictly conditioned by the cheese making technology. The higher cooking temperature and the dimension of the cheese are able to determine the formation of a temperature gradient, which decrease from the inner to outer side of the cheese mass, and which remains for about 24 hours after the extraction of the cheese mass from the vat. This temperature gradient affects the development of microbial populations and, consequently, of their connected enzymatic activities, with important repercussions on the evolution of glicolytic and proteolytic processes in the outer and in the inner zones of the cheese during ripening.

1.5.1 Lactose and lactate metabolisms

Most of the lactose in milk is lost in the whey as lactose or lactate during cheese manufacture. However, low levels of lactose remain trapped in the curd at the end of manufacture (e.g. 0.8–1.0% for Cheddar at milling; Huffman and Kristoffersen 1984). Residual lactose is quickly metabolized by the glycolytic pathway to L-lactate during the early stages of ripening. Fermenting lactose into L-lactic acid is a primary function of any starter culture in cheese manufacture and the fermentation rate is largely determined by temperature and the salt-in-moisture (S/M) levels of the curd (Turner and Thomas 1980; Parente and Cogan 2004). As S/M levels in Grana Trentino and other brine-salted varieties increase slowly, starter activity is not stopped at the end of manufacture. Depending on starter type, lactose is metabolized by the glycolytic (most starter bacteria) or phosphoketolytic (e.g. *Leuconostoc sp.*) pathways (Mucchetti and Neviani, 2006b). The principal products of lactose metabolism are L- or D-lactate or a racemic mixture of both, although some strains, e.g., *Leuconostoc* spp., may produce other products as ethanol (Vedamuthu, 1994). Acid productivity is critical for controlling cheese quality because the culture determines the **final pH** and **mineral content of the curd.** The pH of cheese is determined by the extent of acidification during manufacture, the buffering capacity of cheese curd and, in some cases, deacidification during ripening. The pH of cheese affects the texture of curd directly by influencing the solubility of the caseins; high pH cheeses are softer than more acidic cheeses. pH also influences texture and flavour indirectly by affecting the activity of enzymes important to ripening and, in the case of the coagulant, the retention of enzyme in the curd during manufacture (Mucchetti and Neviani, 2006c). Lactose that remains unfermented by the starter is probably metabolized by NSLAB. If starter bacteria rapidly deplete residual milk lactose in the curd, they can help to prevent its use as a substrate for undesiderable adventitious bacteria, such as, for example, heterofermentative bacteria, that can produce serious flavour and texture

defects by gas production (Mucchetti and Neviani, 2006e; Beresford et al. 2001).

Lactate produced from lactose by the growth of the starter is an important substrate for a range of reactions that occur in cheese during ripening. See a simplified scheme of the pathways of lactate metabolisation in cheese during ripening by LAB and yests in Figure 1.

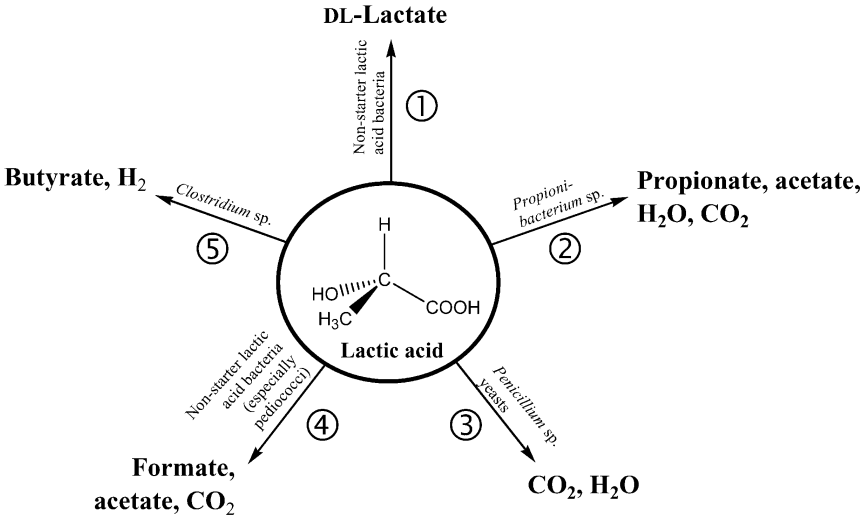

Figure 1. A simplified scheme of the pathways of lactate metabolisation in cheese during ripening (reprinted from McSweeney, 2004)

For example, lactate, can be further **catabolized** by propionic acid bacteria during the ripening to propionate, acetate, H_2O and CO_2. Lactate may be **racemized** by NSLAB, for example pediococci racemize lactate faster than lactobacilli (Thomas and Crow 1983) and racemization is probably faster in raw milk cheeses than in the ones made from pasteurized milk (McSweeney and Sousa 2000). The pathway for lactate racemization involves oxidation of L-lactate by L–lactate dehydrogenase to form pyruvate, which is then reduced to D-lactate by the action of D–lactate dehydrogenase. Racemization of lactate is significant because the Ca-D-lactate is characterised by a lower solubility than Ca-L-lactate, and therefore it may crystallize in cheese forming white specks, particularly on cut surfaces (Thomas and Crow 1983; Dybing *et al*. 1988). These crystals are harmless but they may cause consumers to reject cheese as being mouldy or containing foreign particles (Dybing et al. 1988). Increased levels of lactose favour the growth of NSLAB and thus crystal formation (Pearce et al. 1973; Sutherland and Jameson 1981). Lactate can be **oxidized** by LAB in cheese to several products including acetate, ethanol, formiate and CO_2. For example, it has been found that some lactobacilli and pediococci isolated from Cheddar cheese can oxidise lactate to acetate and CO_2 under aerobic conditions, and this lactate oxidation system of lactobacilli and pediococci may be operative under the ripening conditions of cheese (Thomas, 1986; Thomas, 1987). Furthermore, in the presence of O_2, some NSLAB,

particularly pediococci, can oxidize lactate to formiate and acetate. However, the extent to which this pathway occurs in cheese depends on the NSLAB population and the availability of O_2, which in turn is determined by the size of the block and the oxygen permeability of the packaging material (Thomas 1987). An hard cheese like Grana Padano or Trentino can be thought as a anaerobic environment. **Anaerobic metabolism** of lactate by *Clostridium tyrobutyricum* to butyrate, O_2 and H_2 results in a defect known as late gas blowing, where O_2 and H_2 cause large holes appearing in the cheese during ripening and butyrate causes the development of off-flavours. Gas blowing is a problem principally in brine-salted cheeses because of the time required for NaCl to diffuse into the cheese and to reach inhibitory concentrations (Beresford et al. 2001). Strategies to avoid late gas blowing usually involve minimizing spore numbers in milk (e.g. good hygiene, avoidance of silage), inhibition of spore germination and the growth of vegetative cells, for example by the use of lysozyme or nitrate or the physical removal of spores by bactofugation or microfiltration (Mucchetti and Neviani, 2006e).

1.5.2 Citrate catabolism

Milk contains approximately 18 g citrate per Kg and about the 90 % is localised in the soluble phase and thus is lost on whey drainage (Alais, 2000e). Nevertheless, citrate is an important precursor for flavour compounds in certain dairy products like Dutch-type cheeses where CO_2 produced by citrate metabolism is responsible for the small eyes often present; even succinate and important flavour compounds, particularly diacetyl are produced during citrate metabolism and contribute to the flavour of these cheeses (McSweeney, 2004). Citrate is metabolized by citrate positive (Cit +) strains of lactococci as *Lactococcus lactis* biovar *dyacetilacyis, Lactococcus lactis* ssp. *lactis*, which contain a plasmid for citrate transport. *Leuconostoc mesenteroides* ssp. *cremoris* and *Ln. lactis* also metabolize citrate. Citrate is not metabolized by other LAB used as starters i.e. thermophilic lactobacilli, *Sc. thermophilus* and most strains of lactococci (Diaz-Muniz and Steele, 2006). Citrate is co-metabolized with a fermentable carbohydrate by Cit + lactococci and *Leuconostoc* spp. Cit + microorganisms may utilize citrate as an energy source (McSweeney, 2004). Conditions required for citrate utilization by *Lactobacillus casei* ATCC334 were recently studied by Diaz-Muniz and Steele (2006). Citrate was utilized by this microorganism in a modified chemically defined media as an energy source, solely in the presence of limiting concentrations of galactose. The presence of glucose inhibited citrate utilization by this microorganism even when added in limiting concentration. This results lead to suppose that *Lb. casei* uses citrate as an energy source in ripening cheese only when the residual levels of carbohydrate post-fermentation are limiting (< 2.5 mM), and lactose or glucose are absent.

1.5.3 Lipolysis and Free Fatty Acids metabolism

Lipids in foods may undergo hydrolytic or oxidative degradation. However, in cheese, oxidative changes are very limited due to the low oxidation/reduction potential of the environment (about 250 mV) (McSweeney and Sousa 2000; Collins et al. 2003a). Milk fat contains high

concentrations of short- and intermediate-chain fatty acids which, when liberated by lipolysis, contribute directly to cheese flavour. In all cheese varieties triglycerides undergo hydrolysis by the action of esterases and lipases that catalyse the hydrolysis or synthesis of esters, depending on cheese A_w and fatty acids and alcohols availability. This enzymatic activity results in the liberation of fatty acids in cheese during ripening. FFA directly affect cheese flavour, and can have further effects by serving as precursors for esters and other flavour compounds which have low flavour thresholds thus contributing significantly to the flavour of many cheese varieties (McSweeney and Sousa 2000; Collins et al. 2003a). (Fig. 2)

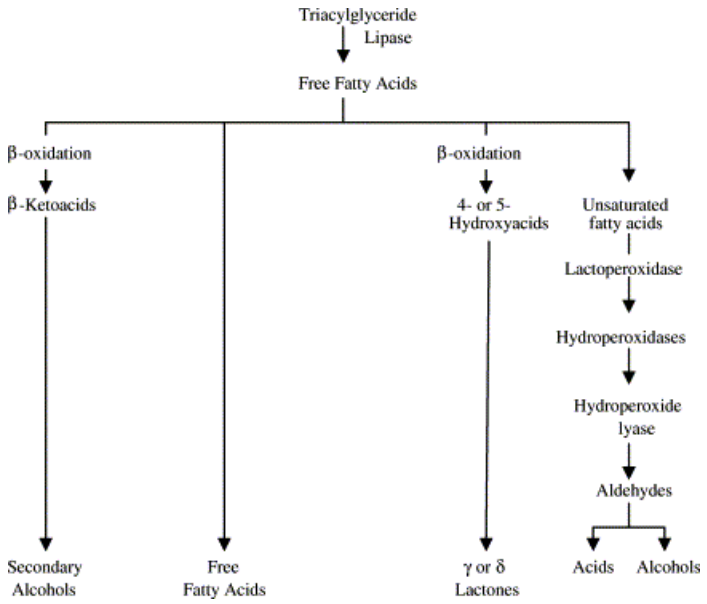

Figure 2: catabolism of Free Fatty Acids (reprinted from Collins et al., 2003a)

Enzymes involved in these reactions may come from rennet, from milk itself, and from SLAB or NSLAB. Excessive levels of lipolysis are undesirable and result in rancidity (McSweeney and Sousa 2000; Collins et al. 2003a). Rennet pastes used in the manufacture of certain hard Italian cheese varieties, such as Provolone and the various Pecorino cheeses, and often in traditional Greek feta, contain much lipase activity. In hard cheeses such as Parmigiano Reggiano that do not use pregastric lipases and esterases, however, flavour notes associated with lipolysis are probably due to lipolytic agents from the milk, and the cheese microflora (starter and non-starter microorganisms). Milk contains a potent indigenous lipase, lipoprotein lipase (LPL), with a molecular mass of 55 kDa, that exists in milk as a homodimer (see above Introduction 1.2.1.1).

Although weakly lipolytic in comparison with some other cheese-related microorganisms (e.g. *Penicillium*), LAB possess intracellular esterolytic / lipolytic enzymes which, in cheese with long time ripening as Grana Trentino, can generate enough free fatty acids and esters to impact flavour (Collins et al. 2003a). The lipase/esterase systems of starter bacteria have received much less attention then their proteolytic systems. Lipolytic enzymes from LAB are intracellular (Fernandez et al. 2000) and hence are

released into the cheese matrix on lysis. The enzymes are optimally active at pH 7–8.5 With few exceptions (Chich et al. 1997), the lipolytic enzymes of LAB have temperature optima at around 35 °C and are most active on substrates containing short-chain fatty acids (Collins et al. 2003). *Lactococcus sp*. are only weakly lipolytic, but lactococci may be responsible for the liberation of quite high levels of FFA when present in high cell number or over extended ripening periods. (Chich et al. 1997). Obligately homofermentative lactobacilli used as starter (*Lb. helveticus, Lb. delbrueckii* ssp.*bulgaricus* and *Lactobacillus delbrueckii* ssp. *lactis*) also produce esterases, some of which have been studied (Khalid and Marth, 1990). Facultatively heterofermentative lactobacilli (e.g., *Lb. casei, Lactobacillus paracasei* and *Lactobacillus plantarum*) which dominate the NSLAB population of many cheese varieties, are weakly lipolytic. *Micrococcus* and *Pediococcus* are also weakly lipolytic (Bhownik and Marth, 1990).

While short-chain fatty acids contribute directly to cheese flavour, long chain fatty acids also contribute indirectly to cheese flavour by acting as precursors for the production of volatile flavour compounds through a series of reactions known collectively as metabolism of fatty acids. Esters are found commonly in many cheese varieties and are produced by the reaction of a FFA with an alcohol. The most common alcohol available for this reaction is ethanol and hence ethyl esters are the dominant esters in cheese (Arora et al. 1995). Ethanol is the limiting reactant in the production of esters; this alcohol is derived from the fermentation of lactose or from amino acid catabolism. Thioesters are compounds formed by the reaction of FFAs with sulphydryl compounds, usually methanethiol (CH_3SH; thus forming methylthioesters) (McSweeney and Sousa 2000; Collins et al. 2003a,). Lactones are cyclic compounds formed from hydroxyacids following intramolecular esterification. Both γ- and δ-lactones (with five- and six-sided rings, respectively) have been found in cheese. The production of lactones during ripening is limited by the levels of their precursor compounds, hydroxyacids. The mammary gland is reported to possess a δ -oxidation system for fatty acids, or hydroxyacids may be produced by reduction of ketones (Collins et al. 2003a).

FFA metabolism is of most significance in blue-mould cheese in which FFAs are converted to 2-methyl ketones (alkan-2-ones) via a pathway corresponding to the early stages of γ-oxidation caused by the action of spores and vegetative mycelia of *P. roqueforti* (Collins et al. 2003a). The rate of production of methyl ketones is affected by a number of factors, including temperature, physiological state of the mould, concentration of precursor FFA and pH: Mycelia oxidize fatty acids over a wide pH range, with an optimum between pH 5 and 7, similar to that of mature Blue cheese. Up to 7 methyl ketones have been identified in cheese and the most common are pentan-2-one, heptan-2-one and nonan-2-one (Collins et al. 2003a).

Evidence for a link between lipolysis in cheese and the starter microflora was given by Collins et al. (2003b), who showed that lipolysis was higher in Cheddar made using a fast-lysing starter culture. Esters have also been claimed to be important contributors to the flavour of Parmigiano Reggiano cheese (Meinhart and Schreier, 1986). Among the 74 compounds

found in Parmigiano Reggiano, the most important esters were ethyl butanoate, ethyl hexanoate, ethyl octanoate, ethyl propanoate, ethyl pentanoate, ethyl heptanoate, and ethyl decanoate. These esters appear to be responsible for the characteristic fruity aroma perceived in this cheese (Qian and Reineccius, 2002).

1.5.4 Proteolysis and Aminoacids metabolisms

Proteolysis and its secondary reactions play the major role in bacterially ripened cheeses. Its relationship to flavour and the enzymes responsible for this process have been reviewed extensively over the past two decades (McSweeney and Sousa, 2000; Yvon and Rijnen, 2001; Sousa et al. 2001; McSweeney 2004; Savijoki et al., 2006). Together, primary and secondary proteolysis of casein influences cheese flavour in at least three significant ways (Fig.3). First, casein network breakdown softens cheese texture, through decreasing the A_w of the curd due to changes in water binding by the new carboxylic acid and amino groups formed on hydrolysis which facilitates the release of flavour compounds when the cheese is consumed. Second, some of the low-molecular-weight peptides produced in these reactions directly affect flavour, but this consequence is generally negative since these peptides impart bitterness. Third, the free amino acids that are liberated can be substrates for a series of catabolic reactions that generate many important flavour compounds. For instance, glutamate and aspartate residues enhance flavour (McSweeney and Sousa, 2000).

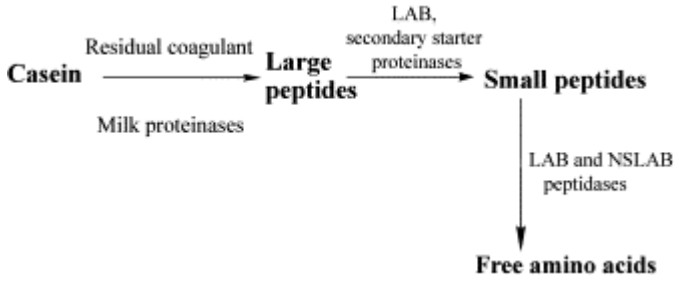

Figure 3 Proteolytic agents in cheese during ripening (reprinted from McSweeney, 2001)

More commonly, released amino acids are precursors for a broad range of potent aroma compounds. These reactions are of particular interest because a growing body of evidence indicates that the LAB conversion of free amino acids into aroma compounds is the rate-limiting step in the development of mature cheese aromas. The major sources of proteolytic enzymes in many cheese varieties are the residual coagulant (e.g. chymosin) and endogenous milk proteinases (e.g. plasmin), while LAB proteinases and peptidases are responsible for producing water-soluble peptides and free amino acids (McSweeney and Sousa, 2000).

Hydrolysis of intact casein during ripening is almost exclusively catalysed by **chymosin**, that remains entrapped in the curd on whey drainage. Up to 15% of the coagulant added to the milk remains active in the curd depending on factors such as enzyme type, cooking temperature and pH at whey drainage (Sousa et al. 2001). The specificity of chymosin on all of the caseins is now known. Chymosin selectivity on α_{S1}- and β-

casein is different in solution or if acting in cheese (Exterkate et al. 1997). Chymosin cleaves α_{S1}-caseins in several sites but only four bonds are cleaved in both conditions (bonds cleaved by chymosin both in cheese and in solution: 23-24, 98-99, 101-102 and 128-129). α_{S2}-caseins are more resistant to hydrolysis by chymosin (McSweeney et al. 1994). Chymosin cleaves also β-casein in 13 sites (five if acting in solution) many of which are located near the hydrophobic C-terminal β-casein, and cleavage of these sites can result in the production of short hydrophobic peptides, which are bitter. The different chymosin modulation in solution and in cheese can be related to the cheese moisture, which can influence the availability of water molecules which contribute to the conformation of the enzyme and/or the substrate. Consequently, the complex interactions between forces regulating binding specificity might change, resulting in a new preference of susceptible sites (Exterkate et al. 1997).

Milk itself is an important source of proteolytic enzymes. The principal indigenous proteinase in milk is **plasmin**, which is a trypsin-like serine proteinase originating in the blood and optimally active at approximately pH 7.5 and 37 °C. The physiological role of plasmin in the blood is in the degradation of fibrin clots during the blood clotting process. Hence, the activity of plasmin in blood must be under tight control and it is thus produced from an inactive precursor, plasminogen, through the action of plasminogen activators (PAs). Inhibitors of plasmin and PAs also form part of this system, which is found in milk. In milk, plasmin, plasminogen and PAs are mainly associated with the casein micelle while plasmin inhibitors and inhibitors of PA are found in the whey. The plasmin activity is higher in hard cheeses because the inhibitors are lost during whey drainage and the PAs inhibitors are inactivated by the high cook temperatures of the curdle. The specificity of plasmin is restricted to peptide bonds of the type Lys–X, to a lesser extent Arg–X, and it degrades the caseins in the order β-casein $\approx$ > α_{s2}- casein > α_{s1}-casein; κ -casein seems to be resistant to the action of this proteinase (Bastian and Brown, 1996). The most important substrate of plasmin in cheese is β-casein, which plasmin hydrolyses at three sites, Lys28–Lys29, Lys105–His106 and Lys107–Glu108, to produce γ1-CN (β-CN f29–209), γ2-CN (β-CN f106–209), γ_3-CN (β-CN f108–209), proteose peptone PP8 fast (β-CN f1–28), PP8 slow (β-CN f29–105 and f29–107) and PP5 (β-CN f1–105 and 1–107). α_{s2}-Casein is also very susceptible to plasmin action and it is likely that the disappearance of this protein, which is often observed in cheese during ripening, is due to plasmin action (Sousa et al. 2001).

Milk also contains other indigenous proteinases originating from the leucocytes of somatic cells. Somatic cells contain many proteinases including **cathepsins** B, D, G and elastase (Sousa et al. 2001). The indigenous cathepsin B is a cysteine proteinase and its significance in milk to proteolysis in cheese during ripening is unknown, although this enzyme has a wide specificity on the caseins. (Considine et al. 2004). The indigenous cathepsin D is an aspartyl proteinase with temperature and pH optima of 37 °C and 4.0, respectively (Sousa et al. 2001).The indigenous cathepsin G is a neutral serine proteinase and may potentially give a significant contribution to proteolysis in cheese from milk with high

somatic cell counts. Some of the cleavage sites of cathepsin G were identical to both elastase and cathepsin B (Considine et al. 2002).

The residual coagulant activity retained in the curd and plasmins (and perhaps cathepsins too) hydrolyse caseins at start of ripening into a range of large and intermediate-sized peptides that are then hydrolysed by **proteinases and peptidases** from the starter LAB and NSLAB to shorter peptides and amino acids. However, the pattern and extent of proteolysis varies considerably among cheese varieties because of differences in manufacturing practices (particularly cooking temperature) and ripening protocols that cause differences in ripening time, pH at draining, moisture content, residual coagulant activity, activation of plasminogen to plasmin, and development of a highly proteolytic secondary microflora. All these factors affect the differences in pH, soluble N content (a widely used index of proteolysis) and peptides which fraction are produced mainly by the action of chymosin and to a lesser extent of plasmin (Mucchetti and Neviani, 2006d). For example in Parmigiano-Reggiano-like cheeses where the high cooking temperature (> 55 °C) used during the manufacture denatures most of the chymosin, plasmin and *Lactobacillus* proteinases are mainly responsible for extensive proteolysis in the cheeses, which are ripened for a long period (even more than 24 months) at elevated temperatures (18–20 °C) (Sousa et al. 2001). Several peptides from Cheddar, Parmigiano Reggiano, blue, Swiss and feta cheeses have been isolated and characterized.

LAB and NSLAB are rich in proteinases and peptidases because they are auxotrophic to obtain all essential amino acids for protein synthesis.

The proteolytic system of *Lactococcus* is initiated by a single Cell Envelope-associated Proteinase (CEP) called lactocepin or PrtP. Lactocepin is a serine proteinase (140 kDa, pH optimum 5.5–6.5), loosely attached by Ca^{2+} to the cell surface. The lactocepins from a number of strains of *Lactococcus* have been studied biochemically and genetically (Kunji et al. 1996). The primary role of lactocepin is to degrade the caseins to provide short peptides which allow the lactococcal cell to grow in milk. However, in cheese, it acts primarily to degrade intermediate-sized peptides produced from the casein by the action of chymosin or plasmin. The specificities of the lactocepins from a range of LAB on the caseins are known. Lactococci lacking this protease can only grow in a free amino acid-rich envioronment or in protein containing media in co-operation with Prt^+ strains (Kunji et al., 1996).

The second step in casein utilization includes transportation of peptides generated by CEP into the cell by the action of oligopeptide permease system (Opp system). Peptide uptake occurs via Opp system and di-/tri-peptide transporters. In addition, various amino acid transport systems have been identified with a high specificity for structurally similar amino acids (Peltoniemi et al., 2002). The Opp system of *L. lactis* transports peptides up to at least 18 residues an the nature of these peptides significantly affects the transport kinetics involved (Savijoki et al., 2006). Described Opp systems for other LAB demonstrate to be similar to that described for *Lactococcus* (Peltoniemi et al., 2002). After the casein-derived peptides are taken up by the cells, they are degraded by a concerted action

of peptidases with differing and partly overlapping specificities (Kunji et al., 1996). The proteinases and peptidases of LAB have been the subject of active studies over the past two decades and the extensive literature on this topic has been frequently reviewed (Kunji et al. 1996; Christensen et al. 1999; Smitt et al. 2005; Savijoki et al. 2006). The proteolytic system of *Lactococcus* has been studied most thoroughly, followed by those of the thermophilic lactobacilli, while the systems of the facultatively heterofermentative lactobacilli that dominate the NSLAB have received less attention.

Several endopeptidases were characterized from LAB and assigned in four types of oligoendopeptidases (Kunji et al. 1996) (Figure 3): intracellular endopeptidases, general aminopeptidases (PepN and PepC), di-/tri peptidases and X-prolyl dipeptidyl aminopeptidase. Carboxy peptidases have never been found in LAB. The **intracellular endopeptidases** are the first enzymes to act on oligopeptides: they are all monomeric metallopeptidases with molecular masses of about 70 kDa with the exception of the *Lb. helveticus* PepE, which was shown to exhibit a thiol-dependent activity (Fenster et al., 1997). A common feature of endopeptidases is their inability to hydrolyze intact casein but they have the ability to hydrolyze internal peptide bonds of casein-derived peptides (Caira et al., 2003). Other peptidases able of acting on oligopeptides are the **broad specificity aminopeptidases** PepN (85–98 kDa) and PepC which are multimeric cysteine metallopeptidase proteins that were characterized from diverse LAB strains. Collectively, these enzymes can remove the N-terminal amino acids from a peptide, the specificity depending on the peptide length and the nature of the N-terminal amino acid residue (Kunji et al., 1996; Christensen et al.,1999). Di/tripeptides generated by endopeptidases, general aminopeptidases, and PepX are next subjected to additional cleavage by the **dipeptidases** and **tripeptidase** (PepT, PepV and PepD). These enzymes prefer peptides containing hydrophobic amino acids including leucine, methionine, phenylalanine, or glycine. Other peptidases with more specific substrate specificities include: PepA, which liberates N-terminal acidic residues (glutammic acid and aspartic acid) from peptides that are three treonine-residue-long; PepP, which prefers tripeptides carrying proline in the middle position; PepR and PepI, which act on dipeptides containing proline in the penultimate position; PepQ, which cleaves dipeptides carrying proline in the second position; and PepS, which shows preference for peptides containing two to five residues with Arg or aromatic amino acid residues in the N-terminal position (Kunji et al., 1996; Christensen et al.,1999; Fernandez-Espla and Rul,1999). The aminopeptidases, dipeptidases and tripeptidases (which are intracellular) are released after the cells have lysed and are responsible for the production of FAA. Caseins are rich in the amino acid proline. Because of its unique cyclical structure, specialized peptidases are needed to hydrolyse proline containing peptides and the LAB produce such enzymes in abundance to enable them to use the caseins fully as growth substrates. **X-prolyl dipeptidyl aminopeptidase** (PepX) releases X–Pro dipeptides from the N-terminus of peptides and is the best characterized proline-specific peptidase of the LAB (Kunji *et al*. 1996). PepXs from most strains are dimeric serine dipeptidylaminopeptidases with molecular masses of 117–200 kDa. LAB

also possess two specialized dipeptidases that cleave proline-containing dipeptides. Prolinase (PepR) cleaves Pro–X dipeptides while prolidase (PepQ) hydrolyses X–Pro dipeptides. Although enzymes of starter and non-starter LAB contribute to the ripening of nearly all cheeses, proteolysis in varieties in which a secondary flora is encouraged to grow is often affected greatly by enzymes from these secondary organisms. For example *Propionibacterium* sp. used as secondary starters in Swiss-type cheeses are weakly proteolytic but are highly peptidolytic (Gagnaire et al. 1999).

The final products of proteolysis are FAA. Their concentration depends on the cheese variety, and have been used as indices of ripening (Aston et al. 1983, Puchades et al. 1989). The concentration of FAA in cheese at any stage of ripening is the net result of the liberation of amino acids from casein and their transformation to catabolic products. Concentrations of amino acids generally increase during ripening, with the exception of Arg, the concentration of which is reported to decrease later in ripening (Puchades et al. 1989). The level of peptides and FAA soluble in cheese in 5% phosphotungstic acid (PTA) has been considered to be a reliable indicator of the rate of flavour development (Aston et al. 1983) because catabolism of FAA can result in a number of compounds, including ammonia, amines, aldehydes, phenols, indole and alcohols, all of which may contribute to cheese flavour.

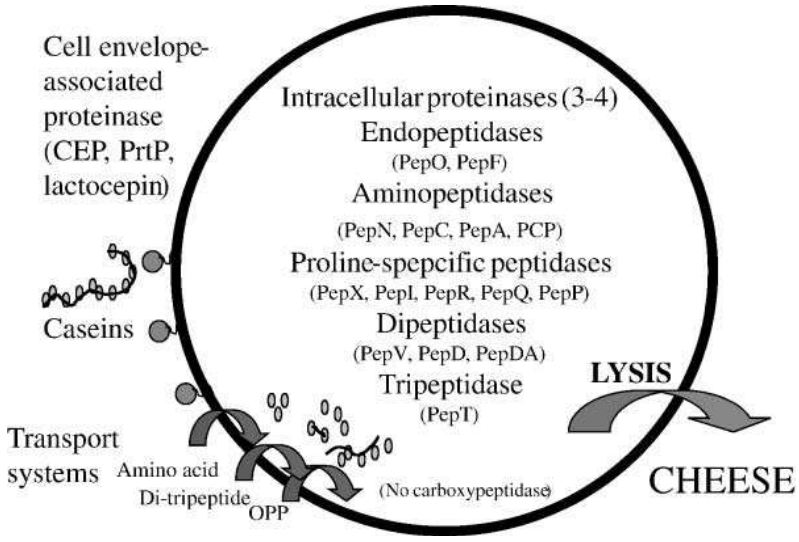

Figure 4. A simplified scheme of proteolitic system of Lactococcus (reprinted from McSweeney, 2004)

Amino acids in cheese appear to be catabolized by the action of an aminotransferase or a lyase, although other catabolic pathways (e.g. deamination or decarboxylation) may also occur (Yvon and Rijnen 2001). The first stage in amino-acid catabolism involves decarboxylation, deamination, transamination, desulphuration or perhaps hydrolysis of the amino-acid side-chains. The second stage involves conversion of the resulting compounds (amines and a-ketoacids), as well as amino acids themselves, to aldehydes, primarily by the action of deaminases on amines.

The final stage of amino-acid catabolism is the reduction of the aldehydes to alcohols, or their oxidation to acids. Sulphur-containing amino acids can undergo extensive conversion, leading to the formation of a number of compounds, including methanethiol and other sulphur derivatives. Volatile sulphur-containing compounds are known to be important for the flavour of a number of cheese varieties (McSweeney and Sousa 2000). As levels of Cys in the caseins are low, sulphur compounds in cheese originate principally from the catabolism of Met. Pathways for the catabolism of Met and the production of important sulphur-containing flavour compounds such as methanethiol, dimethyldisulphide, dimethyltrisulphide and thioesters (produced by the reaction of a thiol, often methanethiol, and a carboxylic acid) have been studied actively (Yvon and Rijnen 2001).

At the beginning of cheese ripening, with a lower pH, amino acids are decarboxylated to amines. In the later stages of ripening when there is an increase in pH, these amines are oxidized to aldehydes via the Strecker degradation (Qian et al 2002). Aldehydes do not accumulate to high concentrations in cheese because they are rapidly transformed to alcohols or to the corresponding acids (Lemieux et al 1992). Aldehydes are thought to contribute to the flavour of many cheese varieties, including Parmesan-like cheeses (Qian and Reineccius 2002). No correlation between the concentrations of Strecker-derived compounds and concentrations of individual FAA have been found in cheese (Lemieux et al 1992).

The first pathway is initiated by the action of **aminotransferases,** pyridoxal-5′- phosphate (PLP)-dependent enzymes, which convert an amino acid to the corresponding α-ketoacid and in turn transfer the amino group of the amino acid to an acceptor molecule, usually α -ketoglutarate, producing a new amino acid, usually glutamic acid. The α -ketoacids produced are then degraded to a range of other compounds by enzyme-catalysed pathways or by chemical reactions. α -ketoacids, produced by the action of aminotransferases, particularly those from aromatic amino acids, are degraded further by cheese-related microorganisms to volatile flavour compounds (Christensen et al.,1999). Methional, 3-methylbutanal, isovaleric acid and benzadheyde are examples of key flavour compounds which are formed through this kind of pathway. Transaminase are widely distributed among micro-organisms and for the conversion of several types of aminoacids, such as the branched chain amino acids (BcAA), specific transaminases have been identified and characterised. The first step in the catabolism of Trp, Tyr and Phe, is catalysed by aminotransferase activity producing the α -ketoacids indole-3-pyruvate, *p*-hydroxyphenyl pyruvate and phenyl pyruvate respectively (phenyl pyruvate can be converted in benzadheyde; Smit et al. 2004). Aromatic amino acid aminotransferases have been studied in a number of cheese related microorganisms including lactococci (Gao and Steele 1998) and lactobacilli (Gummalla and Broadbent 2001). The α-ketoacids produced from aromatic amino acids degrade by a number of pathways to produce volatile compounds important to cheese flavour. The branched-chain amino acids Leu, Ile and Val are degraded by aminotransferases, producing a corresponding α-ketoacid that can then be degraded to other compounds. Aminotransferases capable of transaminating branched chain amino acids have been studied in lactococci (Atiles et al. 2000) and *Lb. paracasei* ssp. *paracasei* (Hansen et al. 2001). α-Keto acids

are central intermediates, and can be hydrogenated to the corresponding α-hydroxy acids, decarboxylated to the corresponding aldehydes and dehydrogenated in the corresponding CoA-esters. All these compounds except hydroxyacids are major aroma compounds.

α -Keto acid reduction to hydroxyacids may cause a lowering of α -keto acid concentrations thereby affecting the flux towards flavours compounds such as aldehydes. The reduction of α -Keto acids derived to hydroxyacids has been observed in many LAB: in lactococci (Gao et al., 199) and in many lactobacilli (Gummalla & Broadbent, 1999).

In LAB α-Keto acids can be converted directly to the corresponding organic acid via an oxidative decarboxilation by a dehydrogenase enzyme complex without transitory formation of aldehydes. During this pathway acyl-CoAs are generated, and further hydrolysed to carboxylic acids either by acyl-CoA hydrolase or by 2 successive steps catalysed by phosphate butyrate-CoA transferase and butyrate kinase (McSweeney PLH., 2004). Various cheese micro-organisms are capable of generating esters from carboxylic acids (or acyl-CoAs) and alcohols. This esterification reaction has been observed in various LAB including lactococci, lactobacilli, *Streptococcus thermophilus*, leuconostocs and pediococci (Liu et al, 1998), but this ability is highly strain dependent. The enzymes involved could be carboxyl-esterases, which have broad substrate specificity, aryl esterases or alcohol acyltransferase. Esterase, that is widely distributed in LAB (Chich et al,1997) might also participate in the ester synthesis although its primary function is in ester hydrolysis. The reaction may also be spontaneous but may require the activation of carboxylic acids to acyl-CoAs. In LAB, aldehydes resulting from α-Keto acid decarboxylation can be further reduced to alcohol by alcohol dehydrogenases (Yvon & Rijnen, 2001), which is often desired in cheese, but which can also generate off-flavours such as the malty or floral off-flavours when their concentration is too high. Aldehydes can be also oxidised to the corresponding carboxylic acids by aldehyde dehydrogenases (Yvon & Rijnen, 2001).

Another important conversion route of amino acids is initiated by **lyases**, like methionine-γ-lyase (which converts Met to α-ketobutyrate, methanethiol and ammonia) cystathionine-β-lyase (which converts cystathionine to homocysteine, pyruvate and ammonia) and cystathionine-γ-lyase (which converts cystathionine to cysteine, α-ketobutyrate and ammonia) have received much attention (McSweeney and Sousa, 2000). In contrast to the transaminase pathway in which methanethiol is formed in several steps, lyase activity on methionine results directly in methanethiol. Threonine aldolase belongs to the class of carbon-carbon lyases, and is able to convert threonine directly to glycine and acetaldehyde (Ott et al., 2000).

Another conversion pathway for amino acids is the **decarboxylation** to amines with the loss of carbon dioxide. Amines often have strong and unpleasant aromas and, more importantly, some of the 'biogenic amines' cause adverse physiological effects in susceptible consumers (Leuschner et al., 1998). Decarboxylases generally have an acid pH optimum (about pH 5.5) and usually require PLP as cofactor (Hemme et al. 1982). The rate of amines production in cheese depends on the concentration of precursor amino acids and, more importantly, the cheese

microflora, which in turn may be affected by factors such as ripening temperature, pH and salt concentration (Joosten H.M.L.J. and Stadhouders J., 1987). Nonstarter lactobacilli and enterococci have been implicated in the production of high levels of biogenic amines in most cheese varieties (Gardini et al. 2001; Roig-Sagues et al. 2002). The principal amines in most cheeses are tyramine and histamine produced by decarboxylation of Tyr and His, respectively (McSweeney PLH, 2004). Histamine present at high levels can result in food poisoning (Santos 1991). No relationship has been found between the concentration of FAA and the production of amines in cheese, probably due to differences in the rates of decarboxylation of individual amino acids or in the rate of deamination of resulting amines (Polo et al. 1985). Simple decarboxylation can explain the formation of most amines found in cheese, but there is no readily available explanation for the formation of secondary and tertiary amines in cheese (Adda et al. 1982).

Amino acids may also be degraded by **deamination** reactions involving the action of dehydrogenases (which use NAD+ as the electron acceptor and produce an α -ketoacid and ammonia) or oxidases (which use oxygen as the electron acceptor and form aldehydes and ammonia) (McSweeney PLH, 2004). Ammonia produced by deamination contributes to the flavour of certain varieties including smear cheeses, and that, if produced in sufficient quantities, may contribute to an increase in pH during ripening.

Studies of the enzyme activities actually present in cheese during ripening are not as numerous as would be expected given the need to understand the enzymology of flavour development. Many studies present data regarding enzyme activities in cheese as supporting evidence for starter autolysis in cheese through release of intracellular marker enzymes. A number of studies have focussed on determining enzyme activity present in extracts of varieties: Spettoli et al. (1985) detected neutral and acid proteinase, aminopeptidase and carboxypeptidase activities in extracts of Provolone and Montasio cheese during ripening. Gobbetti et al. (1997) detected increasing Pep N and Pep C aminopeptidase activities and proline iminopeptidase activities in the rind and core of Pecorino Umbro cheese during ripening. However, carboxypeptidase activity was not detected throughout ripening. Increasing levels of proteinase, endopeptidase, dipeptidase, tripeptidase activities were noted with ripening time and in general, enzyme activity was higher in the rind than in the core of the cheese. A survey of the levels of aminopeptidase and Pep X activities in extracts of 32 commercial cheeses including Swiss, Italian and Dutch types and mould or smear ripened varieties was carried out by Gatti et al. (1999). Highest aminopeptidase activity was detected against arginine, lysine b-naphtyylamide (bNA) substrates, with less activity against leucine-bNA and little or no activity against proline-b NA or glutamate-bNA. In agreement with other studies, significant Pep X activity was detected in most of the varieties surveyed, including those with extended ripening periods, such as Parmigiano Reggiano and Grana Padano.

1.6. THE *TRENTINGRANA* CHEESE

"Trentingrana" cheese is produced in the alpine area of Trento. It is comprised in the "Grana Padano" consortium, which includes several northern Italian regions (Trentino Alto Adige, Veneto, Emilia Romagna and Lombardia) and enjoies a Protected Designation of Origin (PDO). This kind of cheese is made from partially skimmed raw cow's milk using a whey culture obtained from the spontaneous fermentation of part of the previous day's cheese-making whey, (Neviani and Carini 1994). Similarly to Parmigiano Reggiano and Grana Padano, it is subjected to a long ripening period (up to 2 years) which involves a sequential breakdown of milk components, such as fat, protein and lactose, by the enzymes of bacteria (Mcsweeney et al., 2000).

Grana Trentino is made with raw milk from cows feeded mainly with forage from the origin area (Trentino province). The milk does not undergo any thermal treatment and the use of additives is strictly forbidden. The milk from the evening milking and from the morning one are delivered to the dairy in no more than two hours from the end of each milking. Milk can be cooled immediately after milking and kept at a temperature not below 18°C. The evening milk is partly skimmed by removing the cream naturally risen to the surface in open-top stainless steel basins. The morning milk, immediately after arriving at the cheese-factory, is mixed with the partially skimmed milk from the previous evening. Starter whey is then added to the milk. This is a natural starter culture of lactic bacteria, most of them acid-producing, obtained from the spontaneous acidification of the remained whey after the previous cheese making day. Thermophilic lactic acid bacteria selected by the process of curd-cooking are the dominant microflora of natural whey starter. In particular natural whey starters contain thermophilic lactobacilli higher than 10^8 cfu / ml and *Lb. helveticus* is usually the dominant species (Gatti et al., 2003). The milk curdling takes place inside copper vats shaped like truncated cones with the exclusive use of calf rennet. After curdling, the curd is broken up into grains and cooked. These curd grains are then left to settle to the bottom of the vat in order to form a compact mass. The cheese mass is subsequently placed into special moulds for the moulding process. After few days, cheeses are salted in a bath of salt solution. Maturation must last at least 9 months starting from the cheese moulding. In figure 5 time and temperature conditions used in Grana Trentino standard production are shown, and in table 1 are shown the main features of a Grana cheese standard wheel.

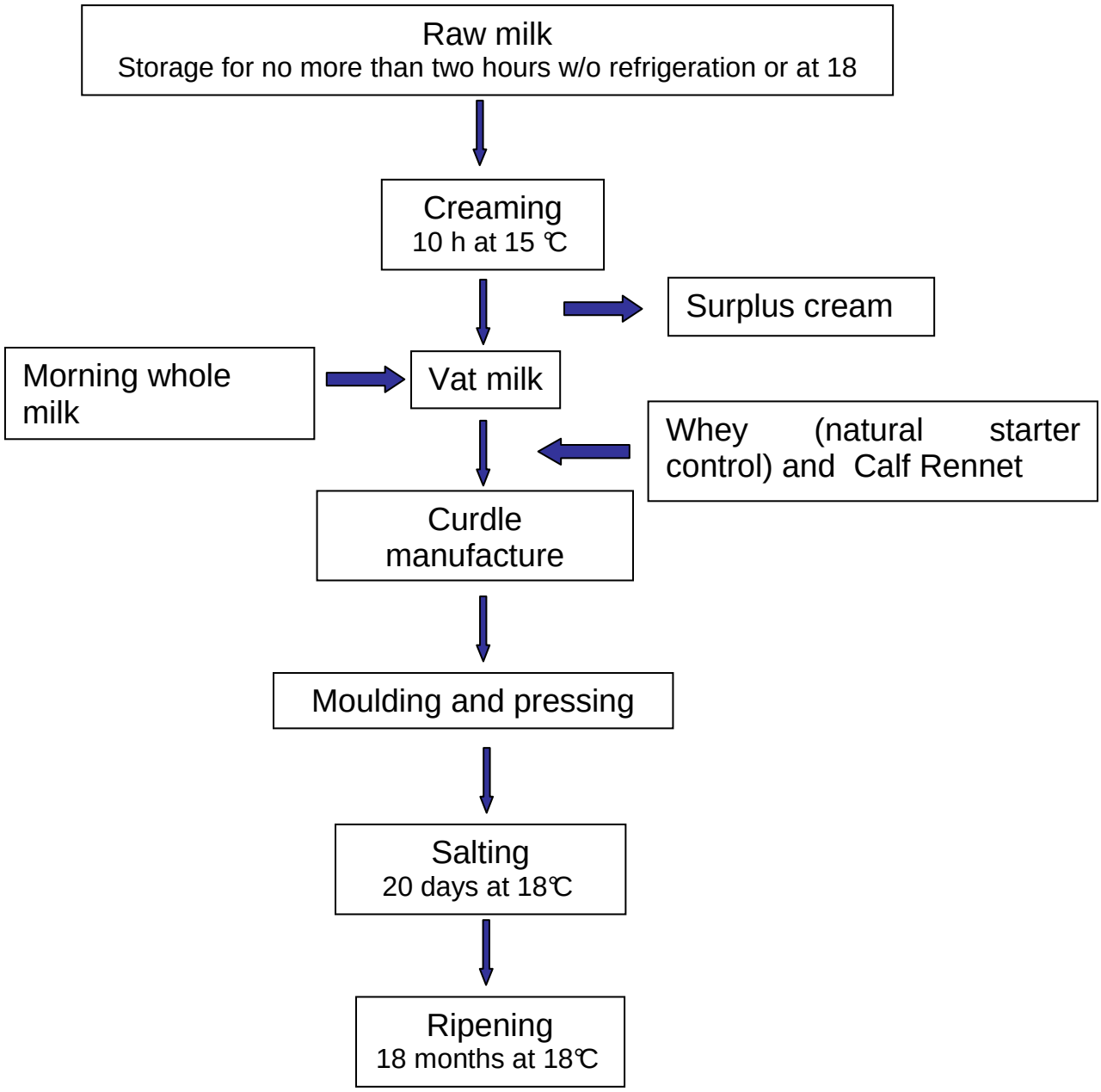

Figure. 5 Grana Trentino cheese-making

Shape	Cylinder shape with slightly convex to straight sides, upper and lower faces slightly chamfered
Diameter of upper and lower faces	From 35 to 45 cm
Side height	From 25 to 30 cm
Minimum wheel weight	35 kg
Rind thickness	From 4 to 7 mm
Paste texture	Fine granules, breaks in brittles
Fat content	Minimum 32 % of dry matter
External appearance	Natural gold-coloured rind
Paste colour	From pale straw-yellow to straw-yellow
Typical aroma	Fragrant
Taste	Delicate

Table. 1 Grana Trentino features

Even if Grana Trentino belongs to Grana Padano Consortium however it is clear distincted in the milk and in the manufacturing technology as follows: in Grana Padano production, lysozyme are used, raw milk comes from Italian Friesian herds breed in Padana valley and every day more than one cheese-making cycle is allowed in the cheese factory. In Grana Trentino production, using of lysozyme is forbidden and lysozyme is known to have significantly influence on the growth not only of butyric acid bacteria but also of lactic acid bacteria and consequently it is able to affect curd acidification during the cheesemaking (Grazia e al. 1984). For Trentingrana production, milk comes principally from Italian Brown herds which are breed in Alpes valleys without ensilage feeding and Malacarne et al. showed that milk from Italian Brown cows is characterised by a higher casein content, better rheological properties and lower losses of fat in the cheese than Italian Friesian cows' milk (Malacarne et al. 2006). This conditions could affect environment during cheese ripening such as pH, oxidation–reduction potential, water activity, and nutrient content which are demonstrated to modify the composition of LAB microflora behaviour (Dicagno et al. 2006). Last but not least, for Trentingrana only one cheese-making cycle is allowed each day in the cheese factory by a traditional artisan way (Regulation CE n. 1107/96). Even the sizes of cheese factories in Trentino are smaller than in other Italian region producing Grana cheeses: a Grana Padano cheese factory usually is able to work in a year more than three fold of milk than a Trentino dairy (Bottazzi 1993). This is because in alpine areas, such as Trentino region, connections among valleys are difficult and roads not allow a fast and comfortable milk transport; for reasons of both costs and milk quality that could be worst after such long and hard transport, dairy plants have to be close to the collection farms. This is why in Trentino province the most common level of milk processing is in

small-sized dairy plants which process limited amounts of milk supplied by a small number of farmers. Beside the difficulty of transport, many other factors led to this kind of solution:

- Cow-farmer cannot ensure the conditions (above all sanitary) in the processing areas required by law and so the milk can not be processed in the same location of milking by the farmer
- A small dairy plant which adopts modern techniques is obviously able to supply a product of high quality, giving greater prestige to both the product itself and the farmers of the area in which it is produced.
- These small plants could be run by farmers co-operatives themselves so that some of the profit from the processing returns to the producers.

Grana Trentino processing represents more than half of the Trentino milk market with 60 thousands milk tons transformed each year in 100.000 cheese moulds in 17 cheese factories using mountain artisan production techniques. This artisan system is able to sustain economic development in less favoured areas and has a significantly better environmental impact than industrial dairy farms. The final quality of the cheese is mainly up to the ability of the cheese-maker to process the different qualities of raw milk (which varies from season to season and from farm to farm) without using any additive but the daily whey as starter culture and the calf rennet (http://www.trentingrana.it).

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2. AIM OF THE THESIS

This PhD research is part of a project addressed to improve the quality of Grana Trentino production. The objectives were to evaluated if milk storage and collection procedures may affect cheese-making technology and quality. Actually the milk is collected and delivered to the cheese factory just after milking in 50 L cans without refrigeration or in tanks cooled at 18 °C. This procedure is expensive (two deliveries each day) and the milk quality is difficult to preserve as temperatures are not controlled. The milk refrigeration at the farm could allow a single delivery to the dairy. Therefore it could be a good strategy to preserve raw milk quality and reduce cheese spoilage. This operation may, however, have the drawbacks of favouring the growth of psychrotrophic bacteria and changing the aptitude of milk to coagulation. With the aim of studying the effect on milk and cheese of traditional and new refrigerated technologies of milk storage, two different collection and creaming technologies were compared. The trials were replicated in three cheese factories manufacturing Grana Trentino. Every cheese-making day, about 1000 milk liters were collected from always the same two farms in the different collection procedures (single or double). Milk was processed to produce 2 wheels of Grana trentino every day. During the refrigerated trials, milk was collected and stored at the farm in a mixed tank at 12 or 8 °C and then was carried to the dairy in truck once a day. 112 cheese making day were followed: 56 for traditional technology and 56 for the refrigerated one. Each one of these two thechnologies lead to different ways of creaming: long time in the traditional one and shorter in the new one. For every cheese making day we recorded time, temperatures and pH during the milk processing to cheese. Whole milk before ceraming, cream and skim milk after creaming, vat milk and whey were sampled during every cheese-making day for analysis. After 18 months ripening we opened 46 cheese wheels for further chemical and microbiological analyses.

The trials were performed with the aim of:

1 estimate the effect of storage temperatures on microbial communities, physico-chemical or/and rheological differences of milk and skim milk after creaming.

2 detect by culture dependent (plate counts) and indipendent (DGGE) methodolgies the microbial species present in whole, skimmed milk, cream and cheese sampled under the rind and in the core;

3 estimate the physico-chemical characteristics, the proteolytic activity, the content of free aminoacids and volatile compounds in 18 months ripened Grana Trentino cheeses from different storing and creaming of milk technologies.

The results presented are remarkable since this is the first in-deep study presenting microbiological and chemical analysis of Grana Trentino that even if belonging to Grana Padano Consortium, it is clearly different in the milk and in the manufacturing technology.

3. RESULTS

3.1 The influence of different temperature and storage regimes on the characteristics of milk to be processed in grana cheese

ABSTRACT

Raw milk used to produce grana cheese followed several technological regimes, including temperature (uncontrolled, 18, 12 and 8°C) and storage: milk from morning and evening milking were conferred to the dairy factory separately or together after storing at the farm for 12 hours. pH values were lower for milk samples kept at room temperature while acidity was higher. Fat/casein (F/C) ratio did not greatly differ among the experimentations. Clotting time (*r*) displayed by the experimentation carried out at 18 °C were higher than the others (19.59 in evening, 20.18 in skim and 19.00 min in vat milk). In all experimentations curd firmness (a_{30}) of evening milk showed lower values than skim and vat milk. Total microorganisms of whole milk samples ranged between 3.5 and 4.0 log cfu/mL. The application of a culture-independent microbiological approach revealed the presence of several milk associated bacteria. Low temperatures are confirmed to be useful in maintaining a high hygiene of raw milk

3.1.1. Introduction

Milk is a rich medium for the development of a wide variety of microorganisms. During the milking procedure the raw milk may be contaminated by the udder surface, the milking equipment and the stable environment. After collection, raw milk may be subjected to further contaminations, e.g. during transport and filling operations and at the dairy factories where milk is left for a while before being processed. Bacterial contamination of milk may also have a mastitic cow origin (Zehner, Farnsworth, Appleman, Larntz, & Springer, 1986). The quality of milk depends strongly on microorganisms living in it, whose ability to grow is also influenced by technological parameters such as cooling and holding temperature, as well as storage time (Heeschen, 1996; Slaghuis, 1996; Murphy & Boor, 2000).

Refrigeration at the farms the main strategy applied to preserve raw milk and the resulting dairy products from spoilage. This operation has, however, the drawback of favouring the growth of psychrotrophic bacteria. Gram-negative proteolytic psychrotrophic bacteria might cause spoilage of milk and milk products, due to their ability to produce thermostable proteases that hydrolyze casein and decrease the yield and sensory quality of dairy products (Dogan & Boor, 2003; Sørhaug & Stepaniak, 1997). Some bacteria also secrete lecithinases and lipases that can play a significant role in cheese-making aptitude of milk as well as in the spoilage of these products (Dogan & Boor, 2003). Thus, the storage temperature of raw milk after milking should be as low to inhibit the growth of pathogenic and spoilage mesophilic bacteria, without allowing the development of psychrotrophic bacteria. Furthermore, the presence of some mesophilic bacteria in raw milk, e.g. non starter lactic acid bacteria (NSLAB), can be desired, since they may play a positive role in artisanal and traditional cheeses (Folquié Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006).

Parmesan and Grana-cheeses, are hard cooked cheeses that undergo a ripening period up to 2 years and are economically relevant in the north part of Italy. “Grana trentino” cheese is produced in the Alpine province of Trento and is a member of “Grana Padano” consortium and enjoys a protected designation of origin (DPR n. 1269, 30 october 1955). The first fermentation of this cheese is triggered, similarly to Parmesan, by adding a Natural Starter Culture obtained from the spontaneous fermentation of part of the previous day’s cheese-making whey (Neviani & Carini, 1994) to partially skimmed raw cows’ milk.

Milk-skimming is a process in which, during milk resting, the fat globules clusterize together moving up and leading to the separation of cream from skimmed milk (Kohnhorst, 2001). Skimming is performed in large and shallow chambers, where due to fat agglutinins, fat globules form clusters and behave like globules with longer diameter, making creaming fast. Any thermal treatment does alter agglutinins slowing down the process, thus, low temperatures allow creaming in shorter times (Salvadori Del Prato, 1998). Besides cream, bacteria can also accumulate at the surface of raw milk because they often associate with the rising fat globules (Belknap, 1978), thus, fat clusters determine a bacterial removing action on milk (Corradini, 1995). This phenomenon greatly improves the

microbiological quality of milk to be processed into cheese, that is typical for spontaneous and natural creaming and is not observed with centrifugation (Corradini, 1995).

This work is part of a project aimed to improve the quality of "Grana Trentino" cheese production. In particular, the objectives of the present study were (1) to evaluate the physico-chemical, rheological and microbiological characteristics of raw cows' milk maintained at different temperatures and storage time at the farms, before delivering it to the dairy (2) to estimate differences in bacterial concentrations during milk maturation and skimming, (3) to detect the totality of prokaryotic species resident in whole, skimmed milk, cream and cheese samples, including the non cultivable bacteria, by denaturing gradient gel electrophoresis (DGGE) and (4) to evaluate physico-chemical composition (fat, protein and NaCl content) of 18 months cheese produced by milk stored and creamed at different temperatures regimes and with different technologies

3.1.2. Materials and Methods

3.1.2.1. Milk supply

Raw cows' milk (Table 1) to be processed into "Grana Trentino" cheese were collected from two farms located in the Trentino region (northern Italy) and delivered to the local dairy factory (Caseificio comprensoriale di Primiero, Mezzano, TN, Italy). Milk bulks were subjected to four thermal regimes after milking: it was stored without any cooling at ambient temperature, or cooled at 18, 12 or 8°C, respectively. In trials with no refrigeration or 18 °C cooling, the milk from morning and evening milkings were delivered separately (double milk delivery) to the dairy factory in 50-L cans (trial without refrigeration) or by a temperature-controlled road tanker (trial with cooling at 18°C). In 12 and 8 °C trials the milk from the morning milking was kept refrigerated under slow stirring for 12 hours then mixed with the evening milk and transferred once a day, at evening, to the dairy factory (single milk delivery) in a temperature-controlled road tanker. When evening and morning milk were delivered separately, overnight skimmed evening milk was mixed with the whole morning milk at the dairy factory. Each day of cheese production with double daily milk delivery, five samples were collected and analysed: evening whole milk (EWM), skimmed milk (SM), cream (C), morning whole milk (MWM) and vat milk (VM) (Table 1). In case of single daily milk delivery, three samples were analyzed: whole milk (WM), SM which is also in this case VM and Cr.

The samples were collected in spring (April-May) and summer (August-September) 2007 during a production period of 16 weeks (four per month). Each experimentation was repeated for four consecutive days per week, one week per month, for a total of 16 sampling days.

3.1.2.2. Skimming process

Two different creaming technologies are used if milk is delivered to the cheese factory once or twice a day. In double milk delivery experimentations, creaming occurred by overnight rest at 15 °C. Evening

milk (ca. 600 kg) was placed into a 700 L-shallow tank in the dairy factory. The day after, skimmed milk (ca. 550 kg) is added to the whole milk (ca. 500 kg) collected in the morning and put in a copper vat for cheese making. Milk delivered once a day (ca. 1180 kg from experimentations at 12 or 8 °C) was placed into a 1200 L-shallow tank and the creaming was carried out without to need temperature control. After creaming, skimmed milk was transferred to the copper vat.

3.1.2.3. Measurements and chemical analysis of milk and cheese samples

Temperatures were recorded with a 175-T2 data logger (Testo, Settimo Milanese, Italy). Values of pH in milk samples were measured with a portable pH meter (Knick Portamess 910, Berlin, Germany) connected to a Cheesetrode (Hamilton Co., Reno, NV, USA) electrode.

Fat and casein contents in milk samples were evaluated by infrared analysis (Biggs, 1978) with a Milko-Scan 134 A/B (Foss Electric, DK-3400 Hillerod, Denmark). Acidity of samples was determined by titrating 100 mL aliquots with 0.25 N NaOH, using phenolphthalein as indicator (end-point pH 8.30) and the results were expressed in °SH.

Measurements of pH and cheese moisture were determined according to the Italian Official Methods for cheese analysis (Anonimous, Metodi Ufficiali di analisi dei formaggi; 1986). Fat content of cheese was evaluated by the Gerber-Siegfeld method (Savini 1946), while NaCl content was determined by titratation with $AgNO_3$ (File, FIL-IDF 88/A:1988). Water activity (a_w) was determined by means of AquaLab® Model Series 3 (Decagon Devices, Inc. Pullman, Washigton, USA) in different sections of grana chesse: inner crust and core.

Total nitrogen (TN) were determined by Kjeldahl according to Butikofer et al. (1993), while the separation of nitrogen matter by fractioning diagram described by Gripon et al. (1975). Soluble nitrogen (NS) at pH 4,6 was calculated by difference.

3.1.2.4. Lactodynamographic analysis

Milk rheological parameters was analysed, without pH standardization, by Formagraph (Italian Foss Electric, Padova, Italy), obtaining the following parameters: r = clotting time (min), time from the addition of rennet to the beginning of coagulation; a_{30} = curd firmness (mm), measured 30 min after the addition of rennet (Zannoni & Annibaldi, 1981).

3.1.2.5. Microbiological analysis

Decimal dilutions of whole milk, skimmed milk, milk mixture and cream samples were prepared in peptone water (0.1% mycological peptone, Oxoid, Basingstoke, UK); cream samples were previously homogenized speed in a Laboratory Blender Stomacher 400 (Seward, London, UK) for 2 minutes at highest to disrupt fat globules. Dilutions were plated and incubated as follows: total bacterial count (TBC) on PCA added with 1 g L^{-1} skimmed milk (SkM), incubated aerobically at 30 °C for 24 h; psychrotrophic bacteria on PCA-SkM, incubated aerobically for 7 days at 7 °C; coliforms on violet red bile agar (VRBA), incubated anaerobically for

24 h at 37 °C; mesophilic rods and cocci LAB on MRS and M17 agar, incubated at 30 °C anaerobically for 48 h and aerobically for 24 h, respectively; thermophilic LAB on whey agar medium (WAM) prepared as reported by Gatti, Lazzi, Rossetti, Mucchetti, and Neviani (2003), incubated anaerobically for 4 days at 45 °C; enterococci on kanamycin aesculin azide (KAA) agar, incubated aerobically for 24 h at 37 °C; pseudomonads on *Pseudomonas* agar base (PAB) supplemented with 10 mg/mL cetrimide fucidin (Oxoid), incubated aerobically for 48 h at 20 °C. Clostridia content was estimated by most probable number (MPN) technique using a 3×3 scheme: undiluted samples and decimal dilutions were pasteurized at 85 °C for 15 min and inoculated into reinforced clostridial medium (RCM) supplemented with 1.4% (v/v) Na-lactate (Merck, Darmstadt, Germany); after that, test tubes were sealed with paraffin:vaseline (1:6) and incubated for 7 days at 37 °C. All media were purchased from Oxoid.

3.1.2.6. Total DNA extraction and DGGE analysis from samples

DNA was extracted from milk and cream samples according to Meiri-Bendek et al. (2002); DNA extracts were then used as templates for PCR reactions.

DGGE samples were prepared by performing PCR amplification of the V3 region of 16S rRNA gene according to Ercolini, Moschetti, Blaiotta, and Coppola (2001a). DGGE was carried out using the DCcode Universal mutation Detection system (Bio-Rad, Hercules, CA, USA) on 16 cm × 16 cm × 1 mm gels. PCR products (8 µL) were loaded onto gels with 8% (w/v) acrylamide (acrylamide-bisacrylamide 37.5:1) and a 25 to 60% of urea and formamide gradient, increasing in the direction of electrophoresis. A 100% denaturing solution consisted of 7M urea and 40% (v/v) deionized formamide. Electrophoresis was conducted in 1× TAE [40 mM Tris, 20 mM acetic acid and 1 M EDTA (pH 8.0)] buffer at 150 V for 5h at 60°C. After runs, gels were stained for 15 min in an ethidium bromide solution, rinsed in distilled H_2O for 20 min and photographed on a UV transilluminator table.

3.1.2.7. DGGE band sequencing and bacterial identification

DGGE fragments found at different positions along the polyacrylamide gel were excised and eluted in 100 µL sterile MilliQ water overnight at 4°C. One microliter of the eluted DNA of each DGGE band was re-amplified as above reported with primers that did not contain the GC-clamp. PCR products were purified using the Exo-SAP-IT kit (USB Co., Cleveland, OH) and sequenced through the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA), following manufacturer's instruction, by means of an ABI PRISM 3100 sequencer (Applied Biosystems). Sequence identities were verified by a BLASTN (Altschul et al., 1997) search against the NCBI non-redundant sequence database located at http://www.ncbi.nlm.nih.gov.

3.1.2.8. Bulk cell DNA analysis

Bulk cell formation for DNA extraction was performed as reported by Ercolini, Moschetti, Blaiotta, and Coppola (2001b). Cell count was carried out on PCA medium. DGGE analysis were performed as above reported.

3.1.2.9. Statistical Analysis

Analysis of variance (ANOVA, StatSoft, Inc. 2008; STATISTICA data analysis software system, version 8.0. www.statsoft.com) (significant level $P<0.05$) was used to evaluate the influence of independent variables such as milk storage temperature (12 and 8°C in the single milk delivery and 18°C or not refrigerated in the double milk delivery) and season of production on the chemical and microbiological parameters measured on milk.

A normalization step was applied to each variable to avoid possible distortions arising from the different magnitudes of the numerical values associated with the different variables. This normalization involved that all microbiological data were expressed as their decimal logarithm to obtain the homogeneity of variance.

3.1.3. Results and Discussion

3.1.3.1 Chemical and technological properties of milk and cream samples

Milk for "Grana Trentino" cheese production was kept at different temperatures during storage before processing. Table 1 reports the means and standard deviations of the physico-chemical and rheological characteristics of milk samples for the different experimentations. In case of no refrigeration, EWM reached the cheese factory at 30.4 and 32.2 °C at evening and morning, respectively. SM and C had lower temperatures after overnight separation in temperature-controlled tanks (15.7 and 17.9 °C, respectively), and final vat milk temperature was 23.7 °C. Similar temperatures were observed even for skimmed SM and C samples from EWM kept at 18.0 °C. When refrigerated at 12 or 8 °C the milk temperature was slightly lower as SM temperature was of 15.0 and 13.4 °C, respectively in vat. In these latter two cases the creaming step was not managed in a temperature control tank. Milk values of pH were always slightly lower in milk and cream samples in not refrigerated trial and the difference was significant in evening and morning whole milk. Probably the storage without refrigeration before delivery did not slow down the activity of mesophilic lactic acidic bacteria, leading to lowered milk pH values. In SM from not refrigerated milk, the acidity was significant higher than in the others trials, probably due to weak acid production by microorganisms that developed during early stages of creaming, when the temperature was still favourable (the temperature decreased from 30.4 to 15.7 °C in about 2 hours). In all other cases the acidity ranged between 3.5 and 3.7 °SH without significant differences ($P < 0.05$) among the different trials.

Fat, protein and casein values and fat/casein (F/C) ratio of milk are reported in Table 1. In the EWM the fat amount did not greatly differ among the experimentations (from 3.56 to 3.62%).Fat values observed in vat milk were always in agreement with those found in Parmigiano Reggiano cheese, reported to be in a range between 2.02 and 3.13%, while proteins and casein contents were higher than values reported by formaggioni et al. (2005) for Parmigiano Reggiano (3.40% and 2.65%,

respectively) Hence, in our vat milk samples, the ratio F/C was lower. Vat milk from refrigeration at 18 °C had higher fat amount (2.48%) and lower casein amounts (2.67%) than other trials, and a F/C significantly higher (0.93) than other experimentations.

Milk casein content affects the characteristics of curd and cheese yield. In milk containing higher amounts of casein, curd formation occurs in a shorter time and is firmer and more contractible, thus facilitating a more uniform draining of whey (Fossa, Pecorari, Sandri, Tosi, & Mariani, 1994).

Generally bulk milk clotting characteristics (r and a_{30}) were not very different among all trials. Only in milk from refrigeration at 18 °C the r value was 19.59 in EWM, 20.18 in SM and 19.00 in vat milk. These values were significantly higher than the mean values of the others trials, e.g. 16.71, 17.61 and 17.84 of EWM of not refrigerated and 12 or 8°C refrigerated milk respectively. However, in all trials, EWM had lower a_{30} value than SM and vat milk. Casein amounts and milk clotting parameters were different from those of "Parmigiano Reggiano" cheese vat milk (Formaggioni et al., 2005), probably because the milk used for Grana Trentino cheesemaking, comes mainly from Brown herds, and has higher casein values and better aptitudes to coagulations than milk produced by Fresian cows, commonly used for "Parmigiano Reggiano" cheese production (Mariani, Pecorari, & Fossa, 1984; Pecorari, Sandri, & Mariani, 1987).

Creaming times for milks refrigerated at 12 or 8 °C were shorter, than those of milks not refrigerated or refrigerated at 18 °C. This determined different percentages of cream in the vat milk after the separating process.

Physico-chemical and rheological parameters of the tested milks showed that no significant differences were linked to the different storage temperatures of milk used for "Grana Trentino" cheese production. Independently on the storage temperature, all the analysed milk had good cheese-making aptitudes, and several technological parameters remained in the range defined for Parmigiano-Reggiano (Formaggioni et al., 2005) and Grana Padano (Pecorari, personal communication) cheese.

Table 1. Physico-chemical and rheological parameters of whole, skimmed and mixed milks and creams from bulks kept at different temperatures after milking[a]: A, room temperature; B, 18°C; 12°C; D, 8°C.

Measurements	Delivery to dairy factory twice a day										Delivery to dairy factory once a day					
	w/o refrigeration					18 °C					12 °C			8 °C		
	EWM	SM	Cr	MWM	VM	EWM	SM	Cr	MWM	VM	WM	SM / VM	Cr	WM	SM / VM	Cr
T (°C)	30.4 ± 2.3 **a**	15.7± 0.7 a	17.9± 1.0 a	32.2 ± 3.2 **a**	23.7 ± 0.9 **a**	18.4 ± 1.1 **b**	15.7± 0.7 a	17.3± 1.1 a	18.7 ± 1.5 **b**	17.7 ± 1.3 **b**	13.6± 1.2 **c**	15.0 ± 1.2 a	15.9± 1.9 a	10.5 ± 0.7 **d**	13.4± 1.7 **b**	15.0± 2.4 a
pH	6.56 ± 0.06 **b**	6.63± 0.05 a	6.67± 0.05 a	6.56 ± 0.06 **b**	6.63 ± 0.05 a	6.65 ± 0.06 a	6.69 ± 0.04 a	6.69± 0.08 a	6.67 ± 0.03 a	6.65 ± 0.06 a	6.66± 0.05 a	6.66± 0.04 a	6.68 ± 0.05 a	6.67 ± 0.04 a	6.69± 0.04 a	6.68± 0.05a
Acidity (°SH)	3.5 ± 0.2 a	3.7 ± 0.1 **b**		3.6 ± 0.1 a	3.6 ± 0.1 a	3.5 ± 0.1 a	3.6 ± 0.1 a		3.6 ± 0.1 a	3.5 ± 0.2 a	3. 6 ± 0.1 a	3.4 ± 0.1 a		3.7 ± 0.2 a	3.5 ± 0.1 a	
Fat%[b]	3.56 ± 0.11 a	1.49± 0.1 a	25.52± 0.93 a	3.45 ± 0.10 a	2.38 ± 0.06 a	3.62 ± 0.08 a	1.67± 0.14 a	24.20± 2.37 a	3.50 ± 0.14 a	2.48 ± 0.12 a	3.60± 0.10 a	2.38± 0.08 a	18.24± 4.49 **b**	3.58 ± 0.07 a	2.38± 0.10 a	19.37 3.80**b**
Proteins%[b]	3.37 ± 0.07 a	3.44± 0.05 a		3.49 ± 0.08 a	3.46 ± 0.04 a	3.39 ± 0.17 a	3.42± 0.13 a		3.53 ± 0.12 a	3.44 ± 0.09 a	3.51± 0.06**b**	3.56± 0.04**b**		3.49 ± 0.05 **b**	3.53± 0.05**b**	
Casein%[b]	2.60 ± 0.06 a	2.67± 0.06 a		2.71 ± 0.07 a	2.68 ± 0.05 a	2.61 ± 0.16 a	2.65 0.13 a		2.73 ± 0.11 a	2.67 ± 0.10 a	2.72± 0.07**b**	2.76± 0.03**b**		2.71 ± 0.05 **b**	2.76± 0.04 **b**	
Lactose%[b]	4.75 ± 0.12 a	4.87± 0.04 a		4.86 ± 0.13 a	4.86 ± 0.12 a	4.73 ± 0.23 a	4.80± 0.18 a		4.82 ± 0.18 a	4.81 ± 0.12 a	4.87± 0.08**b**	4.95± 0.04**b**		4.90 ± 0.02 **b**	4.97± 0.22**b**	
Fat/casein					0.89 ± 0.03 **b**					0.93 ± 0.03 **a**		0.87± 0.04**b**			0.87± 0.04**b**	
r (min)	16.71± 2.68 a	17.04± 2.37 a			17.04± 2.77 a	19.59± 3.26 a	20.18± 4.28 **b**			19.00± 3.00 a	17.61± 3.01 a	17.67± 2.05 a		17.84± 1.86 a	17.06± 1.75 a	
*a*30 (mm)	20.83± 8.46 a	24.50± 4.27 a			24.50± 5.33 a	21.18± 7.33 a	21.18± 8.48 a			23.92± 7.54 a	24.71± 4.56 a	25.53± 4.27 a		23.06± 5.05 a	23.44± 4.16 a	
Creaming duration	10 h 30' ± 30' **a**					9 h 45' ± 20' **b**					7 h 30' ± 30' **c**			7 h 30' ± 30' **c**		
Cream% (w/w)[c]	10.68 ± 0.80					9.94 ± 1.73					5.70 ± 0.32			6.92 ± 0.32		

Abbreviations are as follows: EWM, evening whole milk; SM, skimmed milk; C, cream; MWM, morning whole milk; VM, vat milk; WM, whole milk; Different letters (a, b) on the same row and only at the same column type (sample) indicate significant differences ($p < 0.05$).

[a] Values are means ± S.D. of analysis from 16 different productions in eight weeks.

3.1.3.2. Microbial counts in milk and cream samples

Microbial populations present in EWM, SM, C and vat milk batches from different temperatures storages are shown in Table 2. Total counts in EWM ranged between 3.5 and 4.0 log cfu/mL. Total microbial counts after the creaming process, decreased but not in a significant way: in SM samples, counts were only 0.3 – 0.7 log cfu/mL lower than those from the corresponding EWM. Higher differences were observed in SM from not refrigerated milk than in SM from all others trials. Total bacteria content in vat milk was similar to that one in the EWM and no statistical significant differences were found among the four experimentations. In all trials, the TBC in milk samples was probably composed mainly by mesophilic cocci, whose content was in a range of ± 0.3 log cfu/mL than the respective TBC count. Instead in C samples the difference between TBC and mesophilic cocci contents were higher: mesophilic cocci counts were in a range of ± 0.8 log cfu/mL than the respective TBC count. Different TBC were found in C samples ranging from 5.3 to 6.6 log cfu/mL; the highest cell counts were found in creams originating from not refrigerated milk. These results clearly showed the effect of creaming on the debacterization: cell concentrations in C samples were always at least two logarithms higher than the ones in SM, reaching the greatest difference of almost three logarithms in C samples from not refrigerated milk, so the milk refrigeration limits the microorganisms proliferation during night resting of milk.

No differences in psychrotrophic bacterial counts were found among EWM samples. After creaming, the psychrotrophic bacteria counts in refrigerated samples were as low as in the EWM before creaming.

Only in SM from milk not refrigerated these bacteria grew up to 3.2 log cfu/mL during the spontaneous milk skimming. During creaming the growth of psychrotrophic bacteria in not refrigerated milk made them reach 5.9 log cfu/mL in C samples, while in the other trials C counts were one log lower (4.5, 4.3, 4.1 log cfu/mL, respectively). Pseudomonads, mesophilic rods and thermophilic LAB represented a minor part of total bacterial community in milk (with count values in the range 1.0- 3.0 log cfu/mL) and were found in higher amounts in creams, without significant differences among the four trials. Enterococci were present in all EWM samples (1.8-2.3 log cfu/mL). These values assessed the good microbiological quality of the milk delivered to the cheese factory, whatever the storage temperature.

After creaming, enterococci counts were higher in SM, C, and vat milk samples obtained from not refrigerated milk than in the corresponding samples from all the others trials. These observations suggested that keeping the milk without refrigeration before delivery to cheese factory allows the growth of this group of bacteria during overnight creaming; this does not occur if milk is refrigerated at 18°C or lower temperatures

Coliforms were found at the same concentration (1.3 – 1.7 log cfu/mL) in all whole milk samples. After creaming, a reduction of 0.4 - 0.9 log cfu/mL was observed in all SM samples. Coliform counts in C samples were higher, and when milk was not refrigerated they were significantly higher ($P > 0.05$) than in all other C samples. Butyric clostridia were found only in cream samples deriving from milks stored w/o refrigeration or at 18°C.

Table 2. Microbial populations (log cfu/mL) of whole, skimmed and mixed milks and creams from bulks kept at different temperatures after milking[a]: A, ambient temperature; B, 18°C; 12°C; D, 8°C.

Microbial populations	Delivery to dairy factory twice a day										Delivery to dairy factory once a day					
	w/o refrigeration					18 °C					12 °C			8 °C		
	EWM	SM	Cr	MWM	VM	EWM	SM	Cr	MWM	VM	WM	SM/VM	Cr	WM	SM/VM	Cr
Total microorganisms	4.0 ± 0.5a	3.7 ± 0.5**b**	6.6± 0.7**b**	3.6 ± 0.2a	4.1 ± 0.4a	3.5 ± 0.6a	3.0 ± 0.5a	5.4 0.4a	4.0 ± 0.7a	3.7 ± 0.5a	3.5 ± 0.3a	2.8 ± 0.5a	5.3 ± 0.9a	3.8 ± 0.5a	3.2 ± 0.6a	5.5 ± 0.8a
Mesophilic Cocci	3.9 ± 0.5a	3.7 ± 0.3**b**	6.0± 0.4a	3.5 ± 0.3a	3.9 ± 0.4a	3.8 ± 0.6a	3.0 ± 0.5a	5.2± 0.3a	4.0 ± 0.5a	3.9 ± 0.3a	3.6 ± 0.3a	2.8 ± 0.4a	6.0 ± 0.2a	3.6 ± 0.3a	3.1 ± 0.6a	4.7 ± 0.4a
Psychrotrophic bacteria	2.6 ± 0.2a	3.2 ± 0.5**b**	5.9± 0.2**b**	2.9 ± 0.3a	3.2 ± 0.5**b**	2.2 ± 0.2a	2.2 ± 0.5a	4.5± 0.5a	3.2 ± 0.7a	2.7 ± 0.8a	2.7 ± 0.6a	2.6 ± 0.6a	4.3 ± 0.2a	2.6 ± 0.7a	1.9 ± 0.5a	4.1 ± 0.8a
Pseudomonads	2.3 ± 0.9a	2.0 ± 0.3a	4.4± 0.5a	2.4 ± 0.6a	2.6 ± 0.6a	2.5 ± 0.6a	2.0 ± 0.5a	4.2± 0.7a	2.8 ± 0.7a	2.5 ± 0.7a	2.5 ± 0.6a	2.0 ± 0.6a	4.0 ± 0.8a	2.2 ± 1.0a	2.0 ± 0.9a	3.6 ± 1.0a
Mesophilic Rods	2.7 ± 0.4a	2.6 ± 0.7a	5.1± 0.2a	3.0 ± 0.2a	3.1 ± 0.5a	2.6 ± 0.5a	2.5 ± 0.5a	4.7 ± 0.1a	2.7 ± 0.6a	2.7 ± 0.3a	2.6 ± 0.3a	2.1 ± 0.8a	5.1 ± 0.4a	2.6 ± 0.3a	1.9 ± 0.8a	4.5 ± 1.1a
Thermophilic LAB	1.7 ± 1.1a	1.8 ± 1.2a	4.7± 1.1a	1.5 ± 1.2a	2.2 ± 1.2a	1.2 ± 1.1a	2.1 ± 1.4a	3.8± 0.9a	2.1 ± 1.2a	2.9 ± 0.9a	2.2 ± 0.6a	1.7 ± 0.8a	2.6 ± 0.1a	1.8 ± 0.6a	0.9 ± 1.0a	2.3 ± 0.1a
Enterococci	2.3 ± 0.6a	2.7 ± 0.5**b**	5.0± 0.0**b**	2.5 ± 0.7a	2.8 ± 0.5**b**	1.8 ± 0.3a	1.2 ± 0.5a	4.1± 0.1a	2.0 ± 0.7a	2.0 ± 0.3a	2.1 ± 0.6a	1.9 ± 0.4a	4.1 ± 0.4a	1.8 ± 0.3a	1.3 ± 0.8a	3.2 ± 0.1a
Coliforms	1.3 ± 0.5a	0.8 ± 0.7a	3.8 ± 0.2**b**	1.7 ± 0.7a	1.5 ± 0.8a	1.4 ± 0.7a	0,6± 0.6a	2.0± 0.2a	1.7 ± 0.8a	1.5 ± 0.9a	1.4 ± 0.7a	1.0 ± 0.0a	2.5 ± 0.2a	1.7 ± 0.7a	0.8 ± 0.9a	2.8 ± 0.1a
Butyric clostridia[b]	N.D.[3] a	N.D. a	2.2± 0.2**b**	N.D. a	N.D. a	N.D. a	N.D.a	2.4± 0.0**b**	N.D. a	N.D. a	N.D. a	N.D. a	N.D.a	N.D. a	N.D. a	N.D. a

Abbreviations are as follows: EWM, evening whole milk; SM, skimmed milk; C, Cream; MWM, morning whole milk; VM, vat milk; WM, whole milk.
Different letters (a, b) on the same row and only at the same column type (sample) indicate significant differences ($p < 0.05$).
N.D., not detectable (< 2.0).
[a] Values are means ± S.D. of analysis from 16 different productions in eight weeks.
[b] 2 As estimated by MPN.

In Grana cheese-making the process of milk-creaming has two effects: i) skimming of milk; and ii) the removal of bacteria from milk. Our results clearly showed that the total number of microorganisms did not greatly vary between EWM and SM samples. This observation is probably due to two opposite phenomenons: microbial growth does take place in milk during overnight standing, but milk creaming contemporarily determines a microbial removal together with fat globules. The higher bacterial cell counts found in cream undoubtedly were the result of the overnight growth of milk flora, but also of the concentration in the upper fatter layer (the cream) of the proliferating microorganisms. As a matter of fact, after spontaneous milk creaming the microbial load in SM samples was limited. Besides the physical action of creaming on removing bacteria from the EWM, the EWM storage temperature before delivery to the cheese factory plays an important role in reducing microbial growth. Not refrigerated storage allows the growth of bacterial groups such as psychrotrophic bacteria, which are potentially dangerous for the coagulation aptitude of vatmilk and, thus, negatively affecting cheese ripening.

3.1.3.3 Physico-chemical features of cheeses

Physico-chemical characteristics of Grana Trentino cheeses produced with milks previously stored at different temperatures are reported in Table 3.

Table 3. Compositional analysis of experimental Grana Trentino cheeses at 18 months of the ripening (mean values ±standard deviation of six independent measures.

Measurments	pH	Moisture %	NaCl %	NaCl / M	Fat %	Protein[a] %	Fat %dry matter	Protein %dry matter	SN %	Ripening Index [b]	Aw core	Aw under-crost
w/o refrigeration	5.47± 0.07	32.30 ± 0.26	1,79± 0.08	0,06± 0,00	26.51± 0.82ab	32.45 ± 1.26	39.15± 1.12ab	47.93 ± 1.73	1.31± 0.08a	25.73 ± 2.31 a	0.925± 0.003	0.907± 0.002
18 °C	5.44± 0.04	32.27 ± 0.54	1,79± 0.11	0,06± 0,00	26.78± 0.55 a	32.28 ± 1.86	39.53± 0.59 a	47.67 ± 2.83	1.33± 0.18a	26.10 ± 1.56 a	0.927± 0.002	0.906± 0.003
12 °C	5.48± 0.07	32.77 ± 0.44	1,67± 0.07	0,05± 0,00	25.82± 0.84bc	33.35 ± 0.55	38.40± 1.10bc	49.61 ± 1.09	1.52± 0.07b	29.06 ± 1.26 b	0.926± 0.004	0.907± 0.002
8 °C	5.47± 0.08	32.38 ± 0.65	1,71± 0.12	0,05± 0,00	25.33± 0.91 c	33.48 ± 0.37	37.46± 1.28c	49.51 ± 0.66	1.54± 0.05b	29.32 ± 1.04 b	0.923± 0.005	0.905± 0.004

SN: soluble Nitrogen; NaCl/M: aqueous salt concentration (g NaCl / 100 g H_2O).
a Total Nitrogen X 6,38
b Calculated as percentage ratio between water-soluble and total nitrogen.

No differences ($P > 0.05$) were registered among pH values of all cheeses, which ranged between 5.44 and 5.48, nor among cheese moisture, which was always in the range 32.27 – 32.77.

NaCl content has been referred both to 100 g of cheese and to 100 g of cheese moisture. The latter expression provides an indication of the saltiness of the aqueous phase, in which cheese enzymes are present. These

enzymes are involved in the chemical and biochemical reactions of cheese ripening.

No significant differences ($P > 0.05$) of cheese salt content were observed among the four different technologies: NaCl content ranged between 1.67 and 1.79 and the aqueous phase saltiness ranged between 5,1 and 5,5 %.

In general, the content of cheese fat underlined differences among the four productions. Fat content was significantly ($P < 0.05$) higher in cheeses processed with milk stored w/o refrigeration or at 18°C (Table 3).

Cheese protein contents were in the range 32.28 – 33.48 and did not differ ($P > 0.05$) among the four trials.

A_w of cheese core (0.923 - 0.927) was always significantly higher ($P < 0.05$) than a_w at inner crust (0.905 – 0.907), while no significant differences were observed among the four milk storage conditions.

The ripening index (RI) is defined as Soluble / Total Nitrogen ratio (SN / TN) and is usually expressed as percentage. It describes the proportion of casein (casein are not soluble) progressively digested during ripening by proteolytic enzymes in peptones, peptides and smaller caseinic fractions (casein fractions are soluble). RI was always higher ($P < 0.05$) in cheeses from single milk delivery (milk refrigerated at 8 or 12 °C): the observed values were 49.51 and 49.61 respectively, and 47.67 and 47.93 for cheese obtained from milk stored w/o refrigeration or at 18 °C (double milk delivery). In our trials that smaller was the RI and the higher was the a_w value. Probably the casein breakdown lead to an increase in carboxylic acid and amino acid compounds and this leads to a different water binding and a decrease in the water activity (a_w). This might also lead to soften cheese texture and to facilitate the release of flavour compounds when the cheese is consumed (Sousa et al. 2001). Casein hydrolysis during ripening is mainly due to the action of chymosin trapped in the curd during whey drainage, and to a lesser extent to indigenous milk proteinases as plasmin (Sousa et al. 2001). In Grana trentino wthe high cooking temperature (> 55°C) during manufacturing is supposed to denature most of the chymosin and plasmin so that bacterial proteinases may be responsible for the extensive proteolysis in the cheeses, which are ripened for a long period (even more than 24 months) at elevated temperatures (18–20°C). In general, we observed a higher fraction of soluble nitrogen lower at the lower milk storage temperature. This could be due to a higher proteolytic activity of enzymes from psycrotrophic bacteria which had more time to work (24 hours in the single milk delivery and about 12 hours in the double one) in trials with milk stored at lower temperatures. This could be very interesting because understanding who is going to lead the ripening could carry to a better control of it and to a better definition of the cheese flavour.

When compared to Parmigiano Reggiano, the Grana Trentino cheese showed great differences in RI, which was higher after 18 months of ripening (Panari et al. 2003) than Parmigiano Reggiano whose RI value was 30,83.

3.1.3.4. Bacterial identification

The presence of different bacterial species in milk and cream samples was revealed by DGGE patterns of the amplified V3 region of 16S rDNA as shown in figure 1A.

Table 3. DGGE band sequence similarities.

Bands	Closest relative	Similarity (%)
1	*Lactobacillus helveticus*	100
2	*Lactobacillus delbrueckii* ssp. *bulgaricus*	99
3	*Streptococcus thermophilus*	100
4	*Escherichia coli*	97
5	*Enterococcus* sp.	<97
6	*Acinetobacter* sp.	<97
7	*Acinetobacter* sp.	<97
8	*Lactobacillus rhamnosus*/*Lactobacillus casei*	99
9	SF	
10	*Acinetobacter* sp.	<97
11	*Pseudomonas* sp.	<97
12	*Staphylococcus saprophyticus*	97
13	SF	
14	SF	
15	*S. saprophyticus*	98
16	SF	
17	*Enterococcus* sp.	<97
18	*S. saprophyticus*	99
19	SF	
20	*L. rhamnosus*/*L. casei*	98
21	*Acinetobacter* sp.	99
22	*Acinetobacter* sp.	98
23	*Acinetobacter* sp.	97
24	*Acinetobacter* sp.	97
25	*Staphylococcus* sp.	<97
26	*Leuconostoc mesenteroides* ssp. *mesenteroides*	100
27	*Lactococcus garviae*	99
28	*Lactobacillus fermentum*	99
29	*Lactobacillus helveticus*	100
30	*Lactobacillus delbrueckii ssp. bulgaricus*	98
31	*Enterobacter ssp.*	<97
32	*Streptococcus termophilus*	99
33	SF	
34	*Escerichia coli*	100
35	*Sphingomonas paucimobilis*	99
36	*Lactobacillus rhamnosus*/*Lactobacillus casei*	99

The identification process was based on DGGE band cut and sequencing, followed by BLAST comparison in GenBank located at http://www.ncbi.nlm.nih.gov (sequence similarities reported in Table 3).

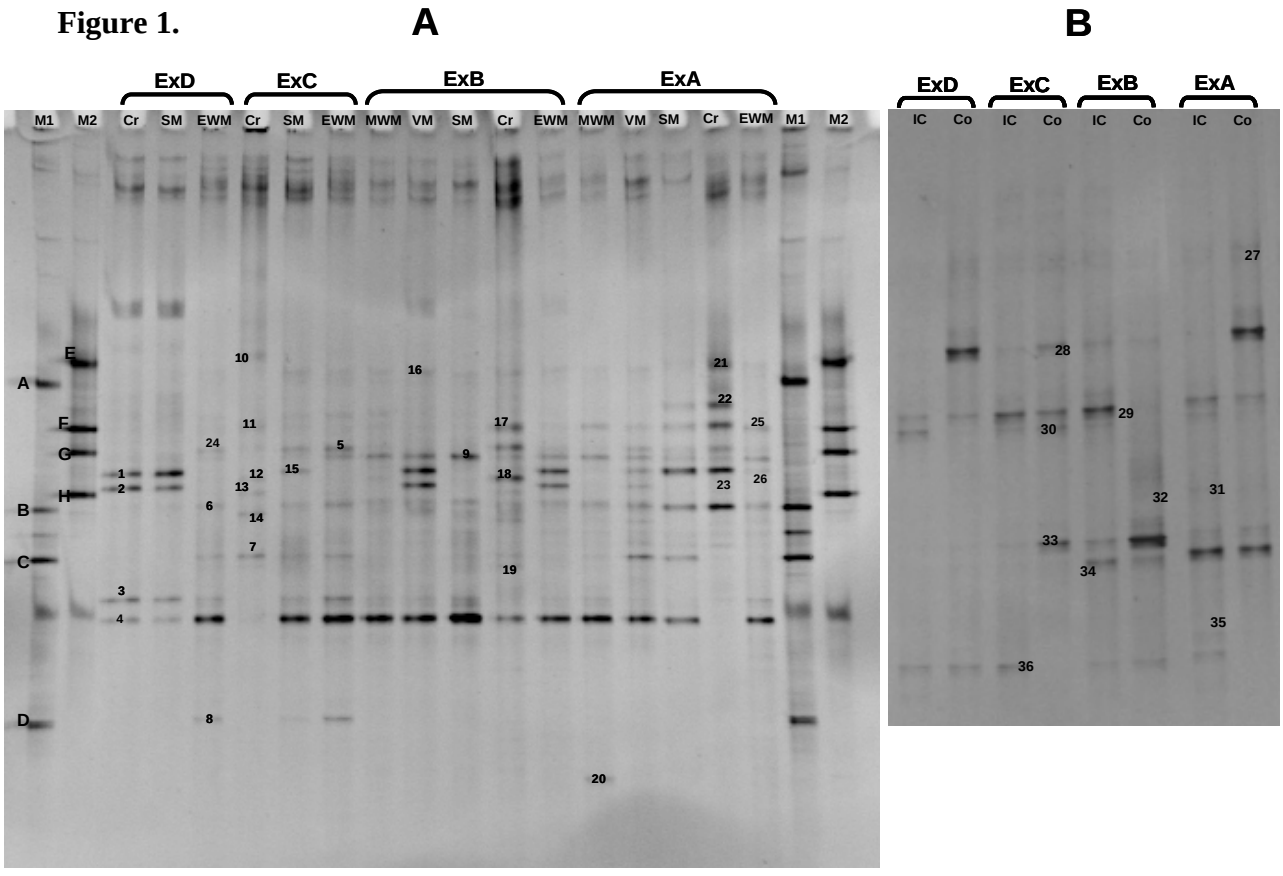
Some bands could not be associated to any bacterial species because of their sequencing failure.

Legend to figures.

Fig. 1A. DGGE profiles of 16S rRNA gene V3 regions obtained from different evening whole milk (EWM), cream (C), skim milk (SM), morning whole milk (MWM) and vat milk (VM) samples from different experimentations: A, ambient temperature; B, 18 °C; C, 12 °C; D, 8 °C. Lanes: M1, marker 1 including *Lactobacillus plantarum* DSMZ 20174^{T} (A), *Streptococcus gallolyticus* ssp. *macedonicus* 15789^{T} (B), *Lactococcus lactis* DSMZ 20069^{T} (C), *Lactobacillus casei* DSMZ 20011^{T} (D); M2, marker 2 including *Lactoccus garviae* DSMZ 20684^{T} (E), *Leuconostoc mesenteroides* DSMZ 20346^{T} (F), *Enterococcus faecium* DSMZ 20477^{T} (G), *Pediococcus pentosaceus* DSMZ 20336^{T} (H).

Fig. 1B. DGGE profiles of 16S rRNA gene V3 regions obtained from grana cheese sections inner crust (IC) and core (Co) from different experimentations: w/orefrigeration; 18 °C; 12 °C; 8 °C.
Bands have been enumerated from 1 to 36 and the corresponding identities are reported in Table 3.

Figure 1.



Escherichia coli band was found in all milk samples; such a dominance decreased in creams and disappeared in milks stored at 12°C or w/o refrigeration. Whole milk samples also showed bands sequenced as *Streptococcus thermophilus*, *Lactobacillus helveticus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. Furthermore, a band sequenced as belonging to *Lactobacillus rhamnosus*/*Lactobacillus casei* species has been detected in EWM samples of from milk refrigerated at 8 or12°C, in SM at 12°C and in MWM of milk not refrigerated. *Leuconostoc mesenteroides* was found only in EWM from milk stored w/o refrigeration. All milk and cream samples showed bands even if faint of enterococci. *Staphylococcus ssp.* has been found in all C and also in whole milk samples. Pseudomonads have been found in all samples and *Acinetobacter* spp. have been detected mostly in non-refrigerated whole milk and C samples.

In refrigerated milk samples at 8 and 12°C, a lower number of bands and a higher presence of lactic acid bacterial bands was found.

By means of a culture-independent approach, thermolabile (*Acinetobacter* spp., *Pseudomonas* spp. and *E. coli*) and thermoduric (*Enterococcus* spp.) bacteria were detected in both milk and cream samples, while no aerobic spore forming bacteria were found. Furthermore, the presence of staphylococci was also revealed, with *S. saprophyticus* clearly recognized. Staphylococci are part of the ubiquitous aerobic mesophilic microorganisms of raw milk (Özer, 2000). These results are in line with what reported in literature for milk that did not undergo a heat treatment (Franciosi et al. 2009b)

The observed Gram-positive rods, generally associated with raw milk (Özer 2000), were mainly represented by LAB. The rod shaped LAB found in all samples included all species of dairy interest: *L. helveticus*, *L. delbrueckii* ssp. *bulgaricus* and *L. rhamnosus*/*L. casei*. Cocci LAB with technological aptitudes were also identified, in particular, *Str. thermophilus* and *Ln. mesenteroides*, both species commonly employed as starter culture (Franciosi, Settanni, Cavazza, & Poznanski, 2009b).

DGGE was also applied to identify the bacterial community in 18-month ripened cheeses (Fig. 1B). *Lactobacillus fermentum*, a species often reported as non-starter LAB in cheese (Chamba & Irlinger, 2000), was detected in all cheese core samples, while only a weak band further sequenced as belonging this species was detected in the inner crust cheese section. The same was observed for *Lc. garvieae* which is a species often isolated form Italian cheeses and whose role in cheese production has been well clarified (Fortina, Ricci, Acquati, Zeppa, Gandini, & Manachini, 2003). *Lactobacillus rhamnosus*/*Lb. casei*, as well as *Lb. helveticus* and *Lb. delbrueckii*, all species of dairy origin, were uniformly distributed among cheese matrices.. Surprisingly, *E. coli* bands were detected in cheese obtained form milk stored w/o refrigeration or at 18 °C. However, the presence of its DNA does not mean that *E. coli* was still viable, probably band detection was due to a high concentration of this species during curd formation. Another atypical species for ripened cheese, found only in the inner crust of cheese from ExA, was *Sphingomonas paucimobilis*, known as a human pathogenic bacterium. For this reason, all cheese samples used for DGGE analysis were subjected to bulk cell DNA extraction as described by Ercolini et al. (2001b). Briefly, cheese samples were subjected to a viable

count, all colonies developed on agar medium were recovered with 1 ml of Ringers solution and subjected to DGGE as above described. All bands detected from viable bacteria belonged to LAB (results not shown).

3.1.4 CONCLUSIONS

In general, cow milking is practiced twice a day on farms throughout the world. In uncontrolled thermal regimes, milk may be kept at temperatures closed to chilling in cold climates, or at very high temperatures in hot climates. Depending on the environmental conditions, milk must be protected from freezing or, on the contrary, from extensive microbial proliferation at high temperatures. Milk storage time, also play a defining role on the number and type of microrganisms that may develop. When temperature and storage time of milk are not maintained under control, microbial concentration of milk is unpredictable. Thus, these two parameters assume a basic importance in the quality of milk.

Different conclusions derived from the present work: (i) some important information have been added to the microbiological knowledge of the creaming process obtained by standing of milk; (ii) low temperatures are confirmed to be useful in maintaining a high hygiene of raw milk and are important for quality of resulting cheeses; (iii) storage of milk carried out at 12°C did not negatively influence the presence of mesophylic LAB important during ripening; and (iiii) microbial culture-independent tools may be used as an alternative and not just as a complement to the culture-dependent detection to quickly compare a high number of samples (256 in this study).

Works are being prepared to isolate and characterize LAB in order to analyse their role during the proteolysis process and amino acid catabolism in Grana Trentino cheese.

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3.2. Changes in psycrotrophic microbial populations during milk creaming to produce Grana Trentino cheese

ABSTRACT
The aim of this study was to study the psycrotrophic microbiota developing during milk creaming in four days of Grana Trentino cheese-making. 138 isolates from raw whole milk, cream and skim milk samples were screened by Randomly amplified polymorphic DNA PCR biotyping and representative strains of each biotype were characterised by partial 16S rRNA gene sequencing and enzymatic activity. *Pseudomonadaceae* were commonly isolated in cream samples while *Streptococcaceae* and *Enterobacteriaceae* in milk samples. *Moraxellaceae* and *Flavobacteriaceae* were found in both cream and milk samples.
More than 80% of Psycrotrophic isolates could grow at 37°C. All *Flavobacteriaceae* and half of *Pseudomonadaceae* biotypes displayed proteolytic activity on milk agar even at low temperatures such as 10°C. All *Streptococcaceae* and some of *Enterobacteriaceae* displayed acidifying activity and almost all *Acinetobacter* spp. displayed lypolitic activity.
Even if psycrotrophic bacteria is not the dominant microbial group in raw milk, their total number increases during creaming and becomes one of the most present group together with Lactic Acid Bacteria. Their enzymatic activities may be key players in determining milk quality for cheese making.

Keywords: Psycrotrophic microflora, creaming, Grana cheese,

3.2.1. INTRODUCTION

Grana Trentino is a hard cooked cheese belonging to Grana Padano consortium, that undergoes a ripening period of almost 2 years. It is produced in Trentino region (Alpine area located in the North of Italy) from raw cow's milk (DPR n. 1269, 30 october 1955). The only allowed additives are calf rennet and natural whey starter cultures. The bulk milk derives from two different milkings mixed approximately in 1:1 ratio. The first is carried to dairy factory the evening before cheesemaking and is put in large shallow tanks for 9-11 hours during which the creaming takes place. After this overnight maturation, the partially skim milk lays under the cream in the tank and is manually drained from the cream fat and put in a vat where is added to an about equal amount of whole milk coming from the morning milking. Cream is used for butter production. During overnight maturation the milk temperature is kept between 15 and 17°C to avoid the growth of undesired microorganisms, and spontaneous creaming occurs. This process is crucial to optimize the fat to casein ratio of milk (Mucchetti and Neviani, 2006a), but has also important microbiological consequences because some microorganisms present in raw milk are removed with the fat globules. The recent development of molecular community fingerprinting methods provided a view of complex microbial ecosystems such as raw milk (Lafarge et al. 2004; Ogier et al. 2004). Raw milk contains bacteria that can have technological relevance, such as lactic acid bacteria, but even spoilage features, as psycrotrophic bacteria (Desmasures et al. 1997; Cousin, 1982). These latter, in particular, can grow and spoil the milk or the cream during overnight maturation, and also affect cheese ripening trough the production of extracellular enzymes, mainly proteases and lipases, which are remarkably heat stable (Cousin 1982; Koka et al. 1991; Lopez-Fandino et al. 1993, Wiedmann et al. 2000). The lipases cause breakdown of fat globules in cream (Craven et al. 1992), consequently lowering the yield in hard cheeses (Chen et al. 2003, Sorough et al 1997), and can also cause the appearance of off-flavours. As proteases can break casein micelles, the final coagulum can be less compact and more fragile (Manfredini & Massari 1989).

The dynamics of microbial population during hard cheeses maturation and ripening are well known (Gatti et al. 2008, Dolci et al. 2008, Randazzo et al. 2006) but the knowledge of the evolution and characterization of psychrotrophic microbiota in milk and cream is limited, although these bacteria play a leading role in the spoilage of refrigerated milk and cheese (Lafarge et al. 2004). Some studies dealing with the creaming process are available, but they are mostly focused on the physico-chemical and rheological properties of milk (Ma and Barbano 2000) or on different maturation conditions (Malacarne et al. 2008). The development of microbial population during spontaneous creaming was studied only *in vitro* (Dellaglio et al. 1969), while the behaviour of inoculated pathogens strains was studied in different creaming conditions (Carminati et al. 2008). The microbial dynamics during creaming in real system was studied by Panari et al. (2007) which considered only the plate counts.

In this study, we monitored the dynamics of psychrotrophic microbiota before and after the skimming of raw whole milk collected in the

evening to cream and skim milk used to produce Grana Trentino cheese in a dairy located in Non valley (Trento, Italy). A total of 138 bacteria were isolated in different steps of milk maturation during 12 different cheese making processes. In order to understand the evolution of psychrotrophic population during creaming, the isolates were characterized by genotypic techniques (RAPD-PCR and sequencing); their spoilage activity was evaluated by testing the presence of lipolytic and proteolytic traits and their lechitinase activity.

3.2.2. MATERIALS AND METHODS

3.2.1 Milk supply and sample collection

All the whole milk (WM), skim milk (SM) and cream (C) samples were collected in a cheese factory producing *Trentingrana* located in Non valley (Trento, Italy). Milk samples were collected in three consecutive days was sampled for four weeks. The WM came always from the same three farms, that were equipped with automated milking facilities. The total WM volume was measured by means of a volumetric pump placed on the tank used to deliver milk to the cheese factory. The milk and cream densities were measured with a volumetric calibrated flask. Samples for microbiological analysis were stored in liquid nitrogen immediately after collection and kept at – 80°C until analyzed.

3.2.2 Milk and cream composition analysis

The following analyses were carried out on all milk and cream samples: pH values using a Portamess® 911 (X) pHmeter (Knick GmbH & Co., Berlin, Germany) connected to a Cheesetrode (Hamilton Co., Reno, NV, USA) electrode after bulk mixing; fat content by infrared analysis (Biggs, 1978) of a bulk sample with a Milko-Scan 134 A/B (Foss Electric, DK-3400 Hillerod, Denmark).

3.2.3 Enumeration of microorganisms and statistical analysis

The first decimal dilution of cream samples was homogenized for 2 min at 260 rpm in a Stomacher Lab Blender 400 BA 7021 (Seward Medical, Worthing, UK) to allow fat globules disruption. Samples were diluted in peptone water (0.1 % mycological peptone, Oxoid, Basingstoke, UK) when necessary and plated in duplicate on following media, all purchased by Oxoid: Plate Count Agar (PCA) incubated at 30°C for 48 h for aerobic mesophilic bacteria counts, and at 7°C for 7 days for aerobic psychrotrophic bacteria counts, De Man Rogosa and Sharp (MRS) Agar incubated at 30°C for 48 h anaerobically for enumeration of lactic acid bacteria (LAB), Pseudomonas Agar Base with CFC supplement incubated at 30°C for 48 h for *Pseudomonadaceae* counts and VRBGA incubated at 37 °C for 24h for *Enterobacteriaceae* counts.

Colonies were randomly picked from psychrotrophic countable PCA plates, streaked onto PCA (incubated at 30°C for 48 h) and the procedure was repeated four times in order to obtain pure cultures. After purification, the isolates were subcultured in liquid Luria-Bertani (LB) medium (Oxoid). On average, 10 colonies per plate and per sample were collected. Gram

reaction was performed using Gregersen's KOH method (Gregersen, 1978), catalase reactions by transferring fresh colonies from an agar medium to a glass slide and adding 3 % H_2O_2, and cytochrome oxidase with Oxidase test (Oxoid). Gram negative, catalase positive and oxidase negative isolates were plated on Violet Red Bile Glucose Agar (VRBGA, Oxoid) and incubated at 37°C for 24–48 h to check their belonging to *Enterobacteriaceae* Family; Gram negative, catalase positive and oxidase positive isolates were plated onto Pseudomonas Agar Base with CFC supplement (Oxoid) to check their belonging to the *Pseudomonas* spp.

Bacterial isolates were stored at -80°C in LB medium containing 20% glycerol.

3.2.4 DNA Extraction and RAPD PCR amplification

Bacterial DNA was extracted from all the isolates after overnight growth in LB Broth at 30°C. DNA from Gram positive bacteria was extracted using the InstaGene Matrix (Bio-Rad, Milan, Italy) according to the manufacturer's instructions, while from Gram negative bacteria DNA was extracted as follows: after centrifugation (10,000 rpm, 10 min.) of the liquide colture, the pellet was suspended in 100 µl of *lysis solution* (0.05 M NaOH and 0,25% sodium dodecyl sulphate [SDS]) and incubated for 15 min at 100°C. The suspension was centrifuged for 1 min at 12,000 g. Five microliters of the twenty-fold diluted suspensions were used in each reaction. All DNA suspensions were stored at -20°C until analysis.

In order to reduce the number of isolates to be identified by partial 16S rRNA gene sequencing, the extracted DNA was subjected to Randomly Amplified Polymorphic DNA–Polymerase Chain Reaction (RAPD–PCR) for biotype screening among the isolates. The partial 16S rRNA gene of different biotypes was sequenced for taxonomic identification.

The amplification reactions were accomplished in a final volume of 25 µl containing 1× buffer 2.5 mM $MgCl_2$, 200 µM deoxyribonucleotide triphosphates (dNTP), 2 µM of each primer, 0,6 U Taq DNA polymerase (BIOTAQ™ DNA Polymerase, Bioline Ltd, London, UK). Three primers were used in the amplification reactions: M13 (5'-GAGGGTGGCGGTTCT-3'), OPA 3 (5'-AGTAGCCCAC-3'), and OPA 9 (5'-GGGTAACGCC-3'). A negative control with sterile water was always included in PCRs. The amplifications were carried out in a PTC 100 thermal cycler (MJ Research, Watertown, MA, USA) using following protocol: 40 cycles consisting of denaturation at 94°C for 1 min, primer annealing at 45°C for 20 s, and extension at 72°C for 120 s. To complete the synthesis of all strands, the procedure was concluded with extension at 72°C for 10 min. The PCR products were separated by electrophoresis on 2,5% (wt/vol) agarose gel (Invitrogen, Carlsbad, CA, USA) and visualized by UV transillumination after staining with ethidium bromide (0,5 g/ ml). Deoxyribonucleic acid ladder 1Kb (Invitrogen) was used as a molecular size marker.

3.2.5 Genetic patterns analysis

The RAPD-PCR profiles were analyzed with the pattern analysis software package Gel Compar Version 4.1 (Applied Maths, Kortrijk, Belgium). Calculation of similarities of band profiles was based on Pearson product moment correlation coefficient. Dendrograms were obtained by

means of the Unweighted Pair Group Method using arithmetic average clustering algorithm.

The repeatability of the method was investigated on 12 different colonies. DNA was extracted from each colony in two independent assays and it was processed as described before. The 24 DNA extracted were amplified with the three primers generating 72 PCR patterns that were analysed in a single dendrogram using the Unweighted Pair Group Method using arithmetic average clustering algorithm, repeatability was determined as the value of similarity between the two replicates with the three different primers (Foschino et al. 2008). Subsequently, the 12 measures were averaged, obtaining the mean repeatability, used as a threshold to define identical genotypes (Foschino et al. 2008).

The discriminatory power of the typing technique combining the fingerprinting obtained with the three primers was evaluated by calculating the Simpson's discriminatory index (Hunter and Gaston 1988).

3.2.6 Identification of isolates using 16S rRNA gene sequencing

A partial 16S rRNA gene of each biotype was amplified by PCR using universal small-subunit rRNA primers corresponding to nucleotide positions 8–17 (5'-AGA GTT TGA TCC TGG CTC AG-3'; Weisburg et al. 1991) and 515–532 (5'- AAG GAG GTG ATC CAG CC-3'; Heilig et al. 2002) of *E. coli*. This fragment includes the V2-V3 region of 16S rRNA gene, commonly used in discriminating different species by DGGE (Walter et al. 2000; Heilig et al. 2002). The PCR mixture (final volume, 25 ml) contained 5 µl of a dilution of DNA template, each primer at a concentration of 0,3 µM, each deoxynucleoside triphosphate at a concentration of 0,05 mM, 2,5 mM MgCl2, 2,5 µl of 10 X PCR buffer and 1,25 U of Taq polymerase (Bioline). PCR conditions consisted of 30 cycles (1 min at 94°C, 1 min at 56°C, 2 min at 72°C). Both RAPD and 16S rRNA PCR products were resolved by electrophoresis on 2,5% (wt/vol) agarose gel (Invitrogen, Carlsbad, CA, USA) and visualized by UV transillumination after staining with ethidium bromide (0,5 g/ ml). Deoxyribonucleic acid ladder 1Kb (Invitrogen) was used as a molecular size marker.

The PCR product (ca. 30 ng) of each strain was purified with Exo-SAP-IT kit (USB Co., Cleveland, OH) and sequenced through the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) as reported by the manufacturer in a ABI PRISM 3100 sequencer (Applied Biosystems). Sequencing was performed for all isolates by using fD1primer (yielding a length of ca. 500 bp). For identification of closest relatives, newly determined sequences were compared to those available in the GenBank (www.ncbi.nlm.nih.gov) databases by means of the standard nucleotide-nucleotide BLAST program (BLASTN; www.ncbi.nlm.nih.gov). The sequences with a homology percentage of 97% or greater were considered to belong to the same species (Palys et al. 1997).

3.2.7 Growth, acidifying activity and production of extracellular enzymes

The temperature growth test was performed by streaking each isolate onto PCA plates that were incubated at 10, 20, 30 and 37°C, respectively. Growth was considered positive if colony diameter was bigger than 1mm

after three days, with the exception of test at 10°C where colony were observed after 7 days.

Enzymes production was detected by agar-diffusion assays, according to the type of enzymatic activity to be detected.

The production of extracellular proteolytic enzymes and acidifying activity was detected on PCA, supplemented with 1% skim milk powder (Harper et al., 1978) and 0,174 g/l bromocresolpurple (Sigma Aldrich, Saint Louis, Missouri, USA). The plates were incubated at 30°C for 72 h at 20°C, 15°C and 10°C: the presence of clear zones around the colonies was considered an indication of proteolysis; the presence of yellow areas around the colonies was considered an indication of acidification.

The production of extracellular lipases was evalueted on Trybutirin Agar (Oxoid), containing 0,5 % (w/v) peptone; 0,3 % (w/v) yeast extract; 0,1 % and (v/v) tributyrin. The plates were incubated at 30°C for 72 h; the presence of clear zones around the colony was considered an indication of lipolytic activity.

The production of extracellular phospholipases (lecithinases) was evalueted on PCA supplemented with 10% egg yolk emulsion (Oxoid), as described by Dogan and Boor (2003). The production of an opaque ring, surrounding lecithinase positive colonies, was recorded after a 72 h incubation at 30°C.

3.2.8 Statistical analysis

One way analysis of variance (ANOVA) was conducted on the viable numbers of the different microbial groups after logarithmic transformation of data by using as data analysis software system STATISTICA, version 8.0. StatSoft, Inc. (2008). www.statsoft.com.

3.3 RESULTS AND DISCUSSION

3.3.1 Microbial counts and isolation

Samples were collected in 12 cheesemaking days in may 2008. About 1000 L of milk are required for each Trentingrana cheesemaking, by which two cheese wheels (about 40 kg each one) are obtained. Approximately the half of this milk is delivered to the cheese factory in the evening and kept for overnight skimming at 15-17°C. The mean amount of milk delivered in the evening was 487.8 (± 20.6) L. After maturation, the cream removed was 38.5 (± 2.7) L, while the partially SM was 450.4 (± 19.4) L.

In Table 1 are reported the results of different microbiological counts of the WM delivered to the dairy in the evening before skimming, of the SM and of the C after maturation. pH values and the fat content are also reported.

The total mesophilic bacteria and LAB counts did not significantly differ in WM and SM samples (count values ranged between 4.4 and 4.9 log cfu/ml). The same counts were considerably higher in cream, where they reached concentrations higher than 7.0 log cfu/ml. Counts of mesophilic bacteria in raw whole milk before creaming were always lower than 10^5 log cfu/ml, reflecting the good microbiological quality of the milk (Barbano et

al. 2006). Raw milks from the same geographical origin showed always similar microbial counts, as already observed by Franciosi et al. (2009).

Tab.1: Variation of the microbial counts (log cfu/ml), pH and fat amount (g/100 g) in evening raw whole milk, cream and in partially skim milk after spontaneous overnight creaming. Each value is the average (mean ± standard deviation) of 12 data from 12 different days. Significant differences were determined by ANOVA test

	Whole milk (log cfu/ml)	*Cream (log cfu/ml)*	*Skim milk (log cfu/ml)*
Total mesophilic Bacteria	4.9 ± 0.5^{a}	7.1 ± 0.5^{b}	4.4 ± 0.4^{a}
Lactic Acid Bacteria	4.9 ± 0.5^{a}	7.5 ± 0.6^{b}	4.6 ± 0.4^{a}
Psycrotrophic Bacteria	3.9 ± 0.7^{a}	6.7 ± 0.5^{b}	4.3 ± 0.8^{a}
Pseudomonadaceae	3.9 ± 0.8^{a}	5.2 ± 0.6^{b}	2.5 ± 0.3^{c}
Enterobacteriaceae	3.1 ± 1.1^{a}	$4{,}8 \pm 1.2^{b}$	2.3 ± 0.9^{a}
pH	6.68 ± 0.04^{a}	6.71 ± 0.03^{a}	6.71 ± 0.03^{a}
Fat (g / 100g)	3.66 ± 0.08^{a}	28.83 ± 0.76^{b}	1.59 ± 0.02^{c}

* A different apical letter indicates the mean value is significant different ($P < 0.05$) from the ones recorded in the others milk fractions

Total psychrotrophic bacteria were lower (approx. 1 log) than total mesophilic bacteria and LAB in the whole bulk milk. This difference between psychrotrophic and total bacteria counts was lower in SM (6.7 and 7.1 log cfu/mL respectively) and C (4.3 and 4.4 log cfu/ml respectively). The counts on PBA and VRBGA in whole evening milk were similar (3.9 and 3.6 log cfu/ml respectively) and decreased significantly in SM. By contrast, these counts increased in cream up to 5.2 and 4.8 log cfu/ml respectively. The pH value was 6.7 and did not significantly change during maturation. More than half of the fat initially present in milk (3.66%) was removed with creaming as expected.

Considering the mean volumes of cream and partially SM and their microbial counts, it was possible to calculate the total number of bacteria cells in each fraction after maturation. This calculation indicated that 90 to 96 % of the total raw milk bacteria were concentrated in the cream, which is less than 8 % of the initial volume. The composition of the microbiota in the WM before creaming, in the SM and in the C indicated also that some psychrotrophic bacteria are able to grow during skimming. In facts, in the whole milk, the psychrotrophic bacteria counts were lower than total mesophilic bacteria and LAB, while were counted at similar values of these latter two microbial populations after maturation, both in SM and cream.

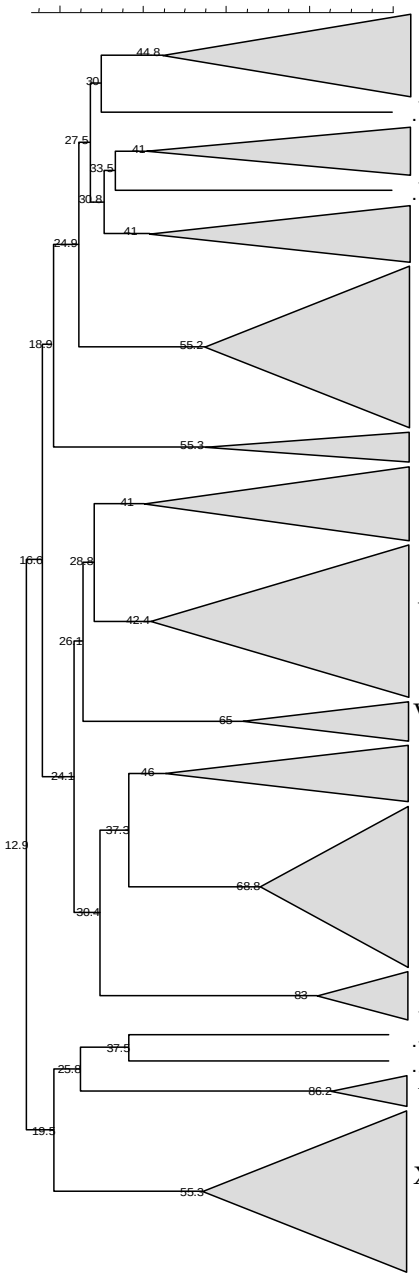
Colonies from plates used for total psychrotrophic counts were randomly picked up and purified for further analyses. A total of 138 isolates

were obtained, 50 from the whole evening milk before maturation, 45 from cream and 43 from partially SM. Among the isolates, 24 were Gram positive, catalase negative and oxidase negative, all cocci-shaped. The remaining 114 Gram negative isolates were catalase positive and among them 15 were oxidase positive and 42 cocci-shaped.

3.3.2. Characterisations by RAPD-PCR and identification of biotypes

The mean repeatability of RAPD-PCR method was estimated to be 93.9% (the lowest being 83.6% and the highest 98.2%), with a discriminatory power of 0.98 (Hunter and Gaston 1988). The technique allowed the typing of all the isolates (100% typeability). The three primers used for genetic RAPD typing of the isolates allowed a optimal insight into strain relatedness, due to their high discriminatory power. This protocol permitted the classification of the 138 isolates in 70 different biotypes, distributed in 13 distinct clusters at a similarity level of at least 40%; only four isolates remained unclustered (Fig. 1). When banding patterns had a level of similarity higher than 83.6% (the lowest value of repeatability) the isolates were considered as a single biotype.

The largest clusters (cluster IV, VII, X and XIII) contained more than 50% of the psychrotrophic biotypes. The biotypes of cluster IV and VII belonged to *Enterobacteriaceae* and were identified as *Enterobacter* sp. (cluster IV), *Citrobacter freundii* and *Hafnia alvei* (cluster VII); the biotypes in cluster XIII were identified as *Chryseobacterium* sp. (*Flavobacteriaceae*) while the biotypes in cluster X belonged to *Moraxellaceae* and were identified as *Acinetobacter* sp.. *Acinetobacter* spp. were the most frequently isolated bacteria and were grouped also in cluster VI (9 biotypes), VIII (5 biotypes) and IX (7 biotypes), while one biotype was unclustered. *Pseudomonadaceae* were found in clusters II (6 biotypes of *P. fluorescens*), III (4 biotypes of *P. fragi* and 3 of *P. syringae*) and one biotype of *P. putida* was unclustered. All the psychrotrophic *Pseudomonas* biotypes were isolated only from C samples. One biotype of *Flavobacter* sp. was also unclustered, as well as one biotype of *Aeromonas sp.* found in milk before maturation. The 24 Gram positive biotypes belonged all to *Streptococcaceae* and were grouped in clusters I (6 biotypes belonging to the species *Leuconostoc lactis* and 4 to *Lactococcus lactis*), V (4 *Streptococcus parauberis* biotypes), XI (6 *Lactococcus raffinolactis* biotypes) and XII (4 *Lactococcus lactis* biotypes). Clusters XI and XII grouped *Streptococcaceae* biotypes found out only in SM samples. All others clusters were composed of biotypes from different samples.



Cluster n°	Number of cluster isolates	Number of cluster biotypes	Family
I	10	4 (SM); 3 (WM); 1 (C)	*Streptococcaceae*
UC	1	1 (WM)	*Flavobacteriaceae*
II	6	3 (C)	*Pseudomonadaceae*
UC	1	1 (C)	*Pseudomonadaceae*
III	7	5 (C)	*Pseudomonadaceae*
IV	19	6 (WM); 3 (SM)	*Enterobacteriaceae*
V	4	1 (C), 1 (EM)	*Streptococcaceae*
VI	9	4 (SM); 3 (C)	*Moraxellaceae*
VII	18	6 (WM); 1 (SM)	*Enterobacteriaceae*
VIII	5	3 (C); 1 (SM)	*Moraxellaceae*
IX	7	3 (SM); 2 (WM); 1 (C)	*Moraxellaceae*
X	20	1 (SM); 2 (C); 3 (WM)	*Moraxellaceae*
XI	6	2 (SM)	*Streptococcaceae*
UC	1	1 (WM)	*Aeromonadaceae*
UC	1	1 (C)	*Moraxellaceae*
XII	4	1 (SM)	*Streptococcaceae*
XIII	19	8 (WM), 2 (C)	*Flavobacteriaceae*

Fig 1. Dendrogram established by the Bionumerics software package using the Unweighted Pair Group Method with arithmetic average clustering algorithm on the basis of the random-amplified polymorphic DNA (RAPD) profiles of 138 isolated obtained with primers M13, OPA3 and OPA9.
The numbers on the clusters branches of the dendrogram indicate the percentage similarities as determined by the Pearson product-moment correlation coefficient (r). On the right side of each cluster are indicated: the cluster number in latin number, the number of isolates belonging to the cluster, the number of biotypes and wherever sample they were found: WM

(whole milk), C (Cream) or SM (Skim milk), and the Family to whom the cluster isolates belongs. UnClustered strains are also indicated (UC).

Tab.2: Closest relative species, identity percentage, closest relative accession number and phenotypical characteristics of the 70 biotypes identified by sequencing chosen among the 138 isolated from whole milk (WM), cream (C) and skim milk (SM) samples.

Biotype (sample)	Number of isolates	Closest relative species	Identity %	Closest relative Accession number	Growth at 37°C	Proteolytic activity.	Acid.	Lyp.	Lec.
Streptococcaceae Family									
1 SM	1	*Lactococcus lactis cremoris*	99	FJ749403	+	-	+	-	-
2 SM	1	*Lactococcus lactis cremoris*	99	FJ749403	+	-	+	-	-
3 SM	1	*Leuconostoc lactis*	99	GU049408	+	-	+	+	-
4 SM	2	*Lactococcus lactis ssp cremoris*	99	FJ749403	+	-	+	+	-
5 WM	1	*Lactococcus lactis ssp cremoris*	99	FJ749403	+	-	+	-	-
6 WM	1	*Lactococcus lactis ssp lactis*	99	FJ915749	+	-	+	-	-
7 WM	2	*Lactococcus lactis ssp lactis*	100	FJ915749	+	-	+	+	-
8 C	1	*Lactococcus lactis ssp cremoris*	100	FJ749403	+	-	+	-	-
9 WM	2	*Streptococcus parauberis*	100	AB446393	+	-	+	-	-
10 C	2	*Streptococcus parauberis*	100	AB446393	+	-	+	-	-
11 SM	3	*Lactococcus raffinolactis*	100	EF694030	+	-	+	+	-
12 SM	3	*Lactococcus raffinolactis*	99	EU091467	+	-	+	+	-
13 SM	4	*Lecuconosctoc lactis*	100	EU419611	+	-	+	-	-
TOT	24				24	0	24	11	0
Flavobacteriaceae Family									
14 WM	1	*Flavobacterium sp.*	98	AF493659	+	+ (30, 20, 15°C)	-	+	-
15 WM	2	*Chryseobacterium sp.*	99	AY468483	+	+ (30, 20, 15, °C)	-	+	-
16 WM	1	*Chryseobacterium sp.*	99	AY468483	-	+ (30, 20, 15, 10°C)	-	+	-
17 WM	1	*Chryseobacterium sp.*	99	AY468483	-	+ (30, 20, 15, 10°C)	-	+	-
18 WM	1	*Chryseobacterium sp.*	99	AY468483	-	+ (30, 20, 15, 10°C)	-	-	-
19 C	7	*Chryseobacterium sp.*	99	AY468483	+	+ (30, 20, 15, 10°C)	-	-	-
19 WM	2	*Chryseobacterium sp.*	99	AY468483	+	+ (30, 20, 15, 10°C)	-	-	-
20 C	2	*Chryseobacterium sp.*	99	AY468483	-	+ (30, 20, 15, 10°C)	-	+	-
21 WM	1	*Chryseobacterium sp.*	99	AY468483	+	+ (30, 20, 15°C)	+	+	-

22 WM	1	*Chryseobacterium sp.*	99	AY468483	+	+ (30, 20, 15°C)	+	+	-
23 WM	1	*Chryseobacterium sp.*	99	AY468483	-	+ (30, 20, 15°C)	+	+	-
TOT	20				14	20	3	10	0
Pseudomonadaceae Family									
24 C	2	*Pseudomonas fluorescens*	100	EU862080	-	+ (30, 20, 15°C)	-	+	-
25 C	2	*Pseudomonas fluorescens*	100	EU862080	-	+ (30, 20, 15°C)	-	+	-
26 C	2	*Pseudomonas fluorescens*	100	EU862080	-	+ (30, 20, 15°C)	-	+	-
27 C	1	*Pseudomonas putida*	100	FJ950573	+	-	-	-	-
28 C	2	*Pseudomonas fragi*	100	AM933514	+	-	-	-	-
29 C	2	*Pseudomonas fragi*	100	AM933514	+	-	-	-	-
30 C	1	*Pseudomonas syringae*	100	DQ017590	-	-	-	+	-
31 C	1	*Pseudomonas syringae*	100	DQ017590	-	-	-	+	-
32 C	1	*Pseudomonas syringae*	100	AJ576247	+	+ (30, 20°C)	-	+	-
33 WM	1	*Aeromonas sp.*	99	AB472989	+	-	+	+	+
TOT	15				7	7	1	10	1
Enterobacteriaceae Family									
34 WM	1	*Enterobacter sp.*	99	FJ587226	+	-	+	-	-
35 WM	1	*Enterobacter sp.*	99	FJ587226	+	-	+	-	-
36 WM	1	*Enterobacter sp.*	99	FJ587226	+	-	+	-	-
37 WM	4	*Enterobacter sp.*	100	FJ897481	-	-	-	-	-
37 SM	5	*Enterobacter sp.*	99	FJ587226	-	-	-	-	-
38 WM	3	*Enterobacter sp.*	100	FJ897481	+	-	-	-	-
39 WM	1	*Enterobacter sp.*	100	AB428448	-	-	-	-	-
40 SM	2	*Enterobacter sp.*	99	GQ891669	+	-	-	-	-
41 SM	1	*Enterobacter sp.*	100	GQ891669	+	-	-	-	-
42 WM	2	*Citrobacter freundii*	99	GU003847	+	-	-	-	-
43 WM	1	*Citrobacter freundii*	99	GU003847	+	-	-	-	-
44 WM	1	*Citrobacter freundii*	100	GU003847	+	-	-	-	-
45 WM	4	*Hafnia alvei*	97	AB244473	+	-	-	-	-
46 WM	1	*Hafnia alvei*	99	AB435609	+	-	+	-	-

47 SM	5	*Hafnia alvei*	99	FM179944	+	-	+	-	-
48 WM	4	*Hafnia alvei*	99	FM179944	+	-	+	-	-
TOT	37				27	0	13	0	0
Moraxellaceae Family									
49 C	1	*Acinetobacter sp*	100	EU375374	-	-	-	-	-
50 SM	1	*Acinetobacter sp.*	96	GQ377756	+	-	-	+	+
51 C	1	*Acinetobacter johnsonii*	100	GQ169068	+	-	-	+	+
52 SM	1	*Acinetobacter johnsonii*	100	EF204269	+	-	-	+	+
53 SM	1	*Acinetobacter sp*	100	GQ369439	+	-	-	+	-
54 C	1	*Acinetobacter sp*	100	GQ369439	+	-	-	+	-
55 C	1	*Acinetobacter johnsonii*	97	EF204269	+	-	+	+	-
56 SM	3	*Acinetobacter johnsonii*	99	FJ263917	+	-	-	+	-
57 C	1	*Acinetobacter sp*	100	EU375374	-	-	-	-	-
58 C	2	*Acinetobacter sp*	100	EU375374	-	-	-	+	-
59 SM	1	*Acinetobacter sp*	100	EU375374	-	-	-	+	-
60 SM	1	*Acinetobacter sp*	99	GQ497289	+	-	-	+	+
61 SM	1	*Acinetobacter sp*	99	AM412163	+	-	-	+	-
62 WM	1	*Acinetobacter sp*	100	AY663435	+	-	-	+	-
63 WM	1	*Acinetobacter sp*	100	AY663435	+	-	-	+	-
64 C	2	*Acinetobacter sp*	99	GU168575	+	-	-	+	-
65 SM	1	*Acinetobacter johnsonii*	100	GQ169068	+	-	-	+	-
66 C	7	*Acinetobacter sp*	100	FN395271	+	-	-	+	-
67 WM	5	*Acinetobacter sp*	99	EU337120	+	-	-	+	-
67 SM	5	*Acinetobacter sp*	99	EU337120	+	-	-	+	-
68 C	1	*Acinetobacter sp*	99	EU337120	+	-	-	+	-
69 WM	2	*Acinetobacter sp*	100	EU337120	+	-	-	+	-
70 C	1	*Acinetobacter sp.*	99	GQ865637	+	-	-	+	-
TOT	42				37	0	1	40	4
TOT	**138**				**109**	**27**	**42**	**71**	**5**

The 70 different biotypes were characterised and identified, by partial 16S rRNA gene sequencing, For each biotype the number and the origin of the isolates, the identification (with the percentage of identity) and the relative accession number are reported in Table 2.

Almost all the 70 biotypes were found only in one of the three analysed matrices (WM, C and SM), with the exception of three biotypes: the 19 identified as *Chriseobacterium* sp. and found out both in WM (2 isolates) and C samples (7 isolates); the 37 identified as *Enterobacter* sp. and found out both in WM (4 isolates) and SM samples (5 isolates) and the 67 identified as *Acinetobacter* sp. and found out both in WM (5 isolates) and SM samples (5 isolates). The biotypes of psycrotrophic population isolated from WM, C and SM samples after skimming belonged to *Pseudomonadaceae, Enterobacteriaceae, Moraxellaceae* and *Streptococcaceae* in the percentage reported in Figure 2 (the only biotype belonging to *Aeromonadaceae* was not considered). These are Families frequently isolated from milk and dairy products (Cousin et al. 1982; Holm et al. 2004; Lafarge et al. 2004; Munsch-Alatossava et al. 2006; Arcuri et al. 2008), however interesting behaviours can be evidenced.

Fig. 2. Percentage of psycrotrophic bacteria isolated in evening whole milk; in the same milk after spontaneous creaming (skim milk) and in cream separated by skim milk after o/n skimming based on partial 16S gene sequencing identification.

Psychrotrophic *Pseudomonadaceae* were never isolated from milk samples (whole evening and SM); by contrast, they were an important

component of cream microbial population (about 33% of the isolates from this source).

Enterobacteriaceae was the Family most frequently isolated in WMpsycrotrophic population (about 50%); members of this Family were isolated even in SM (slightly lower than 30%) but never from cream samples. In spite of the fact that many *Enterobacteriaceae* can have health implications, the psychrotrophic biotypes isolated from milk and cream used for Grana Trentino production belonged to the species *Citrobacter freundii* and *Hafnia alvei*, that are not known to be enteropathogenic (Olsvik and Kapperud, 1982). The presence of *Enterobacteriaceae* is very common in raw milk, and *Hafnia alvei* is one of the most frequently isolated species in these environments (Tornadijo et al. 2001, Martins et al. 2006; Kagkli et al. 2007; Ercolini et al. 2009). The origin of psycrotrophic *Enterobacteriaceae* in milk is not necessary linked to a faecal pollution, but could be related to the water at the farm as Kagkli et al (2007) showed in a recent study. This microbial group is of technological interest, because some psychrotrophic *Enterobacteriaceae* species can produce proteolytic and lipolytic enzymes that negatively affect the organoleptic characteristics of cheeses. Nevertheless, the enzymatic activity of these organisms seems to be strain specific and highly depending on milk storage conditions (Santos et al. 1996). In addition, independently on their eventual pathogenicity, members of this group can cause early blowing of cheese (Mucchetti and Neviani, 2006b).

Psychrotrophic *Streptococcaceae* were isolated rarely from WM and C samples, in which they were 9 and 7% respectively, while they were the dominant group (about 38% of isolates) in the SM immediately before cheese-making. This may have a technological relevance because milk for Grana Trentino cheese-making is not subjected to any thermal treatment: the curd is cooked to a temperature of 55°C which is not for these LAB; in this way they may contribute, at some extent, to the biochemical processes characterizing the cheese ripening carried out at a temperature of about 18°C.

Flavobacteriaceae composed about 21% of the isolates of WM and were found in C at about in the same concentration (24%) but were never isolated from SM. Nine of the 10 biotypes of *Flavobacteriaceae* were identified as *Chryseobacterium* spp. Among *Flavobacteriaceae*, species belonging to the genus *Chryseobacterium* have been frequently isolated from different food products (Hantsis-Zacharov el al 2008), where they may cause defects or alterations. *Chryseobacterium* may occur also in milk (Hantsis-Zacharov et al. 2007; Giannino et al 2009) New species of *Chryseobacterium* were recently isolated and characterized from raw milk (Hugo et al. 2003).

The frequency of isolation of *Acinetobacter* sp. (*Moraxellaceae*) in WM was 19% and was lower than in SM (33%) and well lower than in C samples, where *Acinetobacter* was the genus most frequently isolated (40%). *Acinetobacter* is an ubiquitarious microorganism common in the psychrotrophic microbiota of many food. Together with *Pseudomonas* spp., it was the genus most frequently isolated from Turkish raw milk (Uraz and Çitak 1998), and, according to Arcuri et al. (2008), is one of the dominant components of the microbiota of refrigerated raw milk.

This different microbial distribution in milk and cream samples could be ascribed to hydrophilic or hydrophobic properties of cell surface: Kiely et al. (1997) recognised that classified two strains of *Brevibacterium linens* had hydrophilic surface, and the adsorption of both of them onto an octyl ligand in hydrophobic interaction chromatography appeared to be influenced by pH and ionic strength of the medium. No data for adsorption onto biological materials or to differences in adsorption between various LAB species, was included. However, Ly et al. (2006) showed important differences in the cell surfaces characteristics among the strains of a same species (*Lactococcus lactis* biovar. *dyacetilactis*) showing that even if belonging to the same species, they may have different affinity and adhesion to cream globules.

3.3.3. Phenotipic characterization of biotypes

All the 70 biotypes having different RAPD–PCR profiles were further studied and subjected to phenotypical tests. In fact, psychrotrophs and their extracellular enzymes can play a major role in the spoilage of refrigerated milk and milk products (Sorhoug and Stepaniak, 1997). For this reason, the biotypes were characterized for their ability to growth at different temperatures and to produce extracellular enzymes.

All the biotypes, isolated from plates used for psychrotrophic counts, were obviously able to grow at 10 and 20 °C and, similarly, all the tested biotypes grew at 30°C (data not shown) while 16 were not able to grow at 37 °C (5 *Flavobacteriaceae,* 5 *Pseudomonadaceae,* 2 *Enterobacteriaceae* and 4 *Acinetobacter sp.*) (Table 2).

None of the 24 Gram positive biotypes (*Streptococcaceae*) showed detectable proteolytic activity at 10, 15, 20 and 30°C, and neither psychrotrophic *Enterobacteriaceae* showed it. The highest proteolytic activity was found in some *Pseudomonadaceae* and *Flavobacteriaceae* biotypes: all the biotypes belonging to the latter Family displayed this ability at 15, 20 and 30°C while six *Chryseobacterium* sp. biotypes were proteolytic even at 10°C. The proteolytic ability of psychrotrophic *Pseudomonadaceae* (10 biotypes) was detected in the 3 *P. fluorescens* biotypes (at 15, 20 and 30°C) and in a biotype of *P. syringae* (at 20 and 30°C) and seemed less diffused than that observed in similar matrices (Arcuri et al. 2008). The absence of this enzymatic activity is common among lactic cocci, while it has been associated with some biotype of *Chryseobacterium* spp. (Arcuri et al. 2008; Hantsis-Zacharov et al. 2008) and *Acinetobacter* spp. (Arcuri et al. 2008; Hantsis-Zacharov and Halpern 2007). By contrast none of the *Moraxellaceae* isolated from milk for Grana trentino production exhibited any proteolytic activity.

The ability to acidify the medium was found in all the *Streptococcaceae* biotypes and also widely diffused among *Enterobacteriaceae* (6 biotypes out of 16) and, to a lesser extent, *Flavobacteriaceae* (3 out of 10), while only one biotype of *Moraxellaceae* and no *Pseudomonadaceae* biotype showed this ability. Also the only one *Aeromonas sp* isolated produced acids.

Lipolytic activity was detected in almost all *Moraxellaceae* (40 out of 42 isolates from that Family) and in high percentage among *Pseudomonadaceae* (10 out of 15) and *Flavobacteriaceae* (10 out of the 20)

isolates, as well as in the only one isolate of *Aeromonas hydrophila*. Also 5 of the *Streptococcaceae* biotypes (about 45% of all *Streptococcaceae* isolates) showed lipolytic activity while this feature was never detected among *Enterobacteriaceae*. The prevalence of lipolytic *Pseudomonas* strain in refrigerated raw milk is quite common (Craven and Macauley 1992). All the biotypes of *P. syringae* and *P. fluorescens* were lipolytic under the studied conditions. The lipolytic activity found among the *Chrysobacterium* spp. isolated was already described in several species of this genus (Hantsis-Zacharov et al. 2008; Arcuri et al. 2008), and among *Acinetobacter* spp. (Arcuri et al. 2008). The characteristics of their lipases has been also reviewed by Snellman and Colwell (2004). On the contrary, the biotypes belonging to *Enterobacteriaceae* had no lipolytic activity, which was sporadically found in some strains (*Yersinia* spp., *Klebsiella* spp.) isolated from raw milk (Arcuri et al. 2008). Eleven out of the 24 psychrotropic *Streptococcaceae* biotypes isolated during these trials had a detectable lipolytic activity. Generally LAB are considered to be weakly lipolytic, with some exceptions regarding high esterase activities against short chain fatty acids (Collins et al. 2003) like tributyrin which was used for the lipolytic determination.

Extracellular lecithinases were produced only by four biotypes of *Acinetobacter* sp. and by the biotype of *Aeromonas* sp.. Lecithinasic activity was not common among the biotypes isolated in this trial, if compared to other works in which this activity was observed with higher frequency among isolates from raw milk belonging to different psycrotrophic strains (Wiedmann et al. 2000; Munsch-Alatossava and Alatossava, 2006).

3.2.4. CONCLUSIONS

Actually the practice of milk creaming before cheese-making is a fundamental step during Grana Trentino production to equilibrate the fat / casein ratio before curdling. For this cheese production no technologies to reduce the initial bacterial counts to low levels is used but refrigeration, and during creaming the milk rests for 10 hours at 15 - 18°C giving a selective advantage of growth to psycrotrophic more than to other microbial groups. This microbial group is known to produce enzymes with different properties that may have technological impact (usually spoilage action) during dairy processes. For these reason, we studied the development of psycrotrophic bacteria during creaming and how psycrotrophic communities shares in cream and skim milk after skimming. The study of psycrotrophic microbial dairy communities is limited to pasteurised milks or bulk milks (Hilton et al. 2002; Holm et al. 2004; Munsch-Alatossava et al. 2006; Sorough et al. 1997).

The psycrotrophic bacteria community in milk and cream samples was identified as belonging to *Acinetobacter* sp. and other Families (*Flavobascteriaceae*, *Pseudomonadaceae*, *Streptococcaceae* and *Enterobacteriaceae*) already isolated in similar environments, however, the enzymatic traits of the bacterial populations showed differences at biotype level among isolates identified as the same species (for example there is not a common enzymatic pattern for biotypes belonging to *P. syringae*,

Chriseobacterium sp., *Hafnia alvei* and *Acinetobacter* sp. species). This result demonstrates the importance of using a culture-dependent method that improves the study of the enzymatic activities of the isolates because studying the population species profile and biotypes is not enough since species with the same identity had different enzymatic properties.

During creaming we observed some important behaviours in the growth patterns of psycrotrophic microorganisms. The most abundant psychrotrophic group (i.e. *Moraxellaceae*) was equally distributed between cream and skim milk. By contrast, the higher concentration of *Flavobacteriaceae* and *Pseudomonadaceae* in cream than in skim milk and vice versa of *Streptococcaceae* and *Enterobacteriaceae* in the skim milk used for cheese-making can have a technological relevance, as evidenced also by the enzymatic activities of the biotypes isolated. In fact, lipolytic activity mostly associated with *Flavobascteriaceae* and *Acinetobacter* sp. found out in cream can negatively affect fat based product, such as butter, while the secretion of proteolytic enzymes by *Pseudomonadaceae* and *Flavobascteriaceae* can affect cheese ripening leading to an accelerate casein digestion and rising the fraction of soluble nitrogen in cheese. This aspect may be relevant in particular in long time ripened cheese such as Grana Trentino and other Italian hard cheeses (Grana Padano and Parmigiano Reggiano) because these enzymes have long time to be active (at least 18 - 24 months ripening time), they are active even at 18- 20 °C which are the temperatures of cheese storage during ripening and because even if the psycrotrophic flora is killed by curdle cooking at 55 °C these enzymes are heat stable and able to work during cheese ripening.

The increasing of psycrotrophic bacteria after creaming was about 3 log and probably the observed rising of mesophilic bacteria too (about 2.5 log) was to tribute to psycrotrophic bacteria whose optimum growth temperature is known to be at 30 °C. This was confirmed from the fact that all the isolated psycrotrophic were able to grow at 30°C and about 85 % were able to grow even at 37 °C. Further investigations have any way to be lead to study the equilibrium between strictly mesophilic and psycrotrophic bacterial population among isolated from mesophilic plate counts and to investigate in *in vivo* systems if the activity of heat-stable proteolytic and lipolytic enzymes originating from psychrotrophic bacteria during creaming are or not a limiting factor in maintaining the coagulation aptitude of milk and the quality of cheese products.

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3.3 Milk storage conditions influences on microbiological and chemical characteristics of *Grana Trentino* after 18 months of ripening

3.3.1 INTRODUCTION

Trentingrana is a an Italian Protected Designation of Origin (DOP) cheese, produced in the Trentino region (Alpine area located in the North part of Italy) from partially skimmed raw cow's milk and commercial rennet with the addition of whey daily produced at the dairy factory and rich in thermophilic lactic acid bacteria (LAB). It is a hard cooked cheese subjected to a long ripening period (up to 2 years), similarly to Parmigiano Reggiano and Grana cheeses.

The ripening period involves a sequential breakdown of milk components, such as fat, protein and lactose, by the enzymes of bacteria (McSweeney et al., 2000) Consequently, knowledge of bacterial microbiota involved in cheese ripening is of prime importance in predicting and determining final cheese quality. Several studies have shown the evolution and the nature of the LAB population during the manufacturing of Parmigiano and Grana cheeses, focusing the attention on the natural whey starter and the microbial population of the product during ripening. *Lactobacillus helveticus* is the dominant species in natural whey starter, but also *Lb. delbrueckii* ssp. *lactis* and *L. delbrueckii* ssp. *bulgaricus* occur (Giraffa et al. 2000; Cocconcelli et al. 1997; Gatti et al. 2004; Coppola et al. 1997). Other LAB species, such as *Lb. casei, Lb. paracasei, Lb. rhamnosus,* and some species of pediococci, become dominant from the first month of cheese ripening. In general, thermophilic lactobacilli decrease and mesophilic heterofermentative LAB become dominant after one month of ripening (Coppola et al. 2000). Coppola et al. (1997) have detected LAB throughout the entire period of ageing with the predominance of facultatively heterofermentative lactobacilli.

No study dealing with cheesemaking and ripening have been performed relative to Grana Trentino, which although belonging to Grana Padano Consortium has nevertheless a clear distinct manufacturing technology beginning from the raw stuff: the milk. In Grana Padano production, raw milk comes from Italian Friesian herds breed in Padana valley, every day more than one cheese-making cycle is allowed in the cheese factory and lysozyme is used. In Grana Trentino production, milk comes principally from Italian Brown herds which are breed in Alpes valleys without ensilage feeding and the use of lysozyme is forbidden which is known to have significantly influence on the growth not only of butyric acid bacteria but also of lactic acid bacteria and consequently it is able to affect curd acidification during the cheesemaking (Grazia e al. 1984). Regarding the milk origin, milk from Italian Brown cows is characterised by a higher casein content, better rheological properties and lower losses of fat in the cheese than Italian Friesian cows' milk (Malacarne et al. 2006). This conditions could affect environment during cheese ripening such as pH, oxidation–reduction potential, water activity, and nutrient content which are demonstrated to affect the composition of LAB microflora and its netabolic activities (Dicagno et al. 2006). Last but not least, for Grana Trentino artisanal production, only one cheese-making cycle is allowed each day in the cheese factory by a traditional artisan way (Regulation CE n. 1107/96).

In this PhD work, the milk was subjected to different storage regimes and collection technologies (no refrigeration or refrigeration at 18, 12 or

8°C). Consequently, variation in the physicochemical and microbiological composition of milk was expected to exert an influence on the final characteristics of the cheese. Milk, natural whey starter and cheeses at 18 months of ripening were sampled from three different cheese factories located in Trentino region. The cheeses were sampled in two positions: under the crust and in the core in order to arrive at a comprehensive view of the microbiology of Grana Trentino. In this work, we provided a global picture of the microbial communities of Grana Trentino cheese and moreover, the study of the cheeses after 18 ripening months allowed to evaluate the influence of different storage milk temperatures on the microbiological and physicochemical characteristics of the Grana Trentino cheese. In particular, physico-chemical characteristics, proteolysis, free amino acids (FAAs) and volatile compunds were evaluated at the end of cheese ripening and cheese microbial population have been followed in different cheese locations.

3.3.2. MATHERIAL AND METHODS

3.3.2.1 Cheesemaking

Three cheese factories were selected for their equipments and technological conditions in order to obtain suitable Trentingrana twin wheel each day. We sampled vat milk, whey and cheeses from 46 production days for about an year (from March 2007 to May 2008). Milk for cheese production was subjected to four thermal regimes after milking: it was stored without any cooling at ambient temperature, or cooled at 18, 12 or 8°C, respectively. In trials with no refrigeration or 18 °C cooling, the milk from morning and evening milkings were delivered separately (double milk delivery) to the dairy factory in 50-L cans (trial without refrigeration) or by a temperature-controlled road tanker (trial with cooling at 18°C). In 12 and 8 °C trials the milk from the morning milking was kept refrigerated under slow stirring for 12 hours then mixed with the evening milk and transferred once a day, at evening, to the dairy factory (single milk delivery) in a temperature-controlled road tanker. For each of the 46 production days, raw milk, delivered at the evening was overnight skimmed at the cheese factory. In the morning all the skimmed milk was heated to about 22°C in a copper vat. About 30 liters of natural whey starter were added in the vat containing about 1030 Kg of milk bringing the pH of the mixture to 6.4–6.5. Coagulation occurred at 32–33 °C due to the addition of calf rennet powder, then the curd was broken up for 2–3 min and cooked at a temperature raised gradually to 42–44 °C, and then more quickly to 55–56 °C in 8–10 min. The curd was left covered by the whey for about 60 min and then was removed from the whey and cut in the middle to obtain two wheels as similar as possible. After moulding the two wheels were held for 2 days in a room at about 20°C and frequently turned to enable the complete whey drainage. They were then salted by immersion in brine at 280-300 g NaCl / L 15–18 °C for 20-25 days. Ripening was held in a store room with 80% relative humidity and about 18°C temperature for 18 months, where it was frequently turned.

3.3.2.2 Sampling and isolation

Aliquots of vat milk and whey starter were sampled. For each of the 46 days of cheese-making one wheel was sacrificed: the wheel was cut along the vertical axis to obtain two symmetrical half wheel, and two dish-shaped samples were taken from one of the two half-wheel along the diagonal from the over-dish to the core of the half-wheel. We took a sample 4 cm far from the crust of the cheese dish and 5 from the crust of the lateral side of the half-wheel and one sample in the core of the section (far about 12 cm from the crust of the cheese dish and 25 cm from the crust of the lateral side of the cheese).

3.3.2.3 Microbiological analyses

10 g of each cheese-sample were withdrawn aseptically and homogenized with 90 mL of sterile quarter-strength Ringer's solution in a blender (Stomacher 400, Seward Medical, London, UK).

Each vat milk, natural whey starter and cheese sample, was serially diluted in peptone water (0.1% mycological peptone, Oxoid, Milano, Italy). Ten-fold dilutions were made in the same diluent. All the plates were seeded in duplicate.

3.3.2.4 Media, growth conditions and isolation

Total bacterial count (TBC) was obtained on plate count agar (PCA, Oxoid) incubated at 30 °C for 24h. Mesophilic LAB were counted on PCA (Oxoid) added with 1 g L^{-1} skimmed milk (Oxoid) after aerobic incubation for 2 days at 30 °C; thermophilic LAB were counted anaerobically onto whey agar medium (WAM) prepared as reported by Gatti et al. (2003), after anaerobic incubation for 3 days at 45 °C. Mesophilic rods and cocci LAB were counted on MRS and M17 agar, incubated at 30 °C anaerobically for 48 h and aerobically for 24 h, respectively. From milk samples we monitored TBC; from whey samples the termophilic bacteria and from cheese samples TBC, mesophilic cocci and rods and termophilic LAB.

3.3.2.5 Measurements and Physico-chemical Analysis

Temperature dynamics were registered with the 175-T2 data logger (Testo, Settimo Milanese, Italy). Measurements of pH were carried out by means of a portable pH meter (Knick Portamess 910, Berlin, Germany) connected to the Cheesetrode (Hamilton Co., Reno, NV, USA) electrode.

Fat and casein contents in milk and whey were evaluated by infrared analysis (Biggs, 1978) with a Milko-Scan 134 A/B (Foss Electric, DK-3400 Hillerod, Denmark). Acidity of milk samples was obtained by titrating 100 ml aliquots with 0.25 N NaOH, using phenolphthalein as indicator (end point pH 8.30) and the results were expressed in ° SH.

The dry matter contents in cheeses were evaluated by dry in oven at 102 °C (FIL-IDF 4/A:1982). The fat contents in cheese by the Gerber-Siegfeld method (Savini 1946). NaCl contents by titrating with $AgNO_3$ (FIL-IDF 88/A:1988).

The Water Activity (a_w) in the under-crust and in the core was evaluated by AquaLab® Model Series 3 (Decagon Devices, Inc. Pullman, Washigton, USA).

Quantification of FAAs was achieved using an Agilent 1100 HPLC (Hewlett-Packard, Germany) system and the Agilent Chemstation software. A Merck Licrospher 100 RP-18 (250 X 4.6 mm; 5 mm) (Merck, Darmstadt, Germany) was used. All of the instrumental analytic conditions were as described by Larcher et al. (2004).

3.3.2.6 Proteolysis evaluation

Total (TN) and non-protein (NPN) nitrogen were determined by Kjeldahl according to Butikofer et al. (1993). The separation of nitrogen matter by fractioning diagram described by Gripon et al. (1975); so we were able to obtain the Soluble Nitrogen values at pH 4.4 (NS), Soluble Nitrogen in Trichloracetic Acid 12% (N TCA) and Soluble Nitrogen in Phosphotungstic Acid (N PTA). The ammoniacal nitrogen (N NH_3) was determined by Savini method (1946). Ripening index was calculated from the ratio of water-soluble nitrogen and total nitrogen.

3.3.2.7 Analysis of volatile compounds through SPME

Volatile compounds were collected using solid phase microextraction (SPME) coupled with GC as described by Carlin et al (2005). The 2 cm fiber used for SPME syringe needle was coated with 50 / 30 µm divinylbenzene / Carboxen on polydimethyl-siloxane bonded to a flexible fused-silica core, (Supelco, Bellefonte, PA, USA). Before the analysis, 50 µl of 4-methyl-2-pentanone solution (0.0509 mg / ml), ethyl-eptanoate (0.06 mg / ml) and iso-butirrate acid (20.021 mg / ml) (Sigma Aldrich Chemical Co., Milwaukee, WI, USA) were added as internal standards.

The container was then thermostated at 40 °C for 30 min. The absorbed volatile analyses were then analysed by GC-MS using a GC Clarus 500 gas-chromatograph (Perkin Elmer, Norwalk, CT, USA). The separation was achieved by a HP-Innovax fused-silica capillary column (30 m × 0.32 mm, film thickness 0.52 µm); the oven temperature program was the following: 40 °C for 3 min, 4 °C min−1 to 180 °C for 6 min, then 5 °C min to 220 °C; the flow carrier gas (He) rate was: 1.0 mL min^{-1}; the injector temperature 250 °C; and the detector temperature 220 °C. The eluted compounds were identified by matching their mass spectra with those of the Wiley 175 library (Wiley & Sons, Inc., Germany), NIST-98 library and those of the pure standard components and then confirmed by their GC

The analyses were conducted in triplicate and the results were expressed as a mean.

3.3.2.8 Statistical analysis

All the chemical and microbiological determinations were carried out in triplicate on each sample. For each time point significant differences between means (experimental vs. control) were evaluated by one-way analysis of variance (one-way ANOVA). Differences were considered significant at $p < 0.05$.

3.3.3 RESULTS and DISCUSSION

3.3.3.1 Microbiological characteristics

The results of microbiological analyses for cheeses with milk delivered once or twice to the dairies A, B and C are presented in Table 1.

All parameters from microbiological analyses were studied with ANOVA test to evaluate the differences among the different cheese factories, the double and single milk delivery and the cheese sampling area (core and under-crust). The "cheese factory" factor did not significantly affect the mesophilic and termophilic lactic acid bacteria (LAB) counts in the cheeses but showed to affect the mesophilic LAB counts in the vat milk: ANOVA showed that the vat milk microbial counts were significantly lower in A cheese factory ($p < 0.05$ for mesophilic cocci, rods and total bacterial counts) and significantly higher in C cheese factory ($p < 0.05$ for mesophilic cocci, rods and total bacterial counts).

The variable "sampling area" (under crost and core of the cheese) affected total bacteria and rods whose counts were significantly higher in the under-crust than in the core ($p < 0.05$ for both total mesophilic bacteria and total mesophilic rods). The same trend was observed in all dairies and in both kind of milk delivery (single and double). This phenomenon could be ascribed to the higher temperatures of the cheese core than in the under-crust area during the first and second day after cheese-making (we recorded a ΔT between the core and the under-crust after 5 hours of about 8 °C -data not shown-).

ANOVA test on cheese samples elicited a delivery effect on total bacteria counts and rod counts in the under-crust where they were always significantly higher if milk was delivered twice a day (milk not refrigerated or refrigerated at 18°C) than in cheeses by milk delivered once a day (milk refrigeration at 12 and 8°C). This difference was significant at cheese factories A and B (single delivery counts lower than double delivery ones ($p < 0.05$) but not in the C one.

Information about microbiological characteristics of Grana Trentino was obtained by means of bacterial counts performed on different media. The number of lactococci (M17 agar) determined in 18 months ripened Grana trentino samples was always lower than that of lactobacilli (MRS agar). This fact highlighted that lactobacilli were the most represented organism of the lactic microflora of this cheese, as confirmed by the similar counts obtained on MRS agar used for lactobacilli evaluation and on PCA agar used for total microflora enumeration. The prevalence of lactobacilli has also been observed for Parmigiano Reggiano cheese (Coppola et al. 1997; 2000)

Table 1. Mean Counts[1] (log CFU/g) of the Principal Microbial Groups in Grana Trentino cheese, vat milk and whey

Microbial population (Log CFU / g or ml)	Cheese factory A								Cheese factory B								Cheese factory C							
	Vat milk		Whey		Under-crust		Core		Vat milk		Whey		Under-crust		Core		Vat milk		Whey		Under-Crust		Core	
	D.D.[2]	S.D.[3]	D.D.	S.D.	D.D.	S.D.	D.D.	S.D.	D.D.	S.D.	D.D.	S.D.	D.D.	S.D.	D.D.	S.D.	D.D.	S.D.	D.D.	S.D.	D.D.	S.D.	D.D.	S.D.
TBC	3.9 ± 0.4ab	2.8 ± 0.5bc			4.7± 0.7d	3.4± 0.3b	2.6± 0.5bc	2.4± 0.3c	4.0± 0.7a	4.2± 0.5a			5.1± 0.5d	3.6± 0.2b	4.2± 0.5a	4.0± 0.1a	4.7± 0.5d	4.7± 0.5d			5.1± 1.3d	5.2± 0.7d	2.5± 0.6bc	3.1± 0.2b
Mesophilic Cocci	3.9 ± 0.4ac	2.8 ± 0.5b			3.9± 0.8ab	2.8± 0.2b	2.6 ± 1.4abc	1.8± 1.5ab	4.0± 0.7c	4.3± 0.8c			2.0± 1.0ab	3.9± 0.8abc	2.7± 0.9ab	2.3± 0.5b	4.8± 0.2c	4.6± 0.1c			2.9± 0.6ab	2.1± 1.0ab	1.4 ± 1.2ab	1.9± 1.8ab
Mesophilic Rods	2.9 ± 0.5a	1.9 ± 0.9bd			4.9 ± 0.6c	3.4± 0.4a	1.7 ± 0.7bd	2.0 ± 0.1b	3.1± 0.5a	3.6± 0.6ac			5.0 ± 0.5c	3.5± 0.8ac	2.4± 2.0ab	1.0± 0.4abd	3.6± 0.5ac	4.3± 0.1c			5.0± 1.4c	5.0± 0.5c	0.7 ± 0.3d	0.0± 0.0e
Thermophilic LAB	2.6 ± 1.1a	1.2 ± 1.0a	7.1± 0.9b	7.7± 0.4b	0.5 ± 1.0a	1.9± 1.4a	0.2 ± 0.6ae	1.7 ± 1.9a	2.1± 0.6a	2.2± 1.0a	7.3± 0.7bc	7.2± 0.5b	4.1 ± 0.7f	2.5± 2.3a	1.5± 1.9ae	0.8± 1.2ae	3.0± 0.6af	3.3± 0.5af	8.2± 0.4c	8.0± 0.1c	1.1± 0.4a	0.0± 0.0e	0.0 ± 0.0e	0.0± 0.0e

[1] Mean values ± Standard Deviation of at least 6 samples; [2] D. D., Double milk Delivery to the cheese factory; [3] S. D., Single milk Delivery to the cheese factory . Different letters (a, b, c, d, e) on the same lane indicate significant differences ($p < 0.05$) for the "single or double milk delivery" or "cheese factory" or "sample" factor

3.3.3.2 Physico-chemical features

Physico-chemical characteristics of *Trentingrana* cheeses from milk stored at different temperatures are reported in table 2.

The cheese moisture was always higher when milk was refrigerated at 12° or 8°C and delivered once a day than in cheeses from milk delivered twice. The moisture was not different among the three cheese factories and was always comparable with the one recorded for Grana Padano cheese (31.3 ± 0.9 g /100g; Mucchetti and Neviani, 2006b).

Table 2. Basic composition of Grana Trentino cheese at 18 months of ripening (g / 100g) in different cheese factories (A, B and C) and with different milk delivery (Double Delivery: D.D. and Single delivery: S.D.). Mean values ± Standard Deviation of at least 6 samples.

	Cheese factory A		Cheese factory B		Cheese factory C	
	D.D.	S.D.	D.D.	S.D.	D.D.	S.D.
Moisture	32.28± 0.40a	32.58± 0.57a	31.10± 0.49b	31.24± 0.71b	32.57± 0.50a	32.88± 0.22a
Dry matter	67.72± 0.40a	67.42± 0.57a	68.91± 0.49b	68.76± 0.71b	67.43± 0.50a	67.12± 0.22a
Fat	26.64± 0.68a	25.57± 0.87b	30.61± 0.53c	29.22± 1.35c	28.06± 0.32d	26.32± 0.31e
Protein	32.36± 1.52a	33.41± 0.45a	31.99± 0.52a	33.05± 0.86a	33.57± 0.52b	35.40± 0.63c
NaCl	1.79 ± 0.09a	1.69 ± 0.10b	1.76 ± 0.04a	1.74 ± 0.15ab	1.65 ± 0.15b	1.37 ± 0.23b
NaCl / Moisture %	5.55 ± 0.23a	5.18 ± 0.35b	5.67 ± 0.21a	5.57 ± 0.53ab	7.53 ± 0.73c	6.19 ± 1.05cb
pH	5.44 ± 0.06a	5.47 ± 0.08a	5.41 ± 0.03a	5.44 ± 0.06a	5.41 ± 0.04a	5.48 ± 0.04a
core a_w	0.926± 0.003a	0.924± 0.004ab	0.923± 0.004ab	0.919± 0.003ab	0.916± 0.005bc	0.913± 0.002c
Under-crust a_w	0.906± 0.03a	0.906 ± 0.03a	0.903± 0.006a	0.901± 0.005a	0.899± 0.006ab	0.893± 0.002b

Different letters (a, b, c, d, e) on the same lane indicate significant differences ($p < 0.05$) for the "single or double milk delivery" or "cheese factory" factor

In general, the content of cheese protein and fat has underlined differences between the two different delivery technologies and among the cheese factories. The protein content was not significant different in cheese factory A and B (it ranged between 31.99 and 33.05 g / 100 g) but it was always lower than in the C one (values ranging between 33.57 and 35.40 g / 100 g). The protein content was always higher in cheeses from single milk delivery. Concerning the cheese fat, its content was lower in cheese factories A and C (it ranged between 25.57 and 28.06 g / 100g) than in the B (it ranged between 29.22 and 30.61 g / 100 g).The values from B cheese factory are more similar to the to fat content in Grana Padano (29.7 ± 1,1 g / 100 g). The fat content average value was always higher if milk was not refrigerated or kept at 18°C (double delivery) than in milk from single delivery.

The content of sodium chloride (NaCl) is reported both on 100 g of cheese and on 100 g of cheese moisture. This latter value represents an

indication of the saltiness of the aqueous phase in which cheese enzymes are present. These enzymes are involved in the chemical and biochemical reactions that are the basis of cheese ripening. The cheese salt content was always higher with double delivery than single one: the NaCl content ranged between 1.37 and 1.79 g / 100 g and the aqeous phase saltiness between 5.18 and 7.53 %. All these values are comparable with the salt content measured in Grana Padano cheese (1.6 ± 0.2 g / 100 g)

pH values were always higher with single milk delivery and no differences ($p > 0.05$) were registered among pH values of cheeses produced in the three different dairies: the pH values ranged between 5.41 and 5.48 and were lower than in Grana Padano (5.7).

Water Activity (a_w) was always lower in cheeses from single delivery than cheeses from double one. In the core, the values ranged between 0.913 at dairy C up to 0.926 at dairy A and were always significantly higher than in the external cheese area (the values ranged between 0.893 at dairy C up to 0.907 at dairy A). No significant differences ($p > 0.05$) were observed between the two different delivery technology (double and single one) while the variable "cheese factory" was significant for the a_w: the a_w values were always significant higher in cheeses from dairy A and lower in cheeses from dairy C, no matter of the delivery technology.

3.3.3.3 Proteolysis

The values of the Ripening Index (RI) of Grana Trentino cheeses obtained from milk differently delivered (twice or once a day) and of the composition of pH 4.6 soluble nitrogen composition of Grana Trentino cheeses are reported in table 3. The RI is represented by the percentage of pH 4.6 soluble nitrogen on the total nitrogen of cheese. This index is descriptive of the proportion of casein which is progressively digested by proteolytic enzymes. The degree of casein solubilisation was always significatively higher ($p < 0.05$) in cheeses from single milk delivery.

The values of the RI were 25.9 and 29.2 respectively in double and single milk collection at cheese factory A, 21.3 and 26.0 at cheese factory B and 26.4 and 28.0 at cheese factory C.

The solubilisation of casein was particularly remarkable at cheese factory A and C (at least 2 percentage unit more than at cheese factory B when comparing cheeses from same milk delivery technology, $p < 0.05$). When compared to Parmigiano Reggiano and Grana Padano, the Grana Trentino showed lower Ripening Index (Pecorari et al., 1997; Mucchetti and Neviani, 2006b).

The Soluble Nitrogen (SN) was in a range between 1.1 and 1.6 g / 100 g in all dairies. These are quite low values if compared to Parmigiano Reggiano (Panari et al. 2003).

The SN-TCA fraction of TN is traditionally regarded as a ''ripening depth'' index, as most peptides that are a part of the SN fraction will precipitate in the presence of 12% TCA. This ratio was always higher in cheeses from single milk delivery than cheeses from the double one and ranged between 19.5 and 23.6 % in cheeses from double milk delivery and between 22.2 and 24.1 % in cheeses from the single one.

Table 3. Ripening Index and soluble nitrogen composition (g / 100 g) of Grana Trentino cheese at 18 months of ripening from different cheese factories (A, B and C) and with different milk delivery (Double Delivery: D.D. and Single delivery: S.D. Mean value ± SD of at least 6 samples.

	Cheese factory A		Cheese factory B		Cheese factory C	
	D.D.	S.D.	D.D.	D.D.	D.D.	S.D.
TN as % dry matter	7.5± 0.4a	7.7 ± 0.1b	7.3± 0.1c	7.5± 0.2d	7.8± 0.1e	8.3± 0.1f
SN[1] as % dry matter	1.3± 0.1a	1.5 ± 0.1b	1.1± 0.1a	1.3± 0.1a	1.4± 0.0ab	1.6± 0.0c
SN % / TN [2] (Ripening index)	25.9± 1.9a	29.2 ± 1.1b	21.3± 1.8c	26.0± 1.8d	26.4± 0.8a	28.0± 0.9bd
SN-TCA as % TN	19.6± 2.0a	22.2 ± 1.5a	19.5± 1.0a	23.3± 1.5b	23.6± 0.7c	24.1± 1.3c
Peptones N % / SN	23.6± 6.5a	23.6 ± 3.6a	7.9 ± 5.1b	10.7± 1.4b	10.5± 2.6b	13.9± 1.7c
Peptides N % / SN	32.4± 7.4a	34.0 ± 4.0a	18.2± 3.3b	22.0± 1.3c	15.3± 2.4b	16.4± 0.5b
SN-TCA % / SN (Peptones + peptide N / SN)	48.3± 14.1a	57.5 ± 3.0b	26.0± 5.8c	32.7± 1.8d	25.7± 4.8c	30.4± 1.8cd
SN-PTA % / SN (AA N / SN)	49.9± 8.5a	37.8 ± 2.8b	65.2± 4.7c	60.1± 1.5d	66.5± 3.5c	63.3± 1.2d
NH_3 N [3] % / SN	5.6 ± 0.6a	4.7 ± 0.5b	8.8 ± 1.2c	7.2 ± 0.8d	7.8 ± 0.4e	6.3 ± 0.2f

Different letters (a, b, c, d, e, f) on the same sample (row) indicate significant differences ($p < 0.05$) for the "single or double milk delivery" or "cheese factory" factor

1 SN = pH 4.6 soluble nitrogen

2 SN % / TN = SN *100 / Total nitrogen

3 NH_3 N = ammonia Nitrogen

Based on the ANOVA results, the cheese factory was found to be statistically significant ($p < 0.05$) toward determination of all Total Nitrogen fractions, so they can be claimed to be directly associated with the heterogeneity observed among the cheeses. The factor "single or double milk delivery" also played a significant role ($p < 0.05$) for the TN % (as dry mater), the RI., and the AA and NH_3 in the soluble fraction.

The water-soluble fraction is very heterogeneous in terms of composition, and includes high- medium- and low-molecular weight peptides, as well as FAA (table 3). The set of reactions normally designated as secondary proteolysis result from the action of indigenous milk proteinases, in addition to adventitious microflora. Residual rennet has no activity during ripening because of the degradation at the curdle cooking temperature (55 °C). The proportion of peptones and peptides among soluble nitrogen fractions (the primary substrate of the proteolytic activity) was significant higher ($p < 0.05$) in cheese factory A where they ranged between 19.4 and 23.0 % than in B and C wher they ranged between 25.7 and 32.7 % ($p < 0.05$). The peptones and peptides amount was significant higher at dairy A and B (not at dairy C) for cheeses produced by milk delivered once a day (single delivery) than twice a day (double delivery). The factor "single or double milk delivery" also played a significant role ($p < 0.05$) for the free amino acids content in the SN fraction of cheeses produced at A and B dairy. The ratio SN-TCA / SN was significant higher in cheeses from double milk delivery than from the single one (+12.00

percentage unit at cheese factory A and +5.00 at the B one). Actually, free amino acids are the final products of the proteolytic processes and, consequently, they accumulate during the ripening of cheese.

The flavor impact caused by the water-soluble fraction of cheese is well established, since it contains the majority of taste compounds as salts, amino acids and low-molecular weight peptides, together with some volatiles (Engels et al. 1997). Lemieux, and Simard, (1992) said that in a first step, long chain non-bitter peptides are obtained from the action of non-starter *Lactobacillus.* Enzymes from Starter bacteria are presumed to further attack these long chain non-bitter peptides and release bitter polypeptides. As suggested by Engels et al. (1997), large and medium-sized peptides are probably not major contributors to cheese flavor. These authors concluded that low-molecular weight compounds (< 500 Da) are responsible for flavor in the water soluble fraction of several cheeses, as accounted for by small peptides, FAA, free fatty acids, or breakdown products of these compounds; however, their direct contribution to flavour is rather limited. In conclusion the variety of cheese peptides makes it difficult to correlate flavour with specific peptides.

3.3.3.4 Free amino acid (FAAs) in Grana Trentino

Table 4 shows the composition on FAAs in 18 months ripened Grana Trentino cheeses from different milk delivery technology.

At the end of the ripening (18 months) glutamate was the most abundant AA in all cheeses followed by leucine, lysine, valine and serine. These five aminoacids constituted 50 % - 55 % of the total FAAs recovered in cheeses from dairy factories A, B and C from all trials. Among them leucine and valine are the chemical precursors of isovaleric acid and isobutyric acid (Dahl et al., 2000) which play important roles in flavour development and were especially pronounced in cheeses made from single milk delivery (table 4). In general FAA amounts were higher in cheeses from single delivery. Refrigeration of milk seemed to lead to some significant difference at cheese factories A and B where significant higher amount ($p < 0.05$) of total FAA and of some aminoacids like aspartate, glutamine, glycine and valine were measured, but, if related with the total protein amount (table 4, late two grey lines), only the differences for aspartate, glutamine and valine remained significant.

Glutamine in particular is a ripening indicator because its amount decreases during the ripening (Bottazzi, 1993), thus higher the glutamine amount and “younger” is the cheese.

The cheese factory was found to be highly significant factor for all FAA concentrations as concluded from the ANOVA: only for Ser the “dairy” factor was not significant at the 5% level of significance.

The developing microflora may be responsible for amino acid release from small- and medium-sized peptides, but the numbers of viable lactic acid bacteria shown in Table 2 were significant higher (at least 1.5 Log CFU / g cheese) in cheeses manufactured from double milk delivery.

Table 4. Free amino acids (AA g / proteins g of cheese) in 18 months ripened Grana Trentino cheese (mean ± SD of at least 6 samples) from different cheese factories (A, B and C) and with different technology of milk delivery (Double Delivery: D.D. and Single delivery: S.D.)

	Cheese factory A		Cheese factory B		Cheese factory C	
	D.D.	S.D.	D.D.	S.D.	D.D.	S.D.
ASP	1.93 ± 0.17a	2.13 ± 0.22b	1.63 ± 0.07c	2.05± 0.22db	1.76 ± 0.14e	1,88± 0.14e
GLU	10.36± 0.75a	11.03± 0.88a	8.75 ± 0.42b	9.95± 0.94c	8.71 ± 0.60bd	9.20± 0.34bd
ASN	2.89 ± 0.30a	3.11 ± 0.19b	2.39 ± 0.10c	2.75± 0.32c	2.05 ± 0.16d	2.10 ± 0.02d
SER	3.05 ± 0.21a	3.03 ± 0.33a	2.63 ± 0.29b	3.14± 0.30ab	2.78 ± 0.24bd	2.64 ± 0.28bd
GLN	0.27 ± 0.07a	0.31 ± 0.02b	0.21 ± 0.05c	0.30 ± 0.06bd	0.40 ± 0.09e	0.41 ± 0.01e
HYS	1.82 ± 0.20a	1.85 ± 0.21a	1.61 ± 0.08b	1.61 ± 0.26b	1.96 ± 0.33a	1.86 ± 0.04a
GLY	1.18 ± 0.08a	1.28 ± 0.10b	0.96 ± 0.05c	1.10 ± 0.12d	0.95 ± 0.12ce	1.00 ± 0.06ce
THR	2.20 ± 0.15a	2.25 ± 0.15a	1.79 ± 0.10b	2.15 ± 0.24ac	1.91 ± 0.08d	2.01 ± 0.05d
CIT	0.19 ± 0.07a	0.18 ± 0.07a	1.57 ± 0.34b	1.14 ± 0.76b	1.39 ± 0.58bc	0.66 ± 0.15c
ARG	2.69 ± 0.24a	2.82 ± 0.20a	0.59 ± 0.51b	1.59 ± 1.01b	1.55 ± 0.37c	2.12 ± 0.32c
ALA	1.65 ± 0.12a	1.72 ± 0.07a	1.42 ± 0.07bc	1.56 ± 0.17bc	1.36 ± 0.09bd	1.45 ± 0.04bd
GABA	0.21 ± 0.13a	0.33 ± 0.17a	N. R.b	0.17 ± 0.1b	0.12 ± 0.04bc	0.06 ± 0.03bc
TYR	1.34 ± 0.08a	1.46 ± 0.09b	1.24 ± 0.08c	1.33 ± 0.19d	0.96 ± 0.12e	0.98 ± 0.19e
NH_4	0.43 ± 0.07a	0.47 ± 0.04a	0.51± 0.08b	0.53 ± 0.09b	1.17 ± 0.29c	0.99 ± 0.05c
TRP + MET	2.24 ± 0.17a	2.47 ± 0.14b	1.84 ± 0.12c	2.20 ± 0.24d	1.50 ± 0.29e	1.38 ± 0.03e
VAL	3.98 ± 0.27a	4.28 ± 0.28b	3.14 ± 0.13c	3.83 ± 0.38d	2.91 ± 1.30ce	3.71± 0.07ed
PHE	2.95 ± 0.26a	3.20 ± 0.18b	2.55 ± 0.15c	2.87 ± 0.33c	1.99 ± 0.84d	2.49 ± 0.06d
ILE	2.90 ± 0.17a	2.95 ± 0.27a	2.40 ± 0.17b	2.86 ± 0.28ac	2.61 ± 0.18d	2.69 ± 0.11d
LEU	5.20 ± 0.26a	5.36 ± 0.22a	4.57 ± 0.23b	5.05 ± 0.47b	3.62 ± 0.28c	3.82 ± 0.11c
ORN	0.06 ± 0.03a	0.05 ± 0.04a	0.46 ± 0.21b	0.11 ± 0.08c	0.13 ± 0.04d	0.09 ± 0.01d
LYS	4.73 ± 0.43a	4.99 ± 0.47a	3.84 ± 0.19b	4.22 ± 0.43b	4.63 ± 0.28a	5.05 ± 0.15a
Σ FAA	50.30± 4.78a	54.50± 3.45b	45.63± 2.70c	49.89± 4.83d	44.45± 2.07ce	46.58± 1.44ce
FAA % / proteins	15.55± 1.02a	16.26± 1.01a	13.63± 0.74b	15.08± 1.14c	13.24± 0.62bd	13.16± 0.64bd

Different letters (a, b, c, d, e) on the same sample (row) indicate significant differences ($p < 0.05$) for the "single or double milk delivery" and "cheese factory" factor

It can be concluded that the higher FAA amounts found out in cheeses from single delivery are associated with the refrigeration process itself, that occurred for single delivery (milk was refrigerated at 12 or 8 °C)

rather than with the intrinsic microbial activity. In a previous work Tavaria et al. (2003) showed that refrigeration favoured the release of certain amino acid residues.

Although the role of FAA in flavour development is well recognized, their direct contribution is probably limited. Specific FAA act as precursors of specific cheese flavour compounds in various cheese varieties through both enzymatic and chemical transformations whereas total FAA contribute to the background flavour which is typical of cheese in general (Christensen et al. 1999). Therefore, there is not necessarily a relation between typical cheese flavour and total FAA content (Engels et al., 1997).

3.3.3.5 Analysis of volatile compounds

More than 600 volatile compounds have been identified so far in different cheese varieties (Curioni and Bosset, 2002). In the present work, 38 compounds were identified by gas chromatography-mass spectrometry analysis of the volatile fraction of 18 months ripened Grana Trentino cheeses: alcohols; aldehydes; ketones; ethyl esters; fatty acids and hydrocarbons. Eleven of the 38 volatile compounds detected in the present work were among the 74 compounds found in Parmigiano Reggiano (Qian and Reineccius, 2002). Thirty-three of the 38 volatile compounds had abundance values under 5 p.p.m. in all samples. As happened with the proteolysis and FAA assays, a great variability in chemical composition was observed between samples obtained from different dairies, probably linked to the non-standardized process of manufacture. In general, these cheeses were characterized by high amounts of fatty acids and esters rather than alcohols, aldheydes and ketones. The pattern of volatiles was qualitatively richer in the cheeses made from double milk delivery (table 5) which showed ethyl esters concentration of almost all fatty acids higher than those in cheeses from single delivery. Esters contribute fruity notes to flavour and their production is usually ascribed to lipolytic activity brought by psychrotrophic bacteria enzymes activity (Molimard & Spinnler, 1996).

In terms of signifcant correlations ($p < 0.05$), it became apparent that lactobacilli in the under crust area of cheese sampling counts were correlated with the amount of all the ethyl esters (with the only exception of ethyl acetate) and with the amount of all free fatty acid with the exception of propionic, isovaleric and nonanoic acids. In a previous work, Morales et al. (2005) found that strains belonging to different species of the genus *Pseudomonas* are capable of survival at temperature of 38 °C cooking, growing during ripening, and producting a large variety of volatile compounds and that these abilities are species-dependent. In our work, Grana Trentino was cooked at about 55 °C and even if we assume that *Pseudomonas* enzymes have still activity after this thermal process, the presence of *Pseudomonas* in vat milk was not significant different in double or single delivery and counts onto PBA were always lower than 2.6 CFU / ml. These considerations indicate that the ethyl esters and free fatty acids are probably secondary metabolites of the lactobacilli microbial group and the differences between single and double delivery technology in the amounts of these latter compounds are up to differences in microbial development during cheese ripening between the two technologies.

3.3.3.5.1 Esters

The determined esters were all ethyl esters (table 5).

Arora et al. (1995) reported that ethanol is the most abundant alcohol in cheese; this probably explains why all the esters identified were ethyl derivatives. In the present study, high concentration of ethyl esters was estimated in all samples.

Table 5. Esters, free fatty acids and alcohols (p.p.m.) in the volatile fraction of 18 months ripened Grana Trentino cheese (mean ± SD of at least 6 samples) from different cheese factories (A, B and C) and with different technology of milk delivery (Double Delivery: D.D. and Single delivery: S.D.)

	Cheese factory A		Cheese factory B		Cheese factory C	
	D.D.	S.D.	D.D.	S.D.	D.D.	S.D.
Esters						
Acetate ethyl ester	0.013 ± 0.004a	0.015 ± 0.009b	0.011 ± 0.007a	0.013 ± 0.003ac	0.021 ± 0.009ac	0.033 ± 0.016c
Iso-butanoic acid ethyl ester	0.128 ± 0.034a	0.066 ± 0.034b	0.060 ± 0.018b	0.057 ± 0.013b	0.026 ± 0.007c	0.026 ± 0.009c
butanoic acid ethyl ester	0.418 ± 0.230a	0.196 ± 0.051b	0.231 ± 0.143c	0.209 ± 0.083bc	0.103 ± 0.069d	0.063 ± 0.030d
Hexanoic acid ethyl ester	0.283 ± 0.126a	0.114 ± 0.036b	0.165 ± 0.086c	0.117± 0.020bc	0.116 ± 0.063bcd	0.066± 0.022d
Octanoic acid ethyl ester	0.024 ± 0.013a	0.007 ± 0.002b	0.014 ± 0.007c	0.009 ± 0.002c	0.012 ± 0.007c	0.007 ± 0.002c
Decanoic acid ethyl ester	0.003 ± 0.001a	Traces b	0.001 ± 0.001cd	0.001 ± 0.001bc	0.001 ± 0.000c	0.001 ± 0.000c
Σ Esters	0.869 ± 0.370a	0.399 ± 0.102b	0.484 ± 0.259c	0.406 ± 0.097bc	0.278 ± 0.137bd	0.195 ± 0.072d
Linear fatty acids						
Acetic acid	49.138 ±11.600a	37.011± 4.190b	30.201 ± 9.875c	24.867 ± 3.485cd	20.978 ± 4.765d	19.967 ± 7.648d
Propionic acid	0.142± 0.110a	0.016 ± 0.006b	0.360± 0.260c	0.073 ± 0.056d	2.174± 1.174c	0.629± 0.224c
3-Methylbutanoic acid (isovaleric acid)	0.693 ± 0.378a	0.279 ± 0.095b	0.478 ± 0.169c	0.543 ± 0.285c	0.287 ± 0.103b	0.329 ± 0.169b
Butanoic acid	99.232 ± 33.530a	34.100± 5.377b	68.411 ± 21.878c	38.189 ± 14.700b	108.650± 59.732a	41.822 ± 14.045b
Valerenic acid	0.341 ± 0.134ac	0.101 ± 0.024b	0.332 ± 0.134ac	0.147 ± 0.024c	0.477 ± 0.293ac	0.200 ± 0.069ac
Hexanoic acid (capronic acid)	93.776 ± 34.109a	29.537± 4.747b	62.515 ± 19.608c	31.131 ± 11.783bd	153.580± 101.844e	59.147 ± 20.678e
Heptanoic acid	0.565 ± 0.224ad	0.141± 0.046b	0.374 ± 0.112d	0.153± 0.097b	0.731 ± 0.491ad	0.322± 0.027d
Octanoic acid (caprylic acid)	19.933 ± 9.625ae	5.673 ± 1.215b	10.676 ± 3.141c	6.075 ± 1.916b	37.076 ± 24.751e	17.701 ± 3.681e
Nonanoic acid	0.899 ± 0.215a	0.643 ± 0.616a	0.440 ± 0.143b	0.648 ± 0.462b	0.997 ± 0.359ac	0.375 ± 0.065c
Decanoic acid	2.707 ± 1.413a	0.953 ± 0.473b	1.090 ± 0.250c	0.697 ± 0.274c	3.724 ± 3.674ab	1.048 ± 0.566bc
Σ Fatty Acids	258.388 75.926a	108.416 11.844b	174.877± 53.587c	102.505± 30.486b	327.674± 195.530ae	141.540± 45.809e
Fatty Acids mEq / 100g fat	97.5 ± 30.3a	42.5 ± 5.3b	57.5 ± 18.5b	34.6 ± 8.9c	134.0 ± 82.5abc	58.9 ± 18.8bc

Alcohols						
1-butanol	Traces a	N.R. a	Traces a	N.R. a	0.008 ± 0.007 b	N.R. ab
3-methyl-butanol (alcohol isoamilic)	0.007 ± 0.006ac	0.004 ± 0.002a	0.011 ± 0.006ab	0.015 ± 0.009b	0.007 ± 0.003ac	0.010 ± 0.000c
2-pentanol	Traces a	Traces a	0.001 ± 0.001b	0.001 ± 0.001b	0.002 ± 0.000 c	0.002 ± 0.001c
1-hexanol	Traces a	Traces a	0.002 ± 0.001a	0.001 ± 0.001a	N.R. b	N.R. b
2-ethyl-hexanol	Traces a	Traces a	Traces a	Traces a	0.006± 0.005b	0.007 ± 0.002b
2-heptanol	0.017 ± 0.010a	0.021 ± 0.009a	0.010 ± 0.004b	0.006 ± 0.005b	0.002 ± 0.001bc	0.003 ± 0.000c
3-heptanol	Traces a	Traces a	Traces a	Traces a	0.004 ± 0.001b	0.004 ± 0.001b
2-nonanol	Traces a	Traces a	Traces a	Traces a	Traces a	Traces a

Different letters (a, b, c, d, e, f) on the same sample (row) indicate significant differences ($p < 0.05$) for the "single or double milk delivery" and cheese factory" factor
Compounds less than 0.001 p.p.m. are indicated by traces
N.R. : Not Revealed

Among these compounds the ethyl butanoate was mostly present (0.42 and 0.20 p.p.m. in dairy A; 0.23 and 0.21 p.p.m. in dairy B and 0.10 and 0.06 p.p.m. in dairy C from double and single milk delivery respectively). With the exception of ethyl acetate, the amount of esters was always higher in cheeses from double milk delivery (table 5) and these differences were significant at the cheese factory A. Esters are mainly produced by the enzymatic or chemical reaction of fatty acids with primary alcohols and the alcohol concentration is usually the limiting factor in ester production.

Esters can also be formed by transesterification of partial glycerides (Engels et al., 1997). Therefore, ester production in the double milk delivery cheeses may be attributable to the parallel increase of the short- and medium-chain of fatty acid concentration that results from the hydrolysis of fat by lactobacilli as previously reported (Dahl et al., 2000) and usually lactobacilli were higher in cheeses from double milk delivery.

Most esters encountered in cheese are described as having floral and fruity (if present in low amounts) or yeasty notes (if present in high amounts) (Ayad et al., 2000) and may contribute to cheese aroma giving fruity, sweet and floral notes. In particular ethyl esters are known for their important role in the formation of a fruity taste in cheese. It must be highlighted that the ethyl hexanoate compound plays an important role in the aroma profiles of aged Cheddar, natural Gorgonzola, Grana Padano, Pecorino, and Ragusano cheeses. In particular, the compound showed to produce orange note in the Pecorino type cheese (Curioni and Bosset, 2002).

3.3.3.5.2 Free fatty acids

Free fatty acids are important components of cheese flavour. The concentrations of free fatty acids (FFAs) with the exception of acetic, isovaleric, nonaoic and decanoic, FFAs were higher in cheeses from double milk delivery in all cheese factories and in dairy A and B this diference was significant (table 5).

Acetic (the highest values were recorded at cheese factory A), propionic (the highest values were recorded at cheese factory C) and isovaleric acids may be derived from oxidative deamination of amino acids. Among these latter compounds the acetic acid was mostly present (about 49 and 37 ppm at dairy A; 30 and 24 ppm at dairy B and 20 and 21 ppm at dairy C from cheeses by double and single milk delivery respectively). Acetic acid originates from a number of processes including the oxidation of lactose by lactic acid bacteria under anaerobic conditions, and the catabolism (oxidative deamination and decarboxylation) of alanine and serine by lactic acid bacteria (Ziino et al., 2005). Acetic acid contributed a strong, pungent, vinegary note in Parmigiano Reggiano cheese (Qian and Reineccius, 2002).

Among linear FFAs, containing four or more carbon atoms, butanoic acid showed the highest area value in all cheese samples, hexanoic, octanoic and decanoic acids followed in decreasing order (table 5). This trend was slighty different at dairy C were the hexanoic acid (capronate) was more than butanoic acid. Butanoic and octanoic acids were always over the odour thresholds as shown in thresholds tables by Dahl et al. (2000). The volatile fatty acids total content (grey lines in table 5) may be used as index to evaluate the lypolitic activity occurred in cheeses samples. It was valued in mEq / 100g fat and was always higher in cheeses from double milk delivery than single one. They were about 98 and 43 mEq / 100 g at dairy A; 58 and 35 mEq / 100 g at dairy B and significant higher at dairy C (about 134 and 59 mEq / 100 g) from cheeses by double and single milk delivery respectively.

In the present study since the plating count results, as well as DGGE profiles (see § 3.1.3.4), revealed the dominance of lactobacilli in all cheese samples, the production of FFAs could be ascribed to their lipolytic activity (Ziino et al., 2005). The general production of higher amounts of FFAs in cheeses from double milk delivery than in the ones from the single milk delivery is probably linked to the presence of more lactobacilli amounts in the cheeses from double milk delivery.

Butanoic acid is generally a major aroma compound with cheesy sharp aroma and plays an important role in the flavour of many cheese types as Camembert, Cheddar, Grana Padano Pecorino, Ragusano and Roncal cheese (Curioni and Bosset, 2002). Even hexanoic acid is a characteristic flavour component of Grana Padano and Roncal cheese, and it is perceived as goat-like smell. Octanoic acid was described as rancid and pungent. Qian and Reineccius (2002) determined that octanoic acid conferred strong sweaty, cheesy, lipolyzed notes, whereas heptanoic and nonanoic acids exhibited weak aromas in Parmigiano Reggiano cheese. Carunchia Whetstine et al. (2003) determined that hexanoic and heptanoic acids contributed not only to volatile aroma of fresh Chevre-style goat milk cheeses but also to the sharp sour taste of these cheeses. They noticed that

octanoic acid contributed sweaty/waxy and nonanoic acid dirty/ sour flavour to these fresh goat milk cheeses.

3.3.3.5.3 Alcohols

Alcohol compounds were generally found in low concentrations in all samples (table 5). Only 2-heptanol and 3-methyl-1-butanol were detected in all cheeses in amounts higher than 1 p.p.b. (2-pentanole and 1-hexanole were found only in cheeses from dairy B) anyway without significant differences among samples. Methylalcohols like 3-methyl-1-butanol are mostly derived from the branched-chain amino acids leucine, isoleucine, and valine (Urbach, 1995). Primary alcohols derive from the reduction of the corresponding aldehydes produced from fatty acids and from amino acid metabolism (Urbach, 1995). Therefore, in the present study the amount of alcohol compounds could be related to the presence of wild lactic acid bacteria.

In some cheese varieties methylalcohols are recognized as key flavor-contributing compounds. In particular, 3-methyl-1-butanol confers a pleasant aroma of fresh cheese, which is considered an important contributor to the general flavour and is associated to fruity taste. The weak aroma intensities of most alcohols indicated that they contributed with very weak alcoholic fruity notes to Parmigiano Reggiano cheese (Qian and Reineccius, 2002). Aliphatic primary alcohols such as 2-heptanol, contribute to green and alcoholic notes (Curioni and Bosset, 2002).

3.3.3.5.4 Aldehydes

Aldehydes were found at low levels in Trentingrana cheeses (table 6); probably they did not accumulate in cheese because they were rapidly converted to alcohols or to the corresponding acids (Lemieux & Simard, 1992).

Some aldehydes may originate from the catabolism of amino acids, e.g., 3-methyl-butanal, from leucine and 2-methyl-butanal from isoleucine (Urbach, 1995). This latter compound, which is generally produced in cheeses manufactured by using thermophilic LAB, such as Parmigiano and Grana Padano-like cheeses (Bossett and Gauch, 1993), was detected in all cheese samples examined showing no significant difference among cheeses from different technology production (table 6).

Aldehydes are considered to be potent odorants in some cheeses (Curioni and Bosset, 2002).

No straight-chain aldehydes were found in cheese samples which are the main products of autoxidation of unsaturated fatty acids: autoxidation goes on via hydroperoxides, which in turn undergoes a further degradation becoming hydrocarbons, alcohols and carbonyl compounds (Bellesia et al., 2003).

3.3.3.5.5 Ketones

Ketones are common constituents of most dairy products. In our samples we found out only methyl-ketones. From double delivery milk the concentration of acetoin (3-hydroxy-2-butanone) was always significant higher than in cheese samples (Table 6) from single milk delivery.

Table 6. Aldehydes, ketones and hydrocarbons (p.p.m.) in the volatile fraction of 18 months ripened Grana Trentino cheese (mean ± SD of at least 6 samples) from different cheese factories (A, B and C) and with different technology of milk delivery (Double Delivery: D.D. and Single delivery: S.D.)

	Cheese factory A		Cheese factory B		Cheese factory C	
	D.D.	S.D.	D.D.	S.D.	D.D.	S.D.
Aldehydes						
2-methyl-butanal	0.008± 0.002a	0.010± 0.003a	0.004± 0.001b	0.005± 0.002b	0.006± 0.002a	0.010± 0.003a
3-methyl-butanal	0.026± 0.008a	0.032± 0.009b	0.011± 0.003b	0.015± 0.004b	0.021± 0.007ab	0.032± 0.010a
2-butenal	Traces a	Traces a	Traces a	Traces a	Traces a	Traces a
Ketones						
2-pentanone	0.196± 0.085a	0.128± 0.044b	0.127± 0.005b	0.105± 0.024b	0.212± 0.143ac	0.200± 0.049c
2-hexanone	0.008± 0.003a	0.006± 0.002a	0.009± 0.000b	0.010± 0.001b	0.009± 0.004ab	0.009± 0.001ab
2-heptanone	0.208± 0.059a	0.167± 0.063b	0.213± 0.046ac	0.225± 0.040c	0.384± 0.113d	0.398± 0.036d
2-octanone	0.001± 0.001a	Traces b	Traces b	Traces b	0.003± 0.001 c	0.003± 0.002c
3-hydroxy-2-butanone (acetoin)	0.002± 0.001a	Traces b	0.008± 0.005cd	0.004± 0.002d	0.004± 0.001d	0.004± 0.003d
2-nonanone	0.018± 0.005a	0.017± 0.004a	0.022± 0.004ab	0.026± 0.007b	0.046± 0.011c	0.044± 0.000c
δ-lactones						
Delta-octalactone	0.001± 0.000a	Traces b	0.002± 0.000c	0.002± 0.000c	0.004± 0.001d	0.005± 0.001d
Delta-decalactone	0.001± 0.000a	Traces b	0.002± 0.001c	0.002± 0.000c	0.001± 0.001a	0.001± 0.000a
Aromatic compounds						
Ethyl Benzene	0.067± 0.038a	0.038± 0.036b	0.021± 0.011a	N.R. c	N.R. c	N.R. c
1.2 dimethyl Benzene	0.021± 0.019a	0.029± 0.023b	0.025± 0.023a	N.R. c	N.R. c	N.R. c
2.6 dimethylpirazine	0.005± 0.002a	0.008± 0.003b	0.004± 0.000c	0.009± 0.003bd	0.007± 0.002e	0.014± 0.002f
Benzaldeide	Traces a	Traces a	0.003± 0.002b	0.003± 0.001b	0.005± 0.003c	0.007± 0.003c

Different letters (a, b, c, d, e, f) on the same sample (row) indicate significant differences ($p < 0.05$) for the "single or double milk delivery" and "cheese factory" factor
Compounds less than 0.001 p.p.m. are indicated by traces
N.R. : Not Revealed

Various aroma notes are associated with methyl ketones such as 2-heptanone (blue cheese like, strong) and 2-nonanone (fruity weak), as a result of the metabolism by lactic acid bacteria (Qian and Reineccius, 2002). It's known that volatile profiles of cheeses made from milk inoculated with *L. lactis* strains were richer in ketones (Morales et al. 2003). The formation of methyl ketones is a result of enzymatic oxidation of FFAs to β-ketoacids and their consequent decarboxylation to alkan-2-ones with the loss of one carbon atom (McSweeney & Sousa, 2000), although 2-butanone derives

from diacetyl that is produced by lactose fermentation and metabolism of citrate (Urbach, 1995).

Methyl ketones are the principal compounds responsible for the flavour of the different types of cheese: blue cheese (Engels et al., 1997), Gouda, Cheddar (Ziino et al., 2005) and Parmigiano cheese (Bellesia et al., 2003). No diacetyl (2,3-butanedione) were found in cheese samples; even if diacetyl is commonly found in dairy products associated with *Lactobacillus casei* and *paracasei* (Menendez et al., 2000); maybe because the diacetyl underwent to a further reduction generating acetoin (Garde et al., 2003) that was found in cheese samples in particular by double milk delivery. Acetoin and diacetyl contributed to sour milk and buttery odour respectively (Curioni and Bosset, 2002).

3.3.3.5.6 δ-lactones

δ – octalactone and δ –decalactone were found to be present in small amounts (table 6). Some authors found δ – octalactone, δ –decalactone and others δ -lactones composed of 6–14 carbon atoms in cows' milk (Bendall, 2001); other authors postulated biosynthesis based on dehydration and cyclization of hydroxy fatty acids that can be generated from unsaturated fatty acids by the action of lipoxygenases or hydratases (Behnke, 1980).

Lactones contribute to cheese flavour giving fruity and sweet creamy fermented notes (Curioni and Bosset, 2002).

3.3.3.5.7 Aromatic compounds

Three aromatic compounds were also found in the volatile fraction of the *Trentingrana* cheese samples examined (table 6). The amount of ethyl benzene and 1,2 dimethyl benzene did not show any significant variation from different milk delivery cheeses ($p > 0.05$). Benzyl compounds may come from the degradation of carotene in milk (Ziino et al., 2005), however we are dealing with ubiquitous substances and a contamination from air, water, or tools cannot be dismissed.

A significant higher amount of 2,6-dimethylpyrazine was always found in cheeses manufactured by milk from single delivery. 2,6-dimethylpyrazine has been identified both in Parmigiano Reggiano and Grana Padano cheese previously and was determined to be contributor for the backed and nutty aroma of these cheese (Qian and Reineccius, 2002). Pyrazines may be formed either via the Maillard reaction (Shipar, 2006) or from lysine and dihydroxyacetone (Urbach, 1995).

3.3.4. CONCLUSIONS

The influence of different storage milk temperatures on the microbiological and physicochemical characteristics of the cheese has been finally evaluated in experimental cheese-making trials. In particular, physico-chemical characteristics, proteolysis, free amino acids (FAAs) and volatile compunds were evaluated at the end of cheese ripening and the accurate way of sampling allowed to follow the specific microbial selection in the under crust and cheese core. In the core, lower microbial load amounts were found than under the crust.

From the values obtained for the nitrogen fractions in Grana Trentino cheese, it can be concluded that lower temperatures of milk storage favorite

a cheeses proteolysis that is higher if observing the Ripening Index, but lower if looking to the PTA-SN. The increase in FAA was correlated with higher microflora amount found in cheeses produced by milk from double delivery (not refrigerated or refrigerated at 18 °C). The higher volatile compounds amount found in cheeses with higher bacteria amount confirmes that LAB and their enzyme activities, coupled with a pool of FAA (which act as substrates) provide conditions that may promote flavor development via amino acid conversion into volatile compounds

Results obtained during this doctorate thesis lead to new findings that advance the understanding of microbial dynamics in an appreciated and economically important cheese like Grana Trentino. The approach utilized, the methods and results obtained, open perspectives for insighting into the microbial evolution in fermented food environment.

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4. GENERAL CONCLUSIONS

During this Ph.D. study, traditional and innovative technologies of milk storage and collection were compared in order to study their influences on maturation of *Trentingrana*, a long ripened cheese similar to Parmigiano Reggiano and Grana Padano. In particular the microbial contribution to the cheese ripening was evaluated by means of microbial analyses and chemical methodologies. An accurate and laborious sampling of milk during more than 100 cheese making days and of Grana Trentino cheese at 18 months ripening allowed to reach the aim of this research.

In the first step, the research was focused on the physico-chemical, rheological and microbiological characteristics of raw cows' milk maintained at different temperatures and storage times at the farm, before delivering it to the dairy. In particular, important differences in bacterial concentrations during milk creaming were observed and the totality of prokaryotic species resident in whole, skimmed milk, cream including the non cultivable bacteria were detected by denaturing gradient gel electrophoresis (DGGE). In particular, the psycrotrophic bacteria development was studied because the milk refrigeration may have the drawback of favouring their growth. This microbial group is known for its spoilage activity in the milk and the cream during overnight maturation, and also for affecting cheese ripening trough the production of extracellular enzymes, mainly proteases and lipases, which are remarkably heat stable. When temperature and storage time of milk are not kept under control, microbial concentration of milk is unpredictable. Low milk temperatures (12 and 8 °C) during storage were confirmed to be useful in maintaining a high hygiene of raw milk without affecting the presence of mesophylic LAB that become so important during cheese ripening. In the whole milk, the psychrotrophic bacteria counts were lower than total mesophilic bacteria and LAB, while were counted at similar values of these latter two microbial populations after maturation, both in skim milk and cream, indicateing that psychrotrophic bacteria are able to grow during skimming. A total of 138 isolates from milk and cream samples were isolated, identified and characterised for their proteolytic and lipolytic activities, that are the most dangerous during mik creaming and cheese ripening. During creaming *Acinetobacter* ssp. was the most abundant psychrotrophic group, equally distributed between cream and skim milk, together with *Flavobacteriaceae* and *Pseudomonadaceae* in cream and *Streptococcaceae* and *Enterobacteriaceae* in the skim milk. Proteolytic and lypolytic activities seemed to be strain specific and highly depending on milk storage temperatures. Lipolytic activity was mostly associated with *Flavobacteriaceae* and *Acinetobacter* ssp. found out in cream, while the secretion of proteolytic enzymes was rather associated to *Pseudomonadaceae* and *Flavobascteriaceae*.

In the second step of the research, a global picture of the lactic microflora of Grana Trentino cheese was provided and the influence of different storage milk temperatures on the microbiological and physicochemical characteristics of the cheese has been finally evaluated. In particular, physico-chemical characteristics, proteolysis, free amino acids (FAAs) and volatile compunds were evaluated at the end of cheese ripening.

The accurate sampling allowed to follow the microbial sharing in different cheese positions (under crust and core). in the core lower microbial loads were found than under the crust; different species were recognised by DGGE approach: usually *Lb. paracasei* was more present immediately under the crust while *Lb. fermentum* was mostly present in the core. The same trend was observed in all dairies and with both kind of milk delivery (single and double). This phenomenon could be ascribed to the higher temperatures of the cheese core than in the under-crust area during the first and second day after cheese-making.

The cheese proteolysis during ripening was evaluated by Total (TN) and Soluble (SN) Nitrogen fractioning. The percentage of pH 4.6 soluble nitrogen on the total nitrogen of cheese is defined as Ripening Index (RI). Lower temperatures and longer times of milk storage seemed to favour higher values of RI. This index is descriptive of the proportion of casein which is progressively digested by proteolytic enzymes from indigenous milk proteinases (plasmins), togheter with adventitious microflora enzymes. This higher proteolytic activity in cheeses from refrigerated milk could be due to enzymes produced by psycrotrophic bacteria which had more time to work (24 hours in the single milk delivery and about 12 hours in the double one) in milk stored at lower temperatures and are known to be able to survive to cheese cooking temperatures. Higher temperatures of milk storage seemed to favour higher values of soluble PTA SN. The water-soluble peptides and free amino acids are the product of a secondary proteolysis by LAB proteases and peptidases that act on intermediate-sized peptides obtained from the first casein proteolysis. It was shown that LAB, and in particular lattobacilli, development was correlated with higher amounts of free amino acids in the soluble fraction (PTA SN).
Higher amounts of volatile compounds (esters and free fatty acids) were found in cheeses having higher bacteria loads. This confirmed that LAB and their enzymatic activities, coupled with a pool of FAA, promote the flavour development via amino acid conversion into volatile compounds

These results allowed a better understanding of microbial dynamics in an appreciated and economically important cheese like Grana Trentino. The approach utilized, the methods and results obtained, open perspectives for insighting into the enzymatic evolution in cheese ripening.