UNIVERSITÁ DEGLI STUDI DI NAPOLI "FEDERICO II"

DIPARTIMENTO DI AGRARIA

DOTTORATO DI RICERCA IN:

SCIENZE E TECNOLOGIE DELLE PRODUZIONI AGRO-ALIMENTARI

XXVIII Ciclo

STUDY OF FRESHNESS MARKERS IN BUFFALO MOZZARELLA PDO CHEESE, THROUGH A MICROBIOLOGICAL AND PROTEOMIC APPROACH

Coordinatore:

Prof. Giancarlo Barbieri

Candidata:

Dott.sa Loredana Biondi

Tutor:

Prof. Raffaele Romano

Cotutore:

Dott.sa Donatella Nava

A mia madre

Sommario

ABSTRACT

Buffalo Mozzarella of Campania PDOis a typical stretched-cheese product, exclusively produced with buffalo milk, following a historically established method of production. Its characteristics make it one of the most widespread and appreciated products in the world, a success story of the Italy's and Campania's agri-food sector.
Its production has to be in accordance with the strict regulations in the product specification; according to the Reg. CE 103/2008 PDO has to be obtained exclusively from buffalo milk, starting from raw, fresh thermized milk, or pasteurized milk, and sent to production within 60 hours from milking. Its PDO symbol was created as a guarantee of freshness and connection to the territory.
Protecting the authenticity and the origin of the product is of paramount importance to increase value added that otherwise risks, in the long term, to be significantly and negatively affected by illicit practices.
The seasonal nature of buffalo breeding does harm to the animal husbandry sector; the limited availability of milk, together with the increase for the request of mozzarella during the summer, has led producers to fraudulent practices such as using milk and or curd (semi-processed product transformed by adding curd to milk), or products that are frozen, semi finished and bought at a low cost, from Italy and abroad.
The fraudulent use of the raw material or the semi-processed material, contrary to the strict rules, collides with a product evoking an image of freshness. The product image is darkened by choices imposed by the frantic rush to lower production costs.
The wide media coverage on episodes such as the dioxin scandal and that of the Land of Fires did not help the cause of the product, ensuing confusion among consumers, more and more disoriented in choosing.
Campania Region, in these years, has undertaken necessary measures to guarantee health and to gain consumer confidence. The spread of BMC in Italy and abroad has increased, and in 2015 an 8,5% growth in production has been recorded in comparison to the previous year.
The objective of this Ph.D. project has been to first and foremost assess the quality of milk and of the different matricess of the cheese making process of Buffalo Mozzarella of Campania PDO, with a microbiological approach and, successively, to research – with a proteomic approach, a possible marker able to detect the illegal use of frozen milk or curd in making Buffalo Mozzarella.
The proteomic study allowed the assessment of the function of the different protein fragments such as molecular markers which can track the freshness of frozen milk and curd. This study has been particularly focused on milk proteins and, more specifically, on the γ-casein produced by hydrolysis on β-casein through plasmin, an endogenous enzyme filtered by blood in the mammary gland (Trieu-Cuot P. *et al*. 1981).
The research for molecular markers has employed electrophoretic methods.
During the freezing of buffalo milk and semi-processed products, proteolytic processes occur borne by the casein fraction, with new formations of a peptide fragment which is otherwise absent in bovine milk. Di Luccia *et al.* (2009) identified a fragment of β-casein, listed in literature as f (69-209), weighing 15,748 Da, deriving from the action of an endogenous enzyme of blood origin: plasmin. The hydrolysis produced by this enzyme on β-casein may possibly form, in correspondence of the Lys68-Ser69 amino acids (absent in bovine milk) a fragment proposed as a possible marker of the freshness of mozzarella. Its presence in raw milk

appears as a weak bandwidth increasing with the time of freezing; since it has been identified also in frozen buffalo curd, it has been proposed as a possible freshness marker.
In this work we want to study the efficacy of such a marker and the increase of its intensity with time. To this aim, three dairies located in Campania Region: Caserta, Salerno, Napoli, named A, B, C. For each dairy 12 matricess from all production processes have been taken, starting from raw milk, thermized milk (68°C), premature curd (pH 6,3 - 6,2), mature curd (5,0 - 4,8)and mozzarella. The matricess have been kept at freezing temperature (-20°C) and first analyzed in their fresh state and then, each month, after thawing, for twelve months.
The good quality of the original milk and intermediate products has been verified first, in order to successfully identify a molecular marker, and to accomplish this both chemical and microbiological analysis have been carried out. Microbiological analysis are relative to pathogenic microorganisms, pro-technological microorganisms and spoiler microorganisms; chemical analysis were: pH, free water (Aw), titratable acidity, somatic cells, fat, proteins and lactose. At the same time, from the same matricess caseins contained in the protein fraction were taken each month, for a year, and underwent proteomic analysis. In particular, protein matricess were dried and kept at - 20°C and underwent electrophoretic investigations for the research of the above mentioned molecular marker.

1 INTRODUCTION

We unfortunately live in a time of economic recession, with a marked decline in consumption. The free market of goods and people, the opening of borders and the rise in the cost of oil and raw materials have negatively influenced the operating costs of companies. Many dairy producers started reducing costs, preferring frozen milk or semi-processed products to fresh raw materials.
The buffalo is a polyoestrous and seasonal species, therefore births mostly occur in autumn-winter and some dairies freeze milk or curd, buying it at a low cost, to use it in the spring-winter period, when the production of milk is scarce and the market request for the product is higher (MozzarelladiBufala.org 2013,Zicarelli 1999). Some dairy farms usually freeze curd instead of milk as it requires less labor, less room taken, less danger of microbial alterations. This policy does not favour the consumer who, buying a typical product such as the BMC, chooses a product in which he recognizes a certain raw material, “fresh milk” and nothing else, and he is instead unknowingly frauded. The BMC Product Specification (103/2008) exclusively permits the use of fresh whole buffalo milk, coming only from the protected geographical area. In the past there have been cases in which low-cost frozen curd was imported from abroad, especially from East European countries such as Lithuania, Romania and Bulgaria, against the regulations and practicing unfair competition against those virtuous producers that process milk to mozzarella in accordance to the law. (Il fatto alimentare “La falsa mozzarella conquista il mercato” 11.06.2010). These numbers are cause for serious thought: the price for a liter of milk costs, between March and August, euro 1,35 - 1,50, while in the winter it costseuro 0,90 compared to euro 0,60 for a liter of imported milk (De Augustinis F. **Link**iesta “Il business gonfiato della mozzarella di bufala” 19.05.2014). The price for BMC to large retailers is euro 6,50/kg, even if it should not cost less than euro9/kg, while the price to the public is between 10 and 15 euro/kg. This great price gap is a significant signal.

1.1 THE BUFFALO DAIRY SECTOR IN ITALY

The buffalo dairy sector represents a significant share of our Country's economy, with a production of more than 41.295.000 kg. of Buffalo Mozzarella of Campania (BMC) in 2015. 25% of this production has been exported, in particular to France, Germany, the U.K., the U.S.A., Switzerland and Spain. We can say that in 2015 the product keeps up with the image crisis caused by the dioxin scandal and the Land of Fires, with a surprising +8,5 % compared to 2014, with its 38.068.000 Kg of BMC produced, and compared to the about 37.000.000 of the previous two years (2012 – 2013).
"From 2012 to today there has been a shift from a turnover of300 million Euros to 330 million Euros, while the consumption raised from 500 to 540 millions Euros(L. Pignataro 2016). These data are certified and official, communicated by the Buffalo Mozzarella of Campania PDO's Consortium, to which we need to add data for products outside the PDO mark.
If a buffalo produces an average 12 lt of milk per day, and the average yield is of 24,6% for milk with an 8,3% average fat content and 4,3% protein content, it is easy to calculate that about 3 Kgof mozzarella are made every day. This is a respectable for each buffalo, if we think that in Italy the number of registered buffaloes amounts to 345.000 (Data provided by the Buffalo Mozzarella of Campania PDO's Consortium, www.MozzarelladiBufala.org- Il latte di bufala 2013).
While many national consortia have to deal with saturation problems if not with a market decline altogether, the Buffalo Mozzarella consortium is worried for the limits to its expansions. The relationship between large dairies and small ones is antithetic: while the former push to widen the market, the latter, thanks to the attention paid by the finest restaurants and especially thanks to the sector of Neapolitan pizza, in incredibly rapid expansion, have found a wider market space. It is the same opposition as that of industrial and artisanal panettone: for the former the revenue comes from the high number of pieces produced, in the second by the quality of artisanal work and the marketing philosophy of "small and beautiful", that always works in Italy (L. Pignataro, 2016).
With such results it is surprising that one still hears of frozen milk or curd. The highest officials of the Consortium have declared the will to better manage the winter months, where the sales decrease compared to production, by concentrating the sales especially in the sectors of catering and pizzerias but excluding the possibility of making the freezing of milk or curd legal.
The sector, in 2015, comprises 1.371 buffalo breeding farms included in the PDO, with a complexive number of 102 certified dairies with a total number of 15.000 workers (Gambero Rosso). The final consumer price of BMC ranges between 9 euro (price recommended by the Consortium) to 18 euro/Kg, depending on transportation costs, distribution, promotions, packaging etc. Anyway, despite the crisis, the scandals, the fraudulent use of frozen milk or curd, the BMC is a healthy and safe product.

1.2 BUFFALO MOZZARELLA OF CAMPANIA: EVOLUTIONS OF THE PRODUCT SPECIFICATION

Buffalo mozzarella of Campania is one of finest and most loved products of the Made in Italy, within and without Italy's borders.
When one talks of a product of excellence it is necessary to know its history, if only to understand its difficult history, from the origins to the present day.

As every Italian PDO product, a Consortium is necessary, so to protect its brand-name: on July 13 1981 the safeguarding Consortium for mozzarella was born.
The name "Buffalo Mozzarella" was established thanks to the DPR of September 28, 1979 on all the national territory: this cheese had to be compliant with the working methods aimed at obtaining a product with specific organoleptic and marketing characteristics starting from buffalo whole milk. The DPR mentions a stretched-cheese product to be kept in a protective liquid, but does not specify the working methods in any way. It just outlines some objective characteristics of the product, such as: maximum moisture content 65% and fat content 50 % of the dry matter, and neither it lists properties such as freshness nor the ultra-thin crust and release of whey when sliced.
The Product Specification has been frequently updated, and it will further change in the future to make this product always up to date and available worldwide.
14 years after its birth in 1979, the Prime Ministerial Decree (DPCM) of May 10, 1993, changed the name "*Buffalo Mozzarella*" in "*Buffalo Mozzarella of Campania*", change completed in "*Buffalo Mozzarella of Campania PDO*" from the Regulation (CE) 1107 of June 12, 1996.
The 1993 DPCM established the area of origin of milk; ten years later, in 2003, the Consortium asked to widen the area of origin with the decree of the Ministry of Agricultural and Forestry Policy (MIPAF) of September 18, 2003. The area has been extended to four communes of Campania's five provinces (Napoli, Avellino, Benevento and Caserta) and some communes of the province of Frosinone, Latina, Foggia and only one in the province of Isernia: Venafro. These communes have been recognized for their link to the area and the tradition in producing Buffalo mozzarella.
The buffaloes have to belong to the Mediterranean race exclusively and bred in the PDO area, as the Specification requires.
The MIPAF decree of September 18, 2003 was received by the European Union with the Regulation CE103/2008. Such a regulation basically modifies 1993's regulation in four points:

1) *fresh whole milk* (fat 7,2% and minimum protein 4,2%) and processed raw, thermized or pasteurized to prevent the use of frozen milk, making the other thermic methods not specified by the DPCM explicit.
2) The milk must be *delivered and processed* in BMC within 60 hours from the first milking, eliminating the obligation of delivering within 16 hours, allowing the storage of milk and dairies to close for a day during the week.
3) The *acidification* of the curd is obtained by adding natural whey rennet from earlier processing. After the milk is heated at a temperature ranging from 33°C to 39°C, coagulation is obtained.
4) The *packaging* must be carried out in the same production site, to make tracking easier.

The problems concerning tracking frozen milk and curd pushed the protection Consortium to request further modifications to the Reg. CE 103/2008, which require all buffalo milk in the production sites to be certified as appropriate for producing BMC and to "forbid" the purchase of any frozen, semi-processed buffalo product.

SHAPE AND ORGANOLEPTIC CHARACTERISTICS OF BUFFALO MOZZARELLA

The Specification lays down different shapes, from the round one to "nuggests", "cherries", "pearls", "braids", "knots", to which successively has been added egg-shaped ones.
The weight can vary from 10 to 800 gr in relation to the shape; the "braid" can weigh up to 3 kg.
External appearance: *porcelain white in colour, ultra-thin crust* (about 1 mm), *smooth surface* which *must never be slimy* (caused by microflora) *nor flaky* (caused by incorrect storage). The surface presents, more or less clearly, some irregular features caused by the manual or mechanical detachment of mozzarella from the cheese *pasta*.
The *pasta* must have a *leaf-like structure*, with the external film protecting the underlying *pasta* from which it must detach with a clean break. The pasta must be *slightly elasticin the first 8-10 hours of production and must tend to turn into a melt-in-the-mouth texture, devoid of flaws such as stains, produced by foreign or gaseous fermentations*. It *must be devoid of dye, preservatives or inhibitors.* The texture felt in the first moments of chewing can vary from elastic to rubbery, depending on the hardness of the *pasta*. *When sliced, it releases a whitish, fat whey which smells of lactic starters.The taste is sweet, slightly acerbic, delicate and characteristic, due to the strong aromas of the original milk which depends on the zootechnical feed of aromatic herbs or silage, depending on the season*.
BMC has a *52% minimum fat content of the dry matter, 65% maximum moisture content and a pH of 5.1 – 5.6.*
BMC contains less calories than other seasoned hard cheese, which contain about 400 Kcal/100g; blue cheese 358 Kcal/100 g against a value of 288 Kcal/100 g in Mozzarella with the presence of: 17 g of proteins, 24 g of fats (especially trans fatty acids and short-chain fatty acids), with a low cholesterol content, never higher than 50/60 gr (lower than the limit set by the World Health Organization, 300 mg a day); lactose is extremely low 0,4 g. (Focus 2013; Dati Consorzio Mozzarella di Bufala del 2015).
The Product Specification risks to undergo further modifications, namely the possibility of producing BMC with a percentage of fresh milk which has been frozen and not used within 60 hours from the delivery to the dairy, in a percentage no higher than 25%. This is the most recent proposal of amendment to the Specification (Agro Notizie – Zootecnia). This amendment is hardly realistic, since the great performance of sales and turnover in 2015, recording an increase of 8,5% compared to 2014, in a sector that despite numerous scandals enjoys a time of great success.

1.3.TECHNOLOGICAL CHEESE-MAKING PROCESS

The technological processing method requires the milk to be filtered and heated, undergoing a thermization between 33°C and 39°C. The Specification requires the addition of natural whey rennet from the processing of the day before, and after a variable time of four-five hours from the adding of natural curd – from the fourth stomach of the calf – a mature curd is obtained. The curd is cut in large cubes, ready to be stretched with boiling water, at a temperature of about 90 °C if measured at the centre of the mass, it is taken from the tables where it is left for the syneresis (the separation of whey from curd), and chopped in nut-sized fragments, before being stretched. The shaping follows the stretching, in the shapes and sizes listed by the Specification, then passing to the cooling phase in drinkable water for some minutes, and to salting, for a variable period, depending on weight and saline concentration of the brine. Immediately after follows the packaging, with a wrapping carrying the BMC PDO marks and the respective logos. An optional smoking can be exclusively carried out with natural and traditional methods: in this case the wrapping shall state “smoked”.

1.3.1. PHASES OF MILK PROCESSING IN BUFFALO MOZZARELLA OF CAMPANIA

The technology employed to make buffalo mozzarella is the same of soft stretched cheese. A traditional outline of the process of Buffalo Mozzarella can be divided in two phases.
In the first phase, the curd is prepared and then it matures in whey until it reaches a sufficient acidity, which allows it undergo the stretching. In the second, the acidified curd is stretched so to acquire the characteristic structure of buffalo mozzarella.
The processing cycle for this type of cheese is completed, in the second phase, by shaping, salting and packaging.
All the processing phases of Buffalo Mozzarella of Campania (BMC) must at all times follow the requirements of the Product Specification for the protection of the “Buffalo Mozzarella of Campania”, PDO.
HEAT-TREATMENT OF MILK
The first operation of cheese making is the preparation of milk. As it arrives on site, milk undergoes filtration to eliminate foreign matter, then it is thermally cooled and sent to production for processing or in tanks for storage at 4°C if put on hold. For processing it enters production again and sent to stainless steel boilers, with an average capacity of 10 quintals and the quantity of added whey is never more than 2,5%. Heating happens with a direct steam flow until reaching an optimal temperature between 33 °C and 39 °C, when curd is added (18-20 ml/ql of milk).

Photo n°1: mature curd

COAGULATION OF MILK

Dairy companies use edible curds of animal origin. Nevertheless, several types exist: vegetal, bacterial and fungal, with different textures: solid, liquid and paste. The term "curd" is reserved to the enzymatic preparation extracted from the fourth stomach of young ruminants which have been slaughtered before weaning. The coagulant enzymes of curd are: chymosin, pepsin and some alkaline protease such as cathepsin (Alais 1984). For the production of BMC, as the Specification requires, only liquid calf curd is used. The coagulating effect derives from the enzyme ability to hydrolyse the Phe_{105}-Met_{106}bond. The bond breaking deprives k-casein of its colloidal-protector effect on casein's micelles, causing the formation of para-k-casein and caseinomacropeptide (CMP). In this way, casein's micelles are destabilized and milk coagulates in the presence of calcium. The coagulation of milk always happens thanks to the combined action of pH, temperature, mineral salts and is preceded by the adding of natural whey rennet. This is the whey obtained from the processing of the day before. This whey contains a indigenous composite microflora, made of streptococci and thermophilic lactobacilli, mesophilic streptococci, micrococci and often yeasts, from which the product takes its typical nature (Salvadori del Prato 1998).

The acidity of the whey rennet usually ranges between 40 and 60 ° SH/100 ml, and the temperature is > 40°C.

The processing phase includes a slow beginning of the coagulation, a slow development of acidity, a long time for the curd to mature (Albd-Ed-Salam *et al*., 1990;Ganguli *et al*., 1974).

We can separate three phases in the enzymatic coagulation.

PHASE I, consists in the *detachment* of k-casein's glycolipid. The detachment can take place in pH conditions ranging between 4,6 and 6,5 and temperatures between 37 and 45 °C. If only this phase takes place, the milk stays in a liquid state. To move to a gel state bonds among the destabilized micelles must occur. Coagulation is not evident until 60-80% of k-casein is hydrolysed by chymosin.

PHASE II, non-enzymatic: a *homogeneous gel is formed*: curd. The destabilized micelles aggregate flocculating and making a tri-dimensional grid that slowly acquires texture. In this phase temperature is of paramount importance: it must never reach less than 18 °C and never more than 40 °C; at least 80 mg/l of ionic calcium must be present.

Photo n° 2 : fragment of mature curds

Temperature is essential for the creation of hydrogen bonds among micelles. The gel has the texture of a viscous-elastic material, constantly changing with time. The texture of the curd is the function of the content in ionic calcium of milk, and the quantity of casein there present. The bonds among the micelles contract so to let micelles merge one into another. The pH of the mature curd is 4,70 - 4,90 ph units.

PHASE III, This phase is called *syneresis*, where whey leaks out. In the dairy, this phase is called *draining* and it is simultaneous and successive to the first two phases. The syneresis is a spontaneous process, which takes place because of the contractions of fibrils in the

proteinaceous graticule. This contraction exerts a pressure on the whey imprisoned in the coagulum graticule, and forces it to leak. The leak of whey determines a narrowing of the net around the globules of fat, and the remaining whey fills the spaces among the globules of fat and the casein net, working as a lubricant (Addeo *et al*., 1996). Once the coagulation is over and the syneresis starts, the secondary draining phase takes place.
Immediately after the curd is BROKEN UP with a simple, traditional cross cut, until it reaches the size of very small grains, whose size depends on the cheese to be made. The breaking of curd is usually made by hand, with a club on whose end there is a metallic spike.
The breaking of curd almost inevitably causes a loss of fat in whey. These losses can be limited by slowly stirring. Once the breaking is complete, the *pasta* is left to mature in whey or outside.
In the artisanal processing cycle acidification takes on average 3-4 hours, even if at times it can last up to 8 hours. The duration of the acidification of the curd under the whey is one of the variables more sensibly affecting the quality of the cheese. What determines the length of this processing phase is: the microbial quality of milk, acidity, enzyme concentration, temperature, degree of crumbling of the curd, the quality of the whey rennet used, the availability of calcium.
The time the curd takes to mature is variable, and it is so in different dairies or even in the same one; this can be attributed to the fact that the BMC process always uses raw milk, whose natural cultures of lactic bacteria have a composition that varies with time; one has also to take into account the methods to obtain whey rennet, from whose variables microorganisms inhibiting cheese-production can develop. Mozzarella whey, differently to that obtained in the making of pressed cheese, does not undergo any thermal treatment, therefore a selection of indigenous microflora only takes place depending on acidity development.
The Product Specification requires that the MATURATION of the curd has to take place exclusively by natural fermentation, so without intervention on the pH – by adding organic (citric, acetic, lactic) or mineral (phosphoric) acids to milk before the curd coagulates. The maturation phase is finished on a table, where all the pieces of curd are cut with a sickle to facilitate the purging of whey. The thickness of the curd should be less than 20 cms, in order to obtain a quick and homogeneous acidification and to allow faster exchanges between whey and curd. Another factor influencing the positive outcome of curd acidification is temperature, which has to remain constant. Once the curd is mature, therefore compact and homogeneous with regular openings, it is usually taken from the tables to be minced and be made ready for the stretching.
STRETCHING The curd undergoes a process of stretching in hot water, at a temperature of about 90 °C in the centre of the *pasta*. The “casaro” dairyman decides the moment when to start the stretching process, based on an empirical test. Such a test consists in melting in hot water about 100 grams of pasta, which is stirred with a stick. The excess water is purged and the molten pasta is put on the stick, so that due to gravity it tends to stretch. If the molten pasta, stretched by hand, reaches a length of about 1 meter, it is considered ready for the stretching process.
Traditionally thepasta is stretched by hand. However, many companies nowadays use a machine, called “Turnbuckle”, based on the movement of screws rotating in opposite direction, so to knead and stretch the curds which have been previously matured and warmed in a sliding tank containing boiling water. How much a curd can be stretched depends on the temperature changes: curd can stretch the most around 68 °C. Molten curd breaks at low temperature, while at high temperature the curd is so weak that the material is unable to sustain its own weight, losing texture.
SHAPING Mozzarella is traditionally shaped by hand by two dairymen: one chops – using the thumb and the index fingers – pieces of stretched pasta of about 3-4 kg , while the other carries the pieces. Most dairies today use mechanical shapers, with which the most various sizes are obtainable.

SOLIDIFICATION The shaped product is put in tanks containing cold water; the time it stays there depends on the size. After, it is moved to the brine.
SALTING The salting is obtained by immersing the product in different saline solutions with a varying degree of concentration, from 10 to 18%. The duration of this operation varies depending on the dairy, but generally it does not last more than 10 hours, for sizes of 400-500 gr. The current trend is to eliminate the salting phase altogether; the alternative is to salt the pastaor stretching with salt water.
During the time the cheese spends in the brine bath, salt penetrates it by diffusion, and the speed depends on the brine's saline concentration, on the temperature and the size of mozzarella.
The process is extremely slow, therefore at the end of the period of the brine bath, the concentration of salt in cheese is not homogeneous; for it to be homogeneous, 72 hours are necessary. Once the product is take out of the brine bath and immersed in the protective liquid, the salt concentration tends to re-equilibrate: from the external layers, where the concentration is high, salt moves towards the internal layers. The protective liquid is usually made of an acidic solution containing 2-3 % sodium chloride. Since the salt concentration on the surface is higher than in the protective liquid, a migration process of salt from the product to the protective liquid, and so it is possible that during the conservation of cheese in the protective liquid the salt level decreases too much, causing a sloughing of mozzarella. Another incident that might occur is the excessive migration of water, that by osmosis moves from cheese to the saline solution, with a consequent increase in texture.
PACKAGING After salting, mozzarella is kept in the protective liquid and packed in it; this liquid is composed of stretching water, salt and diluted acid whey. In the summer, the composition of the protective liquid is changed in function of the product destination and the period of permanence on the market. To limit protective liquid fermentations, in case long storage periods, sub-neutral pH buffered water is used, about 1 liter per kg of cheese.

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2. BUFFALO MILK

Milk is defined as: “an opaque white or yellowish liquid, sweet in flavour, coming from the regular, uninterrupted and complete milking of the mammary gland of animals in good health and feeding state”. RD May 9, 1929 n°. 994 and smi. (fig. 1)

Figura 1 Buffalo

Milk is a heterogeneous, complex mixture, in which we can separate three different phases:

- The **true solution** phase, composed of substances soluble in a watery solution, among which carbohydrates, in particular lactose, mineral salts, soluble proteins, non-proteinaceous nitrogenous substances, enzymes, water-soluble vitamins;
- the **colloidal suspension** phase, composed of casein micelles, albumin, globulin dispersed in the watery solution, part of phosphate and calcium citrate;
- the **emulsion** phase, composed of fat globules in aqueousphase and fat-soluble vitamins (Del Prato 1998).

These different phases are in a state of unstable equilibrium. The storage of milk at room temperature causes the fat emulsion to rise to the surface within 12-24 hours, and after the biological action of microorganisms, the casein suspension separates with the formation of a coagulum (flake).
(Charles Alais nuova ed. 1985) (tab. 1)

MIK IS AHETEROGENEOUS SUBSTANCE FROM THE PHYSICO-CHEMICAL PERSPECTIVE

BUFFALO MILK

81,9 % WATER

6,8-7,8% FAT ⟶ EMULSION

4,3 % PROTEIN ⟶ SUSPENSION

5,0% CARBOHYDRATES

→ IN SOLUTION

0,8% MINERALS

Tab. 1 - Composition of buffalo milk

Physico-chemical composition of buffalo and bovine milk

CHEMICAL COMPOSITION	BUFFALO MILK	BOVINE MILK
Energetic value Kcal/l	1100	700
Water %	80,7	87,5
Dry residue %	19,2	12,5
Fat %	8,8	3,5
Protein %	4-5	3,2
Casein %	3,8	2,6
Serum protein %	1,1	0,6
Lactalbmumin Lactoglobulin%	0,7	0,6
Lactose %	5,0	4,5
Ash%	0,8	0,75
pH	6,67	6,5
Acidity (°SH)	10,12	7,1
Density (15°C)	1,031	1,029
Specific gravity	1,033	1,031
Ca mg/l	190	119
P mg/l	124	88
Ca/P	1,61	1,31
Mineral salts	0,24-1,17	1

Tab. 2 - Source: **Institute for Food and Nutrition Research**

COMPOSITION OF TOTAL NITROGENOUS MATTER (TNM)

95% PROTEIN
- CASEIN about 80% → α_{S1} - α_{S2} - β - κ - γ CN
- SERUM PROTEIN about 20% →
  - IMMUNOGLOBULINS
    - β-lactoglobulin
    - α-lactalbumin
    - Serum albumin
    - Immunoglobulin
  - Enzymes

5% NITROGENOUS SUBSTANCES → UREA, α-AA, CREATINE, CREATININE,

NON PROTEINACEOUS	AMMONIA, URIC ACID,
OTHER MINOR NITROGEN COMPUNDS	
SUCH AS PROTEOSE PEPTONE	

Tab. 3 - Total nitrogenous substances

2.1. PROTEIN COMPONENT

Nitrogenous substances in milk represent the most complex and heterogeneous part, with a fundamental nutritional, biological and technological importance.
The content of total nitrogenous substances is very variable, on average there is a 4-5% (g/lt) and this content is heavily influenced by race, lactation stage, age of animals, season, somatic cells and nutrition state (McLean *et al*, 1984; Ng-Kwai-Hang *et al*., 1982).
95% of this 5% of nitrogenous substances in milk is composed of protein nitrogen, while the remaining 5% is composed of soluble nitrogen compounds with a low molecular weight (NPN).
Protein nitrogen is in turn composed by casein (80%), present in milk under the form of a micellar suspension of phosphocasein of insoluble calcium, and by serum protein (20%),, made of β-lactoglobulin, α-lactalbumin and other soluble polypeptides (Tab.3). β-lactoglobulin, α-lactalbumin and casein come from the mammary gland, while serum albumin and immunoglobulin come from blood.
5% of the non-protein nitrogenous compounds with a low molecular weight are made of urea, free amino acids, creatine, creatinine and other minor nitrogen compounds, soluble in lactic plasma (Salvadori del Prato 1998).
Milk proteins are made of – just like bovine ones – insoluble casein with a pH of 4,6 and serum protein with the same pH.
Serum protein contain much more sulphur amino acids (cystine, cysteine, methionine) than casein, making them highly nutritious for man. The most distinctive difference between serum protein and casein is their lower molecular weight (~ 18.000 β-lactoglobulin, ~ 14.000 α-lactalbumin), which allows this protein not to undergo isoelectric precipitation (Salvatori del Prato, 1998). Serum protein are not protein aggregates such as casein, but are present in milk as monomers or polymers that precipitate due to high heating or salting but not by an enzymatic action: this property is exploited in the making of ricotta cheese.
Milk also contains a group of small peptides, listed as proteose-peptone – which mainly derive from casein decomposition through the action of protease. These are enzymes acting on protein and causing its breakdown to produce smaller fragments or single amino acids.

2.1.1. CASEIN IN BUFFALO MILK

Casein is made of a group of phosphoprotein which can precipitate by curd's enzymatic action or by the acidification of milk with a pH of 4.6 (casein isoelectric point). Casein is constituted by an acid, globular proteinaceous complex of milk, present in four protein fractions genetically determined, with a molecular weight ranging between 19.000 and 25.000 Da (Tab. 5), called αs_1-casein (αs_1-CN), αs_2-casein (αs_2-CN), β-casein (β-CN) andκ-casein (κ-CN). A fifth fraction, γ-casein (γ-CN), is a peptide fragment deriving from the proteolysis of β-casein by plasmin.

The family of αs_1 and αs_2 casein differs in the amino acid sequence. The αs_1-CN are particularly sensitive to calcium (actually the letter "s" point exactly the sensitivity to calcium) at all temperatures. Casein is synthesised in the mammary gland and is largely present in the form of micelles, appearing – in the Golgi apparatus – in its distinctive shape thanks to the casein-calcium-phosphate bonds and is similar to bovine casein.
Buffalo milk seems to have a higher percentage of κ-CN and αs_2-CN compared to bovine milk and a lower content of β-CN and αs_1-CN. The higher content of αs_2-CN is due to its higher sensitivity to ionic calcium, influencing the texture of curd (Addeo *et al.* 1977) under a technological profile.
Buffalo **αs_1-CN** is a polypeptide chain of 199 amino acid residues, and compared to the variant C of the bovine homologous, differs for the substitution of 10 amino acids that determine its lower negative charge and so its lower electrophoretic mobility through UREA PAGE (pH8.6) (Chianese *et al*., 1996).
Buffalo αs_1-CN is more heterogeneous for the presence of at least threephosphorylated components αs_1-CN 6P,7P,8P, compared to the bovine homologous 8P, 9P. Due to a mutation, there is a phosphorylate residue instead of a hydrophobic one, which highlights the non-polar character of a protein trait. In particular in bovine milk, αs1 casein possesses a phosphoserinic group in position 115, surrounded by hydrophobic amino acids. The lack of phosphoserine 115 in buffalo αs_1 casein reinforces the non-polar character of this protein (Ferranti *et al*., 1998).
Through isoelectric focusing, buffalo αs_1-CN is shown as made of 3 bands, of which the fastest has the same mobility of its bovine homologous (Ferranti *et al*. 1998). Its molecular weight is 23,616 Daltons (Salvadoridel Prato 1998).
Buffalo **β-CN** is a single chain polypeptide, whose primary structure is made of 209 amino acids, like its bovine counterpart. It's more hydrophobic due to the presence of proline and non-polar α-amino acids. It has a molecular weight of 23,982 Daltons, similar to αs_1-CN (Corradini, 1995). There are 9 known gene variants, A_1, A_2, A_3, B, C, D, E, F, G. Variants A and B have been studied and are listed as β-CN A and β-CN B, and through alkaline PAGE analysis show different electrophoretic mobility A>B for the mutation of the Thr (A) ⟶Met (B)in position 41 (Ferranti *et al*. 1998).
Variant A has been found in the Venezuelan Buffalo, compared to Variant B, more common in the Mediterranean race, bred in Campania. Variant B presents an amino acid sequence in position 68of the Asn(A) ⟶Lys(B).
β-CN is a *plasmin* substrate, an endogenous enzyme of blood origin, derived from milk, active on other types of casein but especially on β-CN(Eigel*et al.* 1984; Fox *et al*. 1994) and on αs_2-CN(Le Bars&Gripon 1989). Medical conditions such as mastitis, enhances the activity of this enzyme, whose action on indigenous proteins determines the formation of the γ_1, γ_2, γ_3-CN by hydrolysing the Lys_{28}-Lys_{29}, Lys_{105}-$His_{106}$and Lys_{107}-Glu_{108} bond, with the formation of the f(29-209) γ_1-CN, f(106-207) γ_2-CN and f(108-209)γ_3-CN of β-CN fragment (Alais 1984).
Apart from plasmin, β-CN is hydrolysed by *chymosin*, exogenous enzyme also active on αs_1-CN (Carles C. &Ribadieau-Dumas B.1984).Faccia *et al*. 2014 reported that the quantification of the αs_1-CN fragment can be effective to reveal the use of frozen curd in the production of Fiordilatte, considering that in the stretching phase (90°C), chymosin is undenatured and hydrolysis is low on αs_1-CN (Sheehan JJ et *al*. 2007, Faccia M. et *al* 2014); reason why this fragment cannot be identified as a possible marker in the finished product.
In fresh cheese such as mozzarella, primary proteolysis of αs_1-CN and β-CN occur during the storage at 4°C (Farkye NY *et al*. 1991; Di Matteo *et al*. 1982).
Trieu-Cuot&Addeo in 1981 studied the formation of a fragment present in buffalo milk and absent in bovine milk which is produced by the action of plasmin on β-CN, identified by Di Luccia *et al.* in 2009 as a fourth fragment deriving from the hydrolysis of the peptide bond in position Lys_{68}-Lys_{69} with the new formation of the fragment f(69-209), named γ_4-CN, first

product whose concentration in milk seems to increases in refrigerated and frozen milk, curd and mozzarella.

αs_2-CN is made of 207 amino acids. It is characteristic as it is bound to a different number of phosphate groups that, during electrophoretic separation, move between αs_1-CN and β-CN. αs_2-CN are highly phosphorylated peptides containing (10P-13P).(Ginger *et al.* 2002).

It is weakly hydrophobic and this separates it from other types of calcium-sensitive casein and just like the other two αs_1-CN e β-CN, it is made stable by the reaction to κ-CN. Its molecular weight varies from 25,150 to 25,390 Daltons because of the change in quantity of phosphoric residues (Salvadori del Prato 1998).

κ-CN is formed by a chain of 169 amino acid residues, like in its bovine counterpart, with a molecular weight of about 19.000 Da. It has the task of stabilize casein micelles present in milk against calcium: it has therefore a colloidal-protector.

As it is widely known, this fraction is specifically hydrolysed in two fragments under the action of chymosin, exogenous enzyme of calf curd, by a specific action on the Met_{105}-Phe_{106} bond, forming insoluble *para K casein* (f N-terminal 1-105) and *macropeptide-casein* (f C-terminal 106-169), the only soluble casein in the presence of calcium ions at any temperature.

Casein in gr/100gr of milk

α s1	**33**
αs2	**11**
ß	**33**
K	**11**
γ 4	**4**

Tabella 4 –Composition of casein micelles. Source: Salvadori del Prato 1998

2.1.2. MICELLAR CASEIN

Casein is found in milk not as free molecules but in the form of micelles, namely spherical aggregates with a porous and spongy appearance, disperse in a continuous phase of water, salt, lactose, whey and protein (Cross *et al*., 2005).Casein micelles are present in large quantities in milk (about 10^{14}/mL), with a variable diameter ranging from 20 to 300 nm, therefore presenting an extremely high surface of action, around 4 m^2/mL. These are composed of many sub-micelles, in turn made of casein molecules, so that the sub-micelles presenting a higher percentage of k-casein are on the outside – since they possess a very polar portion, they form a kind of protective dome which allows the micelle to remain in suspension (Alais 1984), (fig. 2). The aggregation of sub-micelles is favoured by the presence of phosphoserinic sites, located in the external part of the sub-micelles. The serine amino acid present in protein chains can bond phosphor groups. It is known that not all types of casein present the same serine values; for example, α_{S2} has many residues, determining a higher presence of phosphoric groups. The bonds between calcium phosphate and phosphoserinic groups determine the formation of bonds among the sub-micelles (Schmidt 1982), (fig. 3). The aggregation of casein molecules inside sub-micelles is made possible by several hydrogen bonds, while the total micellar structure is made stable by apatite bonds, made of ionic calcium interacting with the phosphates linked to casein. It is therefore essential for calcium to keep the system stable, as essential is a stable pH.

An excessive acidification destroys the stabilizing bonds between calcium and phosphorus, which become a solution, causing the disintegration of the micelle and a consequent flocculation.

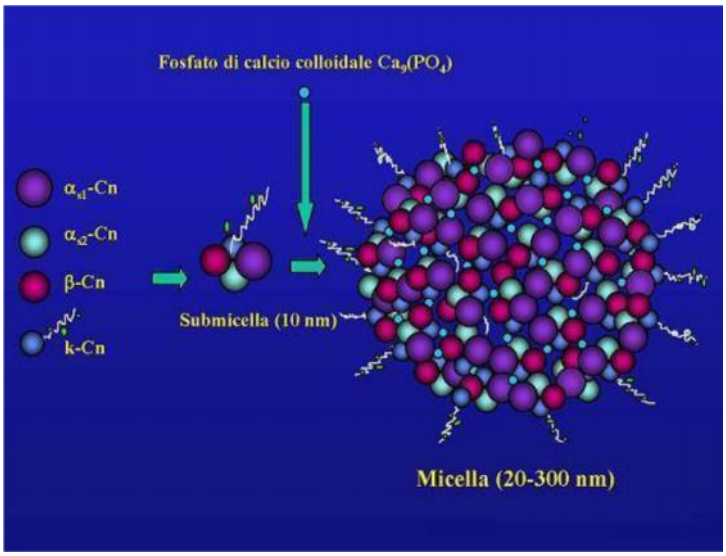

Fig. 2: Formation of a casein micelle Source: **Martini P. 1995**

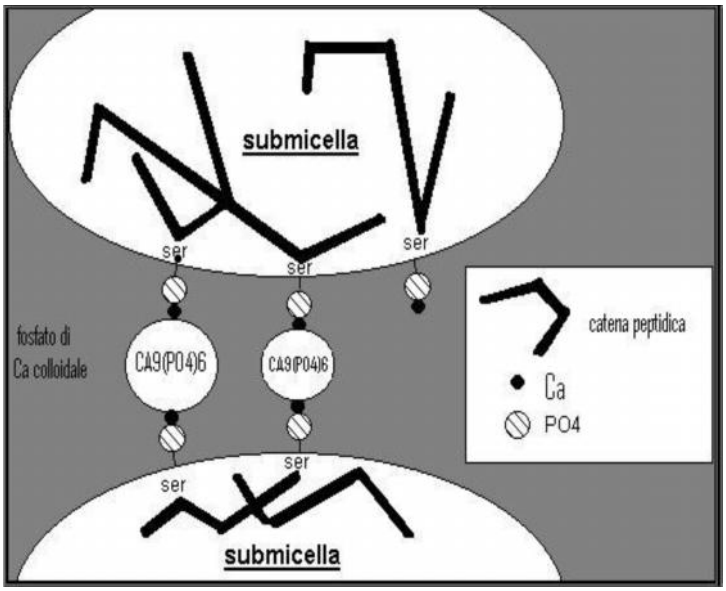

Fig. 3: Outline of the union of two sub-micelles by calcium phosphate Source: Schmidt 1982

Tabella 5 - Properties of the main types of casein. Source: Sciancalepore 1999

	α_{s1}	α_{s2}	β	K
Number of amino acids	199	207	209	169
Molecular weight (Da)	23.600	25.200	24.000	19.000
Cysteinic residues	0	2	0	2
Phosphore groups	8-9	10-13	5	1-2
Carbohydrates	None	None	None	galactose glucosamine . acetylneuramic acid
Types of binding in the associations	hydrogen hydrophobic	elecotrstatic	hydrophobic	not well defined
Sensitivity to chymases	+	-	+	+ + +
Sensitivity to calcium	+ +	+ + +	+	-

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3. ENZYME ACTIVITY IN MILK

Milk contains a large number of active enzymes, of which more than sixty have been identified. Around twenty enzymes have been isolated and characterised from milk. These are enzymes present in large quantities and in most cases, they are significant from a technological perspective. These enzymes play a very important role on the degradation of milk substances, with consequences on the technological characteristics and also as a cause for the possible occurrence of flaws in the products.
Some enzymes are important as indicators of a sufficient degree of pasteurization of milk (alkaline phosphatase) or mastitis (N-acetylglucosaminidase, acid phosphatase) or can provide clues on how healthy the milk is (catalase and lactoperoxidase).

Some enzymes are concentrated on the external layer of the fat globule (aldehyde-reductase, phosphatase) in somatic cells or in casein micelles, where they precipate to the isoelectric point (pH 4.6) (protease and lipase). Our attention now focuses on protease.
These can have favourable effects such as the development of flavour or changes in texture during the maturing period of cheeses. Protease of bacterial origin are able to produce undesired change in milk or in dairy products, such as those cause by *psychrophilic* bacteria o *Pseudomonas* (Alais 1984).

3.1 PROTEASE IN MILK

The protein system of milk is a fundamental element, both regarding the nutritional value of milk, both for the implications on the technological characteristics of milk. It is a dynamic system: lactoproteins synthesised by mammary cells are subjected to the action of proteolytic enzymes, with important repercussions on technological and nutritional aspects of milk. Enzymatic proteolysis of protein can have positive effects on aspects such as the development of flavour or change in texture during the maturing phase of cheese. However, an undesired proteolysis can negatively affect the quality of milk, milk products and their shelf-life.
Proteolytic enzymes in milk are divided into *endogenous*, such as plasmin, cathepsin D, cathepsin B and elastase, and *exogenous*, such as chymosin, pepsine and microbial enzymes accidentally introduced, or after a technological process.
Proteases contained in milk degrade proteins, reducing them to small fragments.
We separate between *alkaline* proteases, with a molecular weight of about 48.000 Da and optimal pH 7,5 containing serine; an important role is played by *plasmin*, which is able to produce the hydrolysis of ß-casein, from which γ-casein originate. Its inactive form is zymogen, whose activation occurs thanks to the function of plasminogen.
Acid proteases, instead, have an optimal pH of 4,0 and a molecular weight of about 36.000 Da, they are not proteases containing serine and they are especially active on αs_1-CN, which is able to hydrolyze it in αs_1-I-CN like *chymosin* does. Some studies have shown that it has a role in the maturing of cheese.
There are different types of proteases in milk, some originate from the contamination of microorganisms and other are transferred to milk from blood. Several are the factors influencing the concentration of such enzymes in milk, such as: race, nutrition, lactation and ill health, i.e. mastitis.
Milk also contains proteases which are not derived from leucocytes (somatic milk cells).
In the rest of this work we are going to deepen the study of the endogenous enzyme present in milk, known as plasmin (Alais 1984).

3.2 PLASMIN AND PLASMINOGEN

Plasmin, the main indigenous milk protease of, is a serine protease as its active site is the serine amino acid. Plasmin is present in blood and in milk in the form of a non-active precursor, plasminogen (PG), which after the action of the plasminogen's tissue activator (tPA) and plasminogen's urokinase activator (uPA), produces its active form, **plasmin** (PL) by the hydrolysis of the peptide bond Arg557-Ile558. Plasmin is made inactive by antiplasmin-alpha 2 and by serine protease inhibitor (serpin).
Plasmin, plasminogens and plasmogen activator are associated to casein micelles, while the inhibitors are located in the whey.

Plasmin is an important enzyme belonging to the hydrolase class, which degrades many proteins; it preferentially splits peptide chains after lysine, an arginine residue, to a lesser extent (Lys-X e Arg-X) (Ueshima, Okada, & Matsuo, 1996). It possesses an optimal pH of 7,5 and a temperature optimum of 37°C (*Fig.4*).

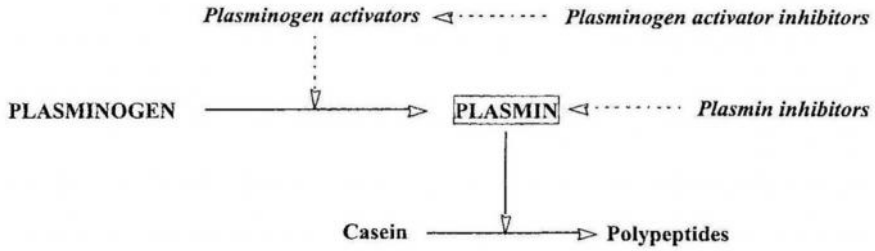

Figura 4 - Outline of the *plasmin* system in milk

Considering its physiological role in blood, it is not surprising that the activity of plasmin is part of complex system of activators and inhibitors (Ismail B & Nielsen SS. 2010). Plasminogen is not present in mammary glands and consequently, in milk, the origin of plasmin and plasminogen is blood (Alichanidis *et al*. 1986); the concentration of plasmin in milk and blood is about 0,3 and 200 mgL^{-1} respectively (Halpaap *et al.* 1977). Plasminogen is a glycoprotein containing 786 amino acid residues and has a molecular mass of 88,092 Da (Schaller *et al*. 1985). The amino acid sequence of human plasminogen differing from bovine plasminogen is longer and is composed of 790 residues, and exists in two variants which have different carbohydrate content (Schaller *et al.* 1985).
Plasminogen possesses five characteristic triple-ring structures (*Fig. 5*) and each of them has a higher affinity for lysine than the others (Bastian and Brown, 1996).

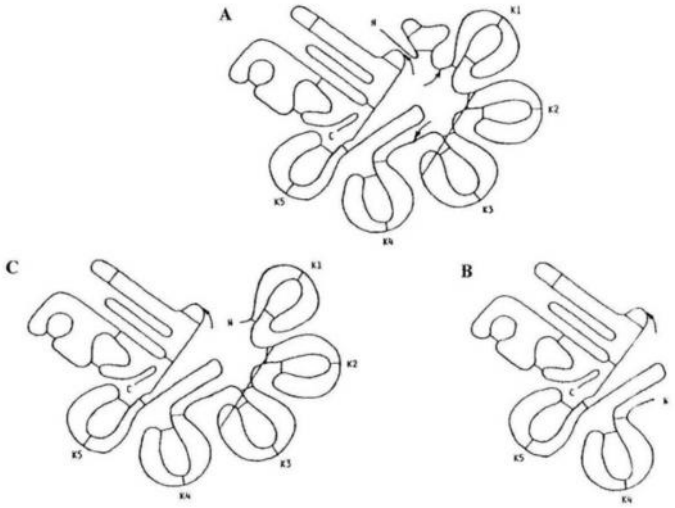

Figura 5 - Models of human plasminogen (A), bovine (B) and human plasmin (C)

Plasmin (PL) starts working when activated, even if it is present in blood in a non-active plasminogen (PG) form. The latter in particular, following the action of the plasminogen tissue activator (tPA) (Zachos *et al.* 1992) and the plasminogen urokinase activator (uPA), by hydrolysis of the peptide bond Arg 557- Ile 558 (While *et al.*1995), leads to the production of its active form, plasmin (Bastian &Brown, 1996). Plasmin is made inactive by antiplasmin-α_2 and by serine protease inhibitor (serpin) (PI), being the amino acid serine its active site.

The concentrations of PL, PG and plasminogen activators (PA) are low in the first stage of lactation (Bastian *et al*. 1991a). Nevertheless, the concentrations of such enzymes in milk increase with the later stages of lactation (Bastian *et al.* 1991a) and are higher during the dry period of the animal. The activity of plasmin in mill is higher in older animals and increases with the lactation number.
Plasmin activity is also influenced by mastitis (Politis *et al.* 1989), as the increase in the number of somatic cells (While *et al.*1995, Heegaard *et al.*1994) contributes to raise the number of plasminogen activators that in turn lead to the production of its active form, plasmin.
Plasminogen converts plasmin faster at body temperature compared to refrigeration temperature; temperature optimum is at 37°C, with pH 7.4-7.5 (Humbert & Alais, 1979). Pasteurization at 72°C for 15 seconds reduces the activity of plasmin in milk only by 10-17% (Bastian & Brown, 1996). However, the storage of pasteurized milk leads to an increase in plasmin activity due to the inactivation of active inhibitors of plasminogen present in milk. To completely inhibit plasmin during storage it is necessary to heat milk at 142°C for 18 seconds or 120°C for 15 minutes.

3.3 PECULIARITIES OF PLASMIN IN CASEIN

The proteolysis in buffalo milk was shown to be caused by an indigenous enzyme, an alkaline serine proteinase, which is a blood enzyme transported from plasma across mammary epithelial cells, this is the plasmin (PL). Its activity is determined by interaction between the inactive precursor, plasminogen, and plasminogen activators, associated with casein micelles (Politis *et al.* 1992).
The PL is an indigenous milk proteolytic enzyme, its importance is as hydrolytic action on casein, the optimum activity at pH 7,5 and 37°C (Humbert & Alais 1979, Gruffery MB, & Fox PF. 1988a, Bastian ED & Brown RJ. 1996, Kelly AL, & McSweeney PLH. 2003).
The enzyme has an affinity for lysine and arginine residues and preferentially claves Lys-X and Arg-X bands (Ueshima S, Okada K, Matsuo O. 1996, Bastian E.D. & Brown R.J. 1996).
PL is associated with casein micelles in milk and degrades αs_1-CN, αs_2-CN e β-CN and proteose-peptones. (Crudden A, *et al*. 2005). In particular, preferentially, it hydrolyzeβ-CN, with production of γ-CN and proteoso-pepton. Also αs_2-CN is readily hydrolyzed by plasmin instate of κ-CN is resistant to proteolysis by plasmin.
There are three plasmin-sensitive bands in β-CN Lys_{28}-Lys_{29}, Lys_{105}-His_{106} and Lys_{107}-Glu_{108}, when plasmin hydrolyzes these bonds, the following peptides are released: γ_1-CN(f29-209), γ_2-CN(f106-209), γ_3-CN(f108-209), proteose-peptone (PP) component 8 fast (f 1-28), β-CN(f 29-105) and (f 29-107), PP component 5 (f 1-105) and (f 1-107). PP component 8-slow was initially (by Eigel & Keenan, 1979) as β-CN (f 29-105 and f 29-107) but this observation was later questioned by Andrews & Alichantis 1983 (Figure 6).
The activity of PL increases in milk, curd and mozzarella cheese when cold stored at 4°C (Somers JM. *et al*. 2002).
Trieu-Cuot P & Addeo F in1981 reported the formation of a fragment, named compound B, produced in river buffalo milk by the action of plasmin on β-CN, which was identified by Di Luccia *et al*. (2009)
As a fourth fragment originating from the presence of plasmin-sensitive Lys band at position 68 that is not present in bovine milk. The presence of β-CN (f69-209) or γ_4-CN, in river buffalo milk, was also reported by Somma *et al*. (2008). Di Luccia *et al.* (2009) established that this fragment was produced during the first fragmentation of β-CN, unlike what occurs in

bovine milk where γ_1 is the first fragment produced, and that fragment γ_4-CN increases in refrigerated and frozen river buffalo milk, in curd and in mozzarella cheese.

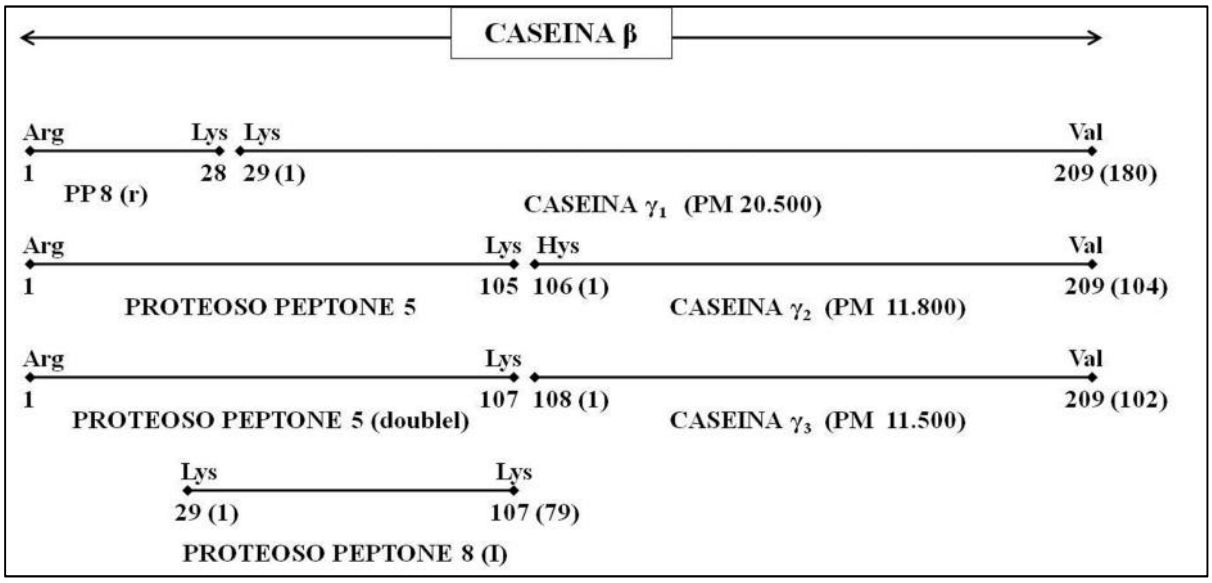

Fig. 6 - Formation of γ caseins and proteoso-peptones from hydrolysis of β-casein. Source:**Alais 1984**

	Buffalo %	*Bovine %*	*Buffalo/Bovine %*
αs1	30.2	38.4	78.6
αs2	17.6	10.5	167.6
β	33.9	36.5	92.9
κ	15.4	12.5	123.2
Total	97.1	97.9	
αs1+αs2	47.8	48.9	97.7

Tab. 6 - Effect of the different casein fractions in cows and in buffalo

3.4 FACTORS INFLUENCING PLASMIN ACTIVITY IN MILK

Plasmin activity is influenced by many factors.

- **Mastitis** Plasmin activity is higher in mastitic milk than in normal milk (Seaman *et al.* 1988, Politis *et al.* 1989 a/b) increased respectively the concentration of plasmin and plasminogen. Politis *et al.* 1989 b reported that all parameters increased during infection, in pre-infection and post-infection period. Most of this increase was from plasmin. They concluded that even after curing mastitis, plasmin activity does not return to its pre-infection level. This may be one reason why milk from older cows has higher plasmin activity than milk from younger cows.
- **Lactation number** Milk from older water buffalo has higher plasmin activity. There is an interaction between age and stage of lactation. Plasmin activity increases in milk from older subjects during lactation (Politis *et al.* 1989 b).
- **Stage of lactation** Plasmin activity and plasminogen increase at the end of lactation (Politis *et al.* 1989 ab, Bastian *et al.* 1991b). Total enzyme (PL and PG) increased during the first six months of lactation, but plasmin in the last three months increased drastically.

- **Milk pH** Plasmin is most active at pH 7,5 to 8,0 and 37°C (Fox *et al*. 1981).
- **Storage conditions** The PL activity is active during cold storage (2-5 °C) the β-CN becomes more soluble at lower temperature. Allow PL proteolysis during refrigerated storage of milk (Crudden *et al.* 2005) with cleavage of Lys68- Ser69 there is the formation of the fragment β-CN (f29-209), has no counterpart in bovine milk. This fragment is a candidate molecular marker to evaluate milk and curd freshness. Changes occur in milk protein micelles during refrigerated storage, in particular β-CN become more soluble at lower temperatures. (Crudden *et al.* 2005). In their study, the authors also observed significant dissociation of β-CN with hydrolysis of Lys 68-Ser 69 by PL and the proteose-pepton complementary β-CN(f1-68) that on presumed to play a physiological role in including milk secretion (Di Luccia *et al.*2009).
- **Bacterial proteases** Numerous factors have been identified that can contribute to proteolysis in milk, one of this is the presence of bacteria proteases. The current trend, today, is lengthened refrigerated storage of milk, enhancing psychotropic bacteria that contribute to proteolysis in storage milk.(Cousin 1982).
  The proteases produced by the psychotropic bacteria can destabilize the casein micelles by hydrolyzing κ-casein (Mitchell and Marshall 1989, Cromie 1992), resulting in reduced cheese quality production of small peptides that contribute to bitter flavour, UHT gelation, and fouling of heat exchangers (Gruffery & Fox, 1988; Champagne *et al.* 1994).
  Gram-negative psychotrophs are responsible for spoilage, in refrigerated raw milk; particularly *Pseudomonas* spp. are predominant in milk after pasteurization. Others microorganism that survive at pasteurization are spore-forming *Bacillus* spp. (Cousin, 1982, Sørhaug and Stepaniak, 1997).
  Several studies have shown that bacterial proteases affect the plasmin system, which in turn will affect the quality of dairy products. Plasmin activity, has been reported to decrease with microbiological growth in storage time. Decreased plasmin activity was observed in fresh raw milk after 4 day of storage at 4°C, with the psychrotrophic bacterial count reaching 10^6 to 10^7cfu/ml (Guinot-Thomas *et al.* 1995) . Further studies have shown that some bacterial proteases can enhance the activity of PA, infact *Pseudomonas fluorescents* protease was shown to enhance PA activity by enhancing its catalytic activity.

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4.INVESTIGATION OF REPRESENTATIVE MICROORGANISMS

According to this literature, the following microbial agents were analysed in milk and semi-finished milk products. The following are the main features:

- **Spoiler Microorganisms**

Mesophilic Total Colony Units

Aerobic mesophilic germs – are a group of different bacteria that live in the presence of oxygen at room temperature used as a generic indicator of hygiene conditions (usually the more there are, the worse the hygiene condition of the foodstuffs, however, without having an absolute relationship with the danger of them).

Enterabacteriaceae

Gram-negative rods (0,3-1,0 x 1,6-6,0 μm); furniture for peritrichous flagella or motionless; non-sporulated; optional anaerobic; catalase positive; oxidase negative. They ferment glucose according to two main metabolic pathways;

- Acid-mixed fermentation with the production of lactic acid, formic, acetic, succinic,ethanol and gas;
- Butandiolica fermentation with production of (acetone), 2,3-butanediol, lactic acid, formic, acetic, ethanol and gas.

The quantification of *Enterabacteriaceae* isparticularly useful as an indicator of process for those foods that are subjected to the technological treatments able to control the pathogenic microorganisms, such as thermal treatments, prolonged freezing, fermentations or additives. Their presence may indicate in these cases the incorrect application of the particular technology (Villani F., 2007).

Coliforms

Faecal coliforms are a group of bacteria belonging to the family of *Enterabacteriaceae* which share some characteristics both morphological and biochemical characteristics. They are gram-negative bacteria, non-sporulated, optional anaerobic, catalase positive, able to multiply in the presence of bile salts of surfactants and ferment lactose with the production of acid and gas in 48 h at temperatures of 30-37°C (Villani F., 2007)

Escherichia coli

Escherichia coli is the species belonging to the family of Enterobacteriaceae more commonly found in the intestinal flora of humans and animals and has been for a long time considered a microorganism which is a non-pathogenic, normal inhabitant of the intestine. It is a gram-negative rod, catalase positive, oxidase negative, anaerobic optional; fermentation of lactose with the production of acid and gas. It is a faecal coliform tested for the ability to produce indole from tryptophan, for reaction to red-methyl for the reation to the test of Voges-Proskauer (production of acetoine) and to ascertain it capacity, or lack of , to grow the citrate agar ground (IMVC Test). It has the following properties: (+); M(+); V(-); C(-). E. coli è, is, in its actual state, the indicator of faecal contamination more widely used, especially for evaluating the potability of water. Moreover the presence of E. coli in processed foods indicates, for the most part, an inappropriate application of particular technology or, more likely, a recontamination of the product post-process resulting, most times, from the process environment (equipment and contaminated utensils, staff or from raw food), as a consequence of the non-application of the rules of good hygiene. In every way the absence of E.coli from food does not necessarily guarantee the absence of other enteric pathogens (Villani F., 2007).

Coagulase Positive Staphylococci

Staphylocci are Gram positive cocci, catalase positive, motionless devoid of flagella. They are optional anaerobic microorganisms arranged in irregular groups, ubiquitous in the sense that they do not prefer a single type of habitat. (Villani F., 2007)

Pseudomonas spp.

Bacteria called Pseudomonas are straight or curved rods, Gram-negative aerobic; catalase and oxidase positive, non-sporulated and generally mobile for polar flagella. They have a respiratory metabolism (never fermentative), even if they may grow in the absence of oxygen, nitrate must be present as the final recipient of electrons in the respiratory chain (anaerobic respiration). They are typical psychotrophic micro-organisms able to grow at temperatures between 4 and 43°C. They are widespread in soil and water and on the surface of plants and animals. They are among the most common food spoilage agents. Their ability to alter foods such as meat, milk and milk derivatives, fish and vegetables, even during cold storage, is in part linked to the production of proteases and lipases. (Villani F., 2007)

Yeasts and Moulds

Yeasts are unicellular fungi that multiply mainly for gemmation or, in some cases, for fission. In some phases of their cellular life they can sexually multiply through formation and fusion of spores. They are aerobic or microaerophilic. The biggest part of yeasts is mesofita even if some can develop even at low temperatures. While being able to regain in certain environments, the surface of the vegetable represents the main habitat of yeasts. Some genre and species are of great interest for the food industry involved in fermentation (production of bread and other bakery products, wine and beer); other species may be responsible for alterations of food.

Moulds are multicellular fungi with a structure called vegetative mycelium. The long, thin filaments of the cells that constitute the mycelium are called 'ifa'. The ifa can be non-set, set uninuclear or multi-nuclear. They are not mobile and the cell wall consists of chitin. The moulds are widely distributed in the environment and are of great interest for the food sector. The majority of them are due to the alteration of a lot of products of either vegetable or animal origin; others are important for the production and maturation of some cheeses and sausages; finally, others can be pathogenic for man since produce micro-toxins in food (Villani F., 2007).

Psychrophilic microorganisms
Psychrophilic microorganisms are microoganisms able to develop in food at temperatures of between -5°C and +7°C. They have great importance as they are responsible for alterations of chilled food. Psicrotrofi microorganisms exist within bacteria, yeasts and moulds. The growth of the microorganisms occurs even at low temperatures due to the fact that the membrane lipids contain a high percentage of unsaturated fatty acids which, by lowering the solidification point, allow the cytoplasmic membrane to carry out their functions of absorption and transportation of nutrients even at low temperatures (Villani F., 2007).

- **Pro-technological microorganisms:**

Lactic bacteria
To give a clear definition of lactic acid bacteria is quite difficult. A general definition, which is widely accepted even if it, too, has its limitations, is the following: gram-positive bacteria with retinal rod, columnar or bacterial-columnar morphology, , negative catalase, non-sporulated, devoid of cytochromes, aerotolerant anaerobes, nutritionally demanding, acid tolerant, and with a narrow fermentative metabolism. The lactic acid bacteria are divided into two groups on the basis of the final principal product of the fermentation of the sugars: homo-fermentation (fermentation of glucose using almost only lactic acid) and heterofermentative (fermentation of glucose producing lactic acid, ethonol/acetic acid and C02). The lactic acid bacteria are microorganisms widespread in nature which prefer a habitat rich in nutrients. They are associated with various food products such as meat, milk and vegetables, and some species form part of the normal microflora of the mouth of the intestine and the female reproductive system (Villani F., 2007).

- **Pathogenic Microorganisms**

Salmonella spp.
The Salmonella genre belongs to the family of Enterobatteriaceae, retinal rod microorganisms, gram-negative bacteria, non spore-forming, generally mobile for the presence of peritrichous flagella, aerobic-anaerobic optional, catalase positive, negative oxidase, mainly lactose and indole negative that grow on common land also in the presence of bile salts (ISTISAN ratios 05/27, 2005). Salmonella finds its optimal conditions in an environment with a pH close to neutral but it has been known to adapt to an even more hostile environment with a pH varying from 4.5 to 9.5. Salmonella seems to possess an aptitude for adaption to acidity. This involves the reduction of the antibacterial efficacy of the pH in the stomach with a consequent decrease of the dose required to induce infection (Mucchetti and Neviani, 2006). The microorganisms of the genre Salmonella demonstrate considerable resistance even at temperatures which differ greatly from ideal ones (37°C): some strains can grow at temperatures close to 54°C, other strains are resistant at temperatures close to those of psychrotrophic microorganisms. Others, still, manage to multiply at temperatures around 2-4°C (Mucchetti and Neviani, 2006).
The outbreaks of Salmonella occurring worldwide were counted against more varied food ranging from meat products to eggs, milk, dairy, chocolate and also vegetables. In every way the particularly incriminated are animal derivative products given that the epidemiological cycle of the disease is centred on the man-animal relationship. An important source of food contamination are the operators of the infected or healthy carriers of asymptomatic infections (Villani F., 2007).

Yersinia enterocolitica

The genre Yersinia is another kind of family of Enterobatteriaceae. The genre belongs to a different species but only Y.enterocolitica has interest in the food microbiology. It is a species recognised as an important pathogen transmitted via food. It is responsible for numerous epidemics of enteritis in humans. It is a short rod, Gram-negative, mobile for peritrichous flagella that are produced only when the microorganism is grown at temperatures of 22-28°C; they are not produced when the incubation is carried out at 37°C (Villani F., 2007).

Y. enterocolitica demands little nutritionally, it colonizes, multiplies and survives on a great variety of food substrates. It is widely distributed in aquatic and terrestial ecosystems. It is frequently isolated in running water, lakes and wells even if the strains of clinical interest are rarely isolated from these water sources. The main subject of the microorganism is represented by warm-blooded animals; in particular pigs are considered a natural reservoir of the germ. Moreover Y. Enterocolitica was isolated from a wide variety of foods. Frequently on carcasses and meat of pork, beef, lamb, raw cow and goat milk, cheese curd, vegetables, oysters and prawns (Villani F., 2007).

Listeria monocytogenes

The presenceof Listeria monocytogenes in different foods intended for human consumption has aroused in the years an increasing interest for this microorganism, classifying it between the most important pathogens transmitted via food. L. Monocytogene is a short rod regulated at 0,4÷0,5 per 0,5÷2,0 μm. It can sometimes arise with curved cells, single or in a short chain with the arrangement in the form of a V or Y. It is Gram-positive (it irregularly stains with old cultures), catalase positive and negative oxidase, does not form capsules and does not produce spores. It is movable by means of peritrichous flagella that impart a motility of type 'rotatory' (Villani F., 2007).

The increasingly frequent isolation of L.monocytogenes from a great variety of foods intended for human consumption located partial justification in some attributes of the pathogen which help explain its ability to adapt and multiply in different technological conditions that characterize the process of production, transformation, preservation and distribution of food. L. Monocytogenes, in fact, presents a certain, though argued, tolerance to thermal treatments. It is able to multiply at refrigerated temperatures in the presence of sodium chloride and nitrate at low values of pH and water activity (Villani F., 2007). Like many other pathogens it is particularly harmful to the health of individuals such as babies, the elderly or people with immune problems or chronic diseases; in which case also a dose smaller than the minimum required to cause infection can cause foodborne illness. Fortunately pasteurization is capable of inactivating Listeria (Mucchetti and Neviani, 2006). It does not multiply in frozen food but survives and multiplies albeit slowly at refrigerated temperatures. With regards to the presence of the germ in food, various types of ready-to-eat foods were analysed in which positivity variables, generally with a low frequency, were found. The highest percentage of positive samples and samples containing more than 100 CFU/g, a level considered as a safety threshold, has been identified in ready-to-eat fish products. Other samples, even in a very small percentage, such as ready-to-eat cheese and meat, have revealed the presence of L. Monocytogenes beyond the 100 CFU/g (EFSA, 2005). Seen from the temperature range the raw milk refrigerated can also be a good substrate for Listeria.

Bacillus Cereus

Bacilluscereus is a retinal rod bacteriumform, gram-positive, an aerobic-anaerobic optional mobile for peritrichous flagella, devoid of the capsule, but susceptible to variations on the basis of those which are immobile and/or capsulated. They form long chains of cells with

sizes of 1.0-1.2x3-5µm. traditionally they are considered mesophilic microorganisms since they normally grow at temperatures between 15 and 50°C, with optimum development at 30-37°C, but have repeatedly demonstrated the possibility of growth of toxin-producing strains between 4 and 37°C and between 6 and 21°C. They produce spores of elliptical shape, central or sub-terminals which do not deform the sporangium. Spore germination occurs between 5 and 50°C with maximum speed at 30°C. Biochemically B.cereus is catalase, oxidase and positive Voges-Proskauer.
Bacillus cereus is widely distributed in nature. It was found in water, the air, plants, and in faecal material of humans and animals. Moreover the genre Bacillus has significant importance in the food industry where its role is linked to the deterioration of the organoleptic characteristics of the product, induced both by the wide and diversified enzymatic activity as well as the toxic action performed by the toxins on the consumer. The environmental strains typical of B.cereus normally do not grow in milk at temperatures lower than 10°C although many are the insulations from milk and milk derivatives of psychrotroph strains able to grow at refrigerated temperatures.

Escherichia coli O157:H7
As previously mentioned, the E.coli is a common inhabitant of the intestinal tract. There are cases, however, where this germ can cause a pathological state both in humans and in animals. It is interesting to note how different strains of E.coli cause several pathological manifestations. E.coli 0157:H7 is between the pathogens of animal origin called 'emerging', one of the most important human pathogens transmitted through food (Villani F., 2007). It is part of the group of enterohaemorragic (EHEC, the most virulent and well known up to now). The pathogenic nature of this strain is not derived from a single factor of virulence but by several which are not yet fully known. It is certain that their pathogenicity is due to the production of potent toxins (verotoxigenic) (Mucchetti and Neviani, 2006) and their capacity of adhering to the cells of the intestinal mucosa. It is for this reason that the strains belonging to the group of EHEC are also indicated by the acronyms VTEC.
A wide range of food may be the source of infection: meat, in particular ground and uncooked; cheeses derived from raw milk, (because of the considerable resistance of the germ at pH close to 4,0) (Mucchetti and Neviani, 2006), and last but not least, raw milk.

Campylobacter spp.
The Campylobacter genre belongs to the family of Campylobacteriaceae which groups anaerobic bacteria, microaerophilic, mobile, helical, Gram-negative bacteria. The species of the genre Campylobacter are small rods of curved or helical mobile; generally for a polar flagellum which can be 2 or 3 times longer than the length of the cell. The morphology is variable, different forms existing within the same species; rod forms, spiral forms, S; in young crops the curved shape cells dominate. If the growth conditions are not favourable for the micro-organism, the rods are transformed into coccoid forms which become predominant in old cultures.
The species of the genre Campylobacter are microaerophilic: requiring an oxygen concentration between 3 and 15% (better 5%) and a concentration of C02 of 3-10%. Occasionally you may have growth under aerobic conditions. They have a respiratory metabolism, they do not ferment in any sugars, they are oxidase positive. The growth temperature varies with the species: all grow at 37°C, some also at 25°C, and others at 42°C (Villani F., 2007). The main food vehicles of Campylobacter are raw milk and poultry, drinking water, fruit and vegetables.

4.1. EFFECTS OF FREEZING THE MICROBIAL FLORA

Lowering the temperature of food to below the point of freezing causes certain damage to the microbial cells. The freezing is generally at a temperature lower than -18°C where no microorganism is able to multiply. Freezing causes a lowering of the water activity (-18°C aw = 0,841) and a concentration of dissolved solids which influence the activity of the microorganisms. During freezing a part of the microbial population undergoes irreversible damage due to the formation of ice crystals which cause an increase of the cell volume, with consequent breaking of the wall and therefore the lysis of the cell. Many microorganisms can survive the freezing process and this resistance depends on a number of factors. In general it can be said that the Gram-negative bacteria are more susceptible than the Gram-positive, while the spores are highly resistant. In addition the cells in the active phase of growth are much more sensitive that when they are in the stationary phase. Moreover, the lethality of the microorganisms during freezing plays a fundamental role in the speed of freezing: the greater the rate of freezing (-18°C in less than 30 min.) the less the cellular damage and this seems to be linked to the formation of ice crystals inside the cell of smaller dimensions than those with the slow freezing (3-72 hours), where the ice crystals are formed on the outside of the microbial cell which undergoes plasmolyisis as a result of the concentration of solutes. A greater speed of freezing generally produces less structural damage to the food, this is also explained by the formation of smaller crystals (Villani F., 2007). The survival of the microorganisms at freezing has great importance for the thawing process. Repeated freezing and defrosting can destroy the bacteria to break the cell membrane; in addition the faster the defrosting the greater the number of bacteria survive, even if the reason for this phenomenon are not yet clear. It has been observed that the defrosting is intrinsically slower that the freezing and has a trend potentially more harmful. Most frozen products do not recommend the refreezing of defrosted food. Although the reasons are linked in particular to the consistency, taste and nutritional quality of the product, the microbiological aspects of food thawed is not of secondary importance. Some researches have noted that the products thawed deteriorate more rapidly than the same food stored in a fresh state (James M. Jay *et al*., 2009).

4.2. FREEZING APPLIED TO BUFFALO MILK AND DERIVATIVES

The application of low temperatures is considered to be a widely used system in the technology industry for the preservation of many products, among them milk and its derivatives as products of easy deterioration for microbial and enzymatic activity.

FAT CONTENT

The lipid fraction is affected by the conditions derived from freezing. In fact, upon the increase of the percentage of milk frozen, there is a progressive loss of fat in the cheese and an increase in the fat content in spun water. The amount of fat that is lost when working frozen milk corresponds to what emerges during the thawing step because the fat fails to be incorporated into the structure of the subsequent processing stages. This is definitely a problem of a technical nature and not an alteration of the frozen milk. From the rest some technological expedients, for example the use of microwaves in the thawing process, could serve to minimize if not to cancel out this drawback which does not seem to significantly affect the organoleptic characteristics of the Mozzarella, but that may have an impact on the yield of processing (Addeo *et al*., 1992).

SOLUBLE SUBSTANCES

Substances soluble in water, lactose and saline substances, increase their concentration in the non frozen portion modifying the equilibrium between the soluble fraction and the insoluble. The conditions resulting from the formation of ice and from the crystallization of lactose (pH fall, increase of the saline concentration, dehydration of the proteins) affect the dispersibility of the proteins and, in particular of the casein, reducing it to the shape of a flocculated precipitate which is a function of the intensity of the 'freezing'.

The protein fraction which is difficult to control and adjust in the freezing process seems to follow the crystallization step of the lactose which may be delayed by means of preheating milk thus avoiding the subsequent formation of crystalized nuclei and protein precipitation.

4.3. STRUCTURE OF MOZZARELLA OBTAINED FROM FROZEN MILK

Observing the structure of Buffalo Mozzarella of the cellular type which can be likened to that of a sea sponge, whose grating is constituted by the interlacing fibre of casein which forms during the coagulation and the cells of which are occupied by the fat globules and from non structured water. A possible variation in the fat content of milk leads to the formation of a structure more compact and less porous which constitutes one of the main differences between Buffalo Mozzarella and Mozzarella from cow's milk. In Figure 5 we see micrographs taken by an electronic microscope both relative to the surface part of the forms of Buffalo Mozzarella: In (A) prepared from fresh milk and in (B) prepared from frozen milk. In both cases we can see the woven fibres of casein formed as a result of the spinning of pasta in water at almost boiling point. It is more dense in the cheese from the frozen milk, and its more disordered and porous in the cheese from the fresh milk.
So we can say that during the process of preservation at low temperatures there is an increase in the consistency and viscosity of mozzarella, while there is a reduction of the elasticity. The cheese prepared from slow, frozen milk is less juicy and more consistent also with a more intense colour tending to a yellow straw colour.

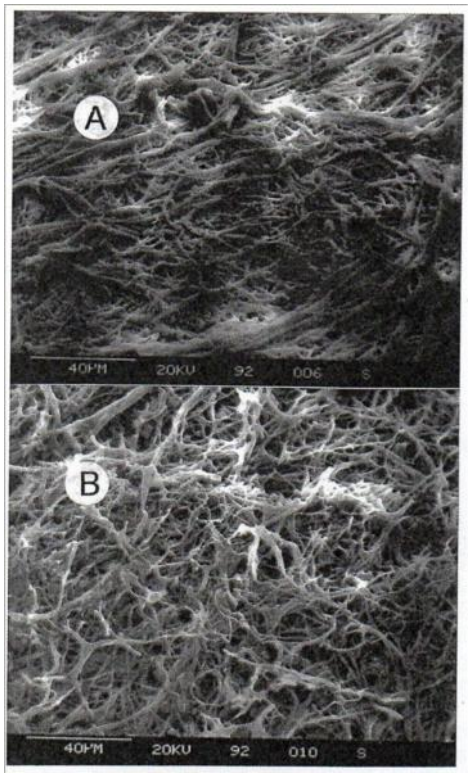

Figura 7- **:** Electronic microscopic scans of the outer part of the Buffalo Mozzarella samples prepared from (A) Frozen Milk and (B) Fresh Milk**. Source**: Addeo F. *et al*., 1992.

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5. ANALYTICAL METHODOLOGIES IN PROTEOMICS

By Proteomics we mean the experimental approach which allows us to decode the information by the sequence of a particular gene and causes the structure, function and possible modifications post-traductional. The proteins are complex macromolecules and, without doubt, are among the most important organic compounds present in the human body; they can be enzymes, hormones, antibodies or receptors.
Proteomics is highly dynamic since the type of protein, its abundance, its location, the type and the amount of post-transduction modifications depend heavily on the environment and on the physiological state in which you are to find the cells.
Proteome Analysis has a very broad range and is usually divided into three subclasses: *Proteomics of the characterization* which takes care to define the proteins present in a cell, tissue of biological fluid; the *differential proteomics* which studies the type and amount of proteins that are in the same tissue but under different physiological conditions; the *functional proteomics* whose purpose is the identification of a group of proteins which carry out the same function.
In this work the thesis takes into consideration differential proteomics. The analysis of the amino acid sequence of a protein which then identifies it, observing this well determined protocol:

1. Homogenization of the biological sample with an appropriate buffer of lysis;
2. Separation of the target protein using an HPLC technique or by exploiting the SDS-PAGE (electrophoresis on polyacrylamide gel with sodium dodecyl sulphate) mono or two-dimensional;
3. Determination, when possible, the molecular weight of the protein as such;
4. Protein digestion with specific enzymes so as to obtain the corresponding peptides;
5. Measurement of the mass of all peptides;
6. Determination of the amino acid sequence of the peptides obtained by enzymatic digestion through experiments in mass spectrometry (MS/MS).

5.1 ELECTROPHORESIS IN THE STUDY OF PROTEINS

Electrophoresis is an analytical separating technique based on the movement, due to an electric field applied by a pair of electrodes to the fluid itself, of electrically charged particles immersed in the fluid. In the case of an electrolytic cell, the cathode takes on a negative charge and the anode assumes a positive charge, for which the particles move towards the electrode with the opposite charge to them; in particular moving towards the cathode if they have a positive charge or towards the anode if they have a negative charge. In electrophoresis SDS-PAGE and UREA-PAGE the negatively charged particles move towards the anode.
Migration is not only due to charge but it can also depend on the charge-to-mass ratio. With SDS-PAGE you have all of the particles of negative charge which differ according to molecular weight, some are larger and others smaller. Clearly the particles which have larger dimensions migrate more slowly compared to those of a lower molecular weight. This means that the molecules with an equal electric charge but a different molecular weight (MW), so with a different charge-to-mass ratio, have a differential migration.
The UREA-PAGE is an electrophoretic technique which separates based on the electric charge instead of the molecular weight. The electrophoresis applies to molecules such as amino acids, peptides, proteins, nucleotides and nucleic acids which possess ionizable groups therefore at every pH value some electrically charged species are present in the solution. Under the influence of an electric field the charged molecules placed in a liquid migrate towards the cathode or the anode. A particle which migrates in an electric field will have its own mobility. The

electrophoretic mobility is a quantity (a mass) which conveys the tendency of a chemical species to move within an applied electric field which can be formed using the Henry equation:

$$U_e = \frac{2 \cdot \varepsilon \cdot \zeta \cdot f(ka)}{3 \cdot \eta}$$

Where U_e is the electrophoretic mobility, ε is the dielectric constant, ζ is the zeta potential $f(ka)$ the function of Henry, and η the viscosity of the solvent.

The electrophoresis can be conducted:

° in free phase (or frontal): the charged particles move through a solution (little friction, high rate of migration);

° of support (or zonal): the particles are moved through a porous medium (agarose and polyacrylamide; paper). On wider application to separate both the nucleic acids and the proteins.

The factors that influence the migration of a charged particle in an electric field are various.

- The first of these is the ***difference in the potential applied to theelectrodes*** equal to v = Rxl, which may be formed from the law of Ohm: I = V/R

  where: I is the intensity of the current in Ampere (A);
  V is the voltage or volts.
  R is the resistance, Ohm Ω;

  By applying a given voltage V, I will depend on R. The difference of potential influences the speed of the electrophoresis; its increase reduces the execution time but involves a dissipation of energy in the form of heat with the risk of denaturing the sample (losing form and in the case of proteins, also function).
- The second factor is the ***sample***, which in turn depends on 3 factors :
  CHARGE→ migration rate increases with the increase of the net charge (linked to pH);
  DIMENSIONS (r) → migration rate decreases with an increase in the molecular weight of the molecule charge;
  FORM → molecules of similar size but with a different shape (eg. Fibrous proteins and globular) have a different migration velocity (frictional force effect and electrostatic).
- The third factor is the ***electrochemical potential***. The electrochemical potential in a protein is the net charge which is located on the molecule and which depends on the ionizable groups (aa-acids and aa-basic) present on the peptide chain. Depending on the pH of these aa may be loaded or not.
- Another important factor is the ***ionic strength*** of the buffer. It can be stated that increasing the ionic strength increases the electromotive force of the cell but if the ionic strength is too high it does not favour the migration of the molecules to be separated because of the increased competition between the sample ions and ions of the pad. The ionic strength is calculated with the following relation

  $(\mu) = \frac{1}{2}\sum CZ^2$

  Where: C is the molar concentration of the ion; Z is the charge of the ion
- The temperature is a very important factor in so far as the increase of the temperature decreases the viscosity of the medium and decreases the forces of friction and resistance. An excessive temperature rise, however, can alter both the support and the migration of the samples by reduction

  The ***Joule effect*** slows the migration speed of the sample. This is because when an electrical current passes through a column of electrolytes it has a uniform heat production. The heat loss is greater in the suburbs where the buffer concentrates slowing the migration speed of the sample.

  The last factor is the support which constitutes the matrices onto which the sample is loaded. The supports for electrophoresis must meet a series of requirements and should not interfere in the analysis. They should be inert, homogeneous and must not cause the electroendosmosis phenomenon . This phenomenon is due to the presence of charged groups on the surface of the

support means (eg. Paper – carboxy groups). When an electric field is applied, the electrolyte near the wall cations migrates towards the cathode dragging with it the electrolyte solution. If, for example, the support consists of negatively charged agarose, it may happen that the H^+ of the median are attracted by the charge holder and thus the negatively charged protein is slowed down on its journey towards the anode.

The supports for electrophoresis are distinguished based on how they separate in supports:

- No sieving:
- Paper,
- Cellulose acetate
- Sieving:
- Polyacrylamide gel
- Agarose gel

The supports most commonly used are those sieving.

The electrophoresis of the agarose is a classic technique used to analyze and separate nucleic acids. The gel acts as a sieve as it forms a network of pores where smaller molecules migrate faster than the larger ones. The DNA for example, being negative, will migrate towards the anode. The Agarose is a linear polysaccharide composed of D-galactose and 3,6 – anhydro-L-galactose linked alternately with glycosidic bonds. The agarose is sugar soluble in water at boiling temperatures, it becomes solid as it cools forming a gel by means of hydrogen bonds between the linear chains.

The polyacrylamide gel is a polmer formed from acrylamide monomers. Acrylamide before polymerization is a liquid and is, in this state, neurotoxic and carcinogenic. The solid polymer is no longer toxic to the operator. It is used to separate proteins due to its controllable porosity: the pores of the gel are more or less tight according to the % of acrylamide at departure. Separate proteins with a molecular weight of 5000 to 200,000 Da. The polyacrylamide gel is prepared at the time of use, preparing a stock solution of acrylamide and bis-f acrylamide diluted in a buffer with curing agents which act as catalysts:

- Ammonium persulphate (APS), which acts as an ***initiator;***
- N,N,N$^-$, N$^-$tetrametilenendiamine (TEMED), which acts as an ***activator.***

The APS is a radical form which is highly reactive and activates the double bond of acrylamide and then activates the polymerization, The TEMED catalysis the decomposition of persulfate and activates the formation of free radical SO_4^- with the unpaired electron.

$S_2O_8^{2-} + 1e^- \rightarrow SO_4^{2-} + SO_4^{-\cdot}$

The radical reacts with the acrylamide monomer and forms a new free radical thus beginning a chain reaction which leads to the polymerization of acrylamide molecules between them (Figure 8).

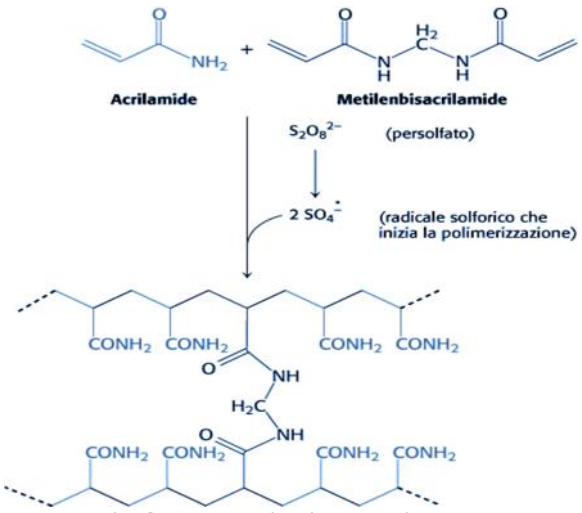

Fig. 8 - Polymerization reaction

The porosity of the polyacrylamide gel can be controlled by knowing the ratio of the total concentration of acrylamide (T) and of the cross-binder concentration (C) expressed as a percentage:

$$\% T. \frac{gr\ acrilammide + gr.bis-acrilammide}{ml\ della\ soluzione\ di\ polimerizzazione} \times 100$$

$$\% C \frac{gr.bis-acrilammide}{gr.acrilammide + grbis-acrilammide} \times 100$$

Acting on T and C we change the size of the pores and thus we can improve the separation.

CHARACTERISTICS OF POLYACRYLAMIDE

The polyacrylamide gel is very versatile, highly reproducible and its porosity can be exactly predicted and chosen according to the molecular loss of the molecules to be separated so as to increase the power of the resolution of the electrophoretic technique (resolution power = the ability to separate the molecules with a smaller molecular weight difference). They have a negligible tendency to absorption and an ease in the analysis of the results, both directly (they do not absorb in U.V), and after staining (Coomassie, silver nitrate).

They have the same considerable mechanical resistance when they are hydrated as they do when dried, with complete transparency in both the visible and the U.V. adhering well to glass avoiding preferential routes (Gadaleta M.N. 2009).

5.2 ELECTROPHORETIC TECHNIQUES

Electrophoretic techniqes are distinguished by:

- Electrophoresis on polyacrylamide gel under denaturing conditions in the presence of sodium – dodecylsulphate (SDS-PAGE);
- Electrophoresis on polyacrylamide gel under native conditions at acid pH or alkaline (PAGE);
- Isoelectric focusing (IEF).

The principle of separation based on electrophoresis differs according to the technique. In the case of SDS – PAGE its based on molecular size. The PAGE, under native conditions, is based on the net charge and therefore depends on the pH. A pH acid has a net positive charge if a basic pH has a negative charge. With the IEF technique the separation is based on the isoelectric point.

5.2.1 SDS – PAGE

The electrophoresis on polyacrylamide gel in the presence of SDS is one of the most common techniques for the analysis of proteins. The principle on which this technique is based is to separate the individual protein species of a complex mixture of proteins according to their molecular weight. So that the separation only takes into account the size of the proteins it is necessary for the polypeptides to all have the same charge and shape; They must be completely denatured. The proteins must be treated with:

.β-mercaptoethanol

.SDS

SDS is a strong anionic detergent which makes soluble almost all denatured proteins (Lopez *et al*., 1991), as it breaks down the hydrogen bonds and hydrophobic interactions destroying the secondary and tertiary structures taking on a rod shape. The SDS molecules, negatively charged, are arranged along the amino acid chain and then spread out thanks to the electrostatic repulsion

between charges of the same sign (Figure 9). The protein then becomes wrapped in a shell of uniform negative charge.

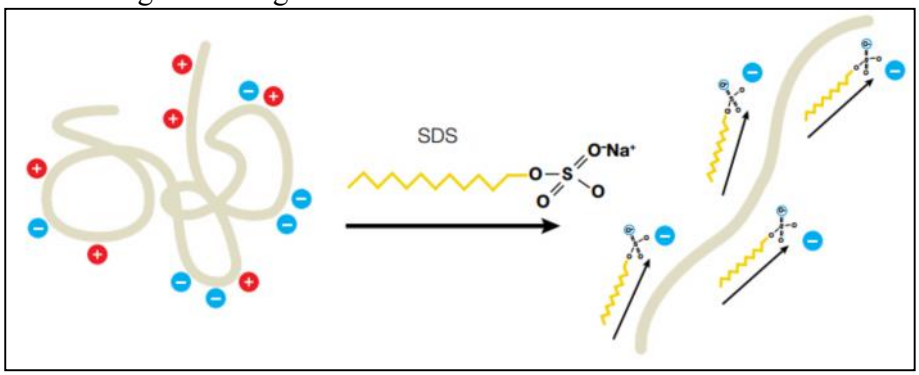

Fig. 9 - Action of SDS along the amino acid chain -

The SDS – protein ratio is greater than 1:1, as a gram of protein binds to 1.4 grams of SDS (1SDS : 2 aa). For the SDS to position themselves around the protein it is necessary that the latter does not have a disorderly spatial conformation but instead must be unrolled; only this way you can 'mask' the charge made by the protein amino acids.

In addition to the SDS it is necessary to use a reducing agent, β-mercaptoetanolo which functions to destroy the disulfide bridges between the cysteines making the linear amino acid chain. The action of β - mercaptoethanol is essential so that the SDS is able to bind to proteins by inserting them with the a polar portion toward the inside and with the negatively charged portion to the outside. (Figure 10).

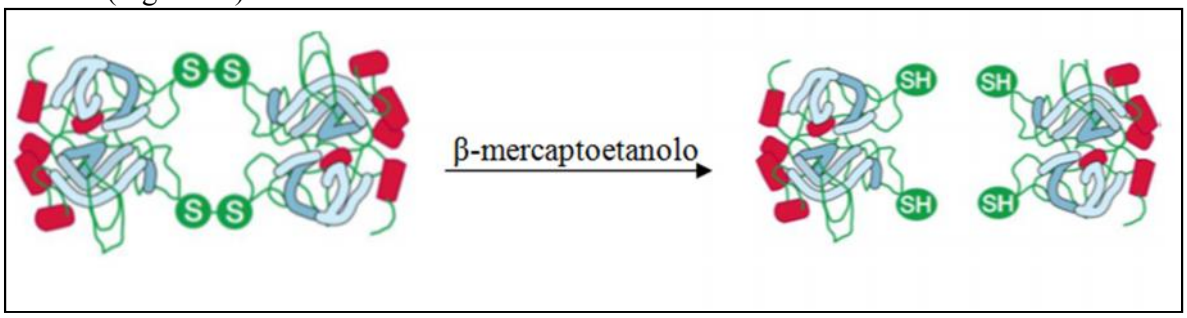

Fig. 10 - Action of β - mercaptoethanol

To completely destroy the tertiary structure and eventually the quaternary structure, the sample solution is heated to a temperature higher than 60°C, usually 100°C for minutes; the heat improves the action of SDS molecules on more hydrophobic and hidden portions of the polypeptide chain.

5.2.2. UREA-PAGE

Electrophoresis on Polyacrylamide gel in the presence of urea for analysis of peptide fragments. The Urea 9 M gives a basic pH 8.6 and a net negative charge capable of separating the peptides depending on the electric charge. Its application serves to highlight in particular the αs1 caseins mainly formed by the action of chymosin, an exogenous enzyme from the rennet added in the technological process of transformation of milk into cheese.

In fact the chymosin is an enzyme which is active in both αs1 and in β casein (Carles and Ribadeau-Duas,1984, Carles and Ribadeau-Duas 1985), in cheese it seems to hydrolyze mainly αs1-CN, yielding the fragment αs1-I-CN. (McSweeney *et al.* 1993). Subsequent studies (Face *et at*.).

2014) have reported that the quantification of the fragment αs1-I-CN can be effective to detect the use of frozen curd in the production of fiordilatte, because the chymosin is denatured at high temperatures (90°C) which are used for the water in the spinning step.

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EXPERIMENTAL PART_ PHASE I:

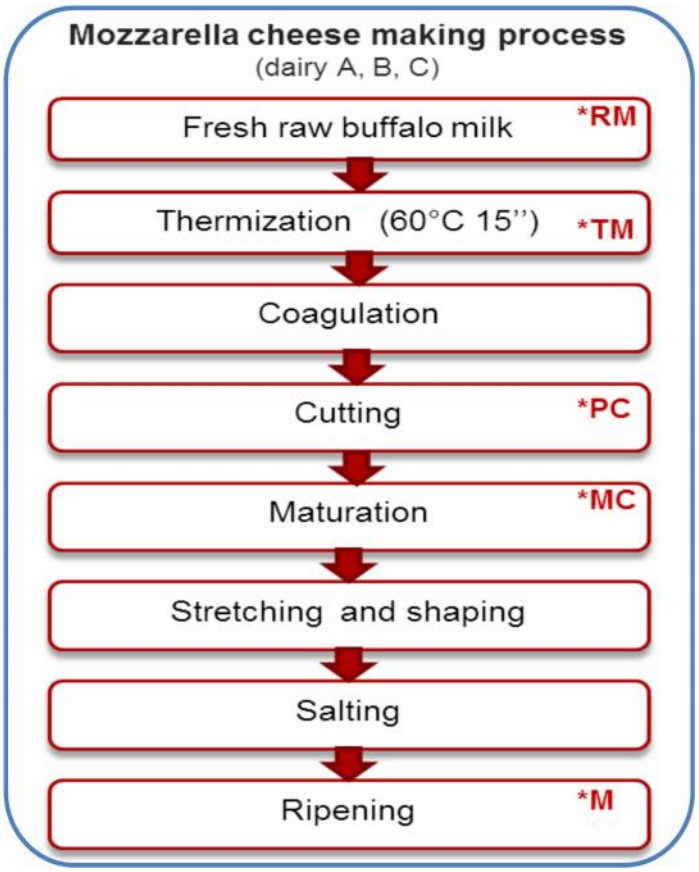
"EFFECTS OF FREEZING ON THE MICROBIOLOGICAL EVOLUTION"

6.OBJECTIVE PHASE I

Buffalo Mozzarella of Campania PDO[1] is a typical stretched-cheese product, appreciated all over the world, where it is exported and consumed. It still represents, for the time being, one of the quality Made in Italy products.
This is a product that has to be obtained starting from milk and exclusively from the Mediterranean buffalo (*Bubalus bubalis*), bred in an enclosed area and worked on – in accordance with the Production Specification – in 60 hours since milking, starting from raw, fresh thermized milk, or pasteurized milk.
The core issue is here represented by the period of maximum production of autumn-winter milk. As a matter of fact, the market request is higher during the spring-summer months, where the production of milk is minimal. A solution to this problem has been to adopt not contemplated practices, such as the use of frozen milk or curd, of dubious source, a low cost, for mozzarella PDO production.
This practice has contributed to damage the image of a prestigious and envied Italian product.
This study, which has been carried throughout a year of analysis, has as aim to know the effect of freezing on the microbiological evolution of all matrices of the entire cheese making process on the Buffalo Mozzarella of Campania PDO.

[1]Protected Denomination of Origin, from the Italian PDO, "Denominazione di Origine Controllata".

7.EXPERIMENTAL PLAN



LEGEND *:

RM: raw milk

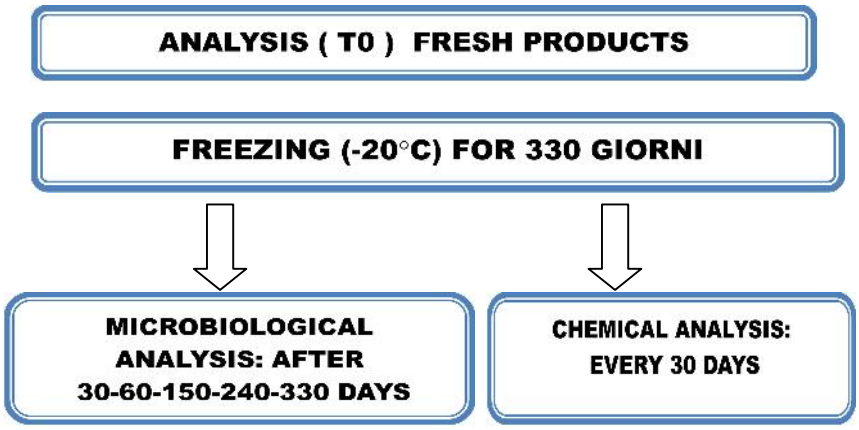
TM: thermized milk (60°C pe 15'')

PC: premature curd (pH 6,3 - 6,2)

MC: mature curd (pH 5,0 - 4,8)

M: mozzarella

Sampled matrices were subjected to:



8.MATERIALS AND METHODS

8.1 SAMPLING

Different dairies with the following characteristics have been selected on the regional territory of Campania:

- ✓ in accordance with the health standards of Reg. CE 852/2004, 853/2004, Reg. (CE) 2073/2005, and Reg. (CE) 2074/2005and s.m.i.
- ✓ the production exclusively concerns Buffalo Mozzarella of Campania PDO;
- ✓ both industrial and small-scale production systems;
- ✓ availability of several production lines for parallel studies;
- ✓ availability of an internal laboratory for manufacturing analysis.

The matrices which have been studied are: raw and thermized milk, premature curd (pH 6.2-6.3), mature curd (pH 4.8-5.0) and mozzarella cheese. Samples were collected in three dairies located in Campania Region: Caserta, Salerno, Napoli, named A, B, C, during cheese making process, in April, May, July 2014 respectively. In each dairy were collected 11 samples for matrices in different stages of the supply chain for total 30 Kg of samples for dairy. The samples were transferred in the IZSM laboratory of Portici, analyzed in the fresh state and then frozen at -20 ° C for 11 months. Monthly, frozen samples were thawed at 20°C and subjected to chemical and microbiological analysis.

The chemical and microbiological analysis are useful to know the parameter variations, for the research and the count of the main pathogenic, spoilage and pro-technological microorganisms that can be observed in the different matrices analyzed, using official ISO methodologies, in accordance to the Reg. CE 2073/05 relative to the limits prescribed by the law. In particular, the analysis have been the following:

- chemical determinations pH, Aw
- research of the pathogen bacteria: *Salmonellaspp.*, *Listeria monocytogenes,* *Campylobacter spp.,* *Escherichia coli O157:H7,* *Bacillus cereus,* *Yersinia enterocolitica,*
- Count of the pro-technological bacteria: *Lactic bacteria*
- Count of spoilage microorganisms: *Total bacterial count,* *Coliforms,* *Enterobacteriaceae,* *Escherichia coli,* *Psychrophilic microorganisms,* *Pseudomonas spp.,* *Coagulase-positive staphylococci* *Yeasts and moulds*

8.2 CHEMICAL DETERMINATION

The samples taken in the different dairies can be divided in two categories:

- Liquids: Raw milk (RM), thermized milk (TM)
- Solids: Premature curd (PC), Mature curd (MC), Mozzarella (M)

For every category specific determinations have been carried out, detailed in the following schematization (Table 7)

Tabella 7- Specific determinations of food matrices

	LIQUIDS		SOLIDS		
DETERMINATION	**RM**	**TM**	**PC**	**MC**	**M**
pH	X	X	X	X	X
A_w			X	X	X
Fat, Protein, Lactose	X	X			
Titratable acidity	X	X			
Somatic cells	X	X			
Microbiological analysis	X	X	X	X	X

We performed the following chemical analysis : pH, a_w , fat, protein, lactose, titratable acidity, somatic cells.

In this work, the pH has been measured on every examined sample thanks to the **FiveEasy™ pHmetre** instrument. For solid samples special penetration probes have been employed. Generally, such probes are equipped with a glass electrode measuring the difference of electric potential on the two sides of a thin glass membrane, located on the far end of the electrode: such a difference in potentialis linked to the difference between the hydrogen ions inside and outside the membrane. In addition to this probe also a temperature probe has been used, which corrects the reading of the electrode depending on the actual temperature of the sample. The instrument has an accuracy of ± 0,02 pH.

To measure the Aw degree an **Aqualab 4TE** instrument has been used, composed of a probe-container useful for the housing and measurement of the sample and of a portable reader. Currently for the determination of a_w the most widely used technique is the one developed by Van den Berg which is based on calculating the value of a_w depending on the concentration of solute in a sample to directly affect the final value. The equipment in use today is based on the *electrical conductivity* of the average in regard to a *lithium chloride* solution reference. The perception is depicted by a sensor that measures the variation of electrical conductivity at a constant temperature in a sealed chamber. The procedure of analysis is simple: the sample is located inside the hermetically sealed probe-container; part of the water inside the sample evaporates, allowing the sensor to detect the degree of free water. This instrument for the measurement of water activity is able to precisely read in about 4 minutes, with an accuracy of ±0.003 Aw at a temperature of 25°C ± 1°C.

The result is conveyed as a percentage of the equilibrium relative humidity (% URE), since $a_w = p/p_0$ where p = the value of steam pressure on the surface of the product, therefore %

URE = a_w . 100 e where p_0 = steam pressure on the surface of pure water at the temperature of the sample. (Salvadori del Prato 1998).

Fat, protein and lactose: are determined with **MilkoScan FT120 della FOSS,** uses the principles of infrared spectrophotometer. When the milk contains organic substance characterizations detected at below 300 nm (ultra-violet UV) and above 750 nm (infra-red IR). The spectrophototometry calculates the absorption of a set molecular group. Infact, because the group absorbs it must have an electric dipole corresponding with a vibration of a set wave length in the area of 5600 nm. Particularly the foreign bond of grease absorbs at a wave length of 5600 nm. The peptide bond of proteins, instead, absorbs around 6400 nm while the hydroxyl group of the lactose is detected at a wavelength of 9600 nm. (Alais 1984).

The tool analyzes small quantities of milk which are drawn by using peristaltic pumps, the milk passes through a heater at 40°C and then to a cell where it is infiltrated by a ray of infrared light (IR). The radiation is absorbed by the functional groups and sent to a detector to be quantified numerically.

To determine the titratable acidity of milk through differential pHmetry, the **EC CL-10 PLUSBiocontrol Italia S.r.l**. has been used. The titratable acidity of a type of milk corresponds to the Na OH ¼ normal milliequivalents (meq) necessary to 100 ml of a sample to arrive at a pH 8.4, or to the turning of the phenolphthalein indicator for direct titration.

The degree of acidity is expressed in °SH/100 ml on a 25 µl milk sample, and the accuracy is ± 0,03; degrees between 9,7 and 10,3 are acceptable.

The somatic cells are components of both the milk source and diverse concentrations and are made up of leukocytes, lymphocytes and flaking cells. The leukocytes from blood represent approximately 10% and carry out the role of defense. The lymphocolytes make up approximately 70% and are derived from the sap indicating the status of the animal's immune system and to a 20% lesser extent flaking epithelial cells of the mammary gland from the milk ducts. The white blood cells are larger presenting a mononuclear or polinuclear form. Lymphocytes are a smaller, globular shape with a large, single core and the epiteliati cells are various shapes and sizes. The number of somatic cells may undergo drastic variations from tens of thousands to a few million such as in a case of udder mastitis. (Salvatore del Prato 1998).

Pasteurization while destroying the somatic cells does not stop their enzymatic action which is neither stopped by even cold temperatures. The value of this parameter must be as low as possible for the milk to make cheese. Italian law (Reg.853/2004) allows Buffalo Milk a somatic value of ≤ a 400.000 x ml * (* rolling geometric average, calculated over a period of three months with at least one sample per month from bulk milk, unless otherwise specified by the appropriate authorities.) The method involves the use of a DNA intercalator solution (reagent C for calibration) by adding 500µl to 500µl of milk: the somatic cells present are revealed through a fluorescence microscope's Core Counter which is a very easy apparatus to use. The sample is loaded into special compartments which are housed in the unit. Within a few seconds the image density of the somatic cells will show on the screen of the computer and the level is calculated automatically by the system's software.

8.3 ANALYTICAL PROCEDURES

An analytical procedure, approved by precise European regulations allows the detection of microorganisms following the same procedures in all the laboratories which have been accredited in carrying out analysis, and therefore appropriately certified.

For example, the ISO 6579:2008 analysis procedure, decreed in the Reg. CE no. 2073/2005, describes the various steps allowing the detection of Salmonella spp. in a food matrices. The ISO-certified laboratories in Europe will follow the same methods for the detection of the microorganism.
In the course of the execution of a trial procedure it is mandatory to create and keep sterility as much as possible. Bunsen burners are used, that with their free flame create a delimited sterile area; individual protection devices (in Italian DPI), are used to prevent the possible microbial contamination of the operator.
All ISO procedures employed – and here explained – share the use of certain instruments.
Employed materials:

– disposable sterile plastic bags, each of them coded, where to weigh the sample units with precision balances;
– sterile pincers and scissors to handle and obtain the sample.

The quantity of the obtained sample depends on the procedure employed; generally for pathogenic microorganism 25g or ml are obtained, in other cases 10 g or ml. Then the procedure continues by homogenizing what has been taken in a stomacher to which the pre-entichment, or only enrichment – depending on the method – medium.

Other instruments used are: thermostats, microwave ovens, water baths, electronic multijets with different sterile pipettes graduated on different volumes, from 1 to 25 ml, sterile tubes (Falcon) for different dilutions, sterile Petri dishes, plastic loops, electronic stirrer (vortex), mediums, solutions and reagents.
Finally, for certain microorganism the optical microscope is used if the research of the bacterium requires a Gram's stain.

A correct quality of analysis requires to:

- start the operations as soon as possible after taking the sample; if this is not possible, freeze the samples before starting the analysis,
- work in a sterile envinronment to prevent sample contaminations,
- solid samples have to be weighed and homogenized with a suitable diluent,
- choose the diluent depending on the method and the nature of the food sample,
- the diluent's temperature has to be close to that of the sample, in order to prevent thermal damage to the microorganisms,
- freeze the excess sample for possible further investigation.
-

8.3.1 PREPARATION OF THE SAMPLE

The aim of the preparation of a sample for a microbiological analysis is to homogenize the product by using a diluent, without damaging the microbial life there present, which instead has to be investigated. It is paramount to work in a sterile environment so to avoid external contaminations that might invalidate the analyisis. The methods of preparation are different, depending on the nature of the product:

- *Solid products*

Sterilely weigh a sufficiently representative quantity of the examined sample (10-25 g) in a sterile bag, add a sterile diluent with a ratio 1:10. Such a dilution, also called first dilution or stock dilution, is usually made by adding 90 ml of diluent to 10 g of sample, then by homogenizing. The homogenization is executed with a Stomacher, a tool that, thanks to the rotational motion of pedals,

crushes the sample and determines its pulping; how long the homogenization lasts depends on the hardness and consistency of the sample. The analysis has to be executed immediumstely; if this is not possible, the diluted sample has to be kept in a fridge (4°) (Figure11).

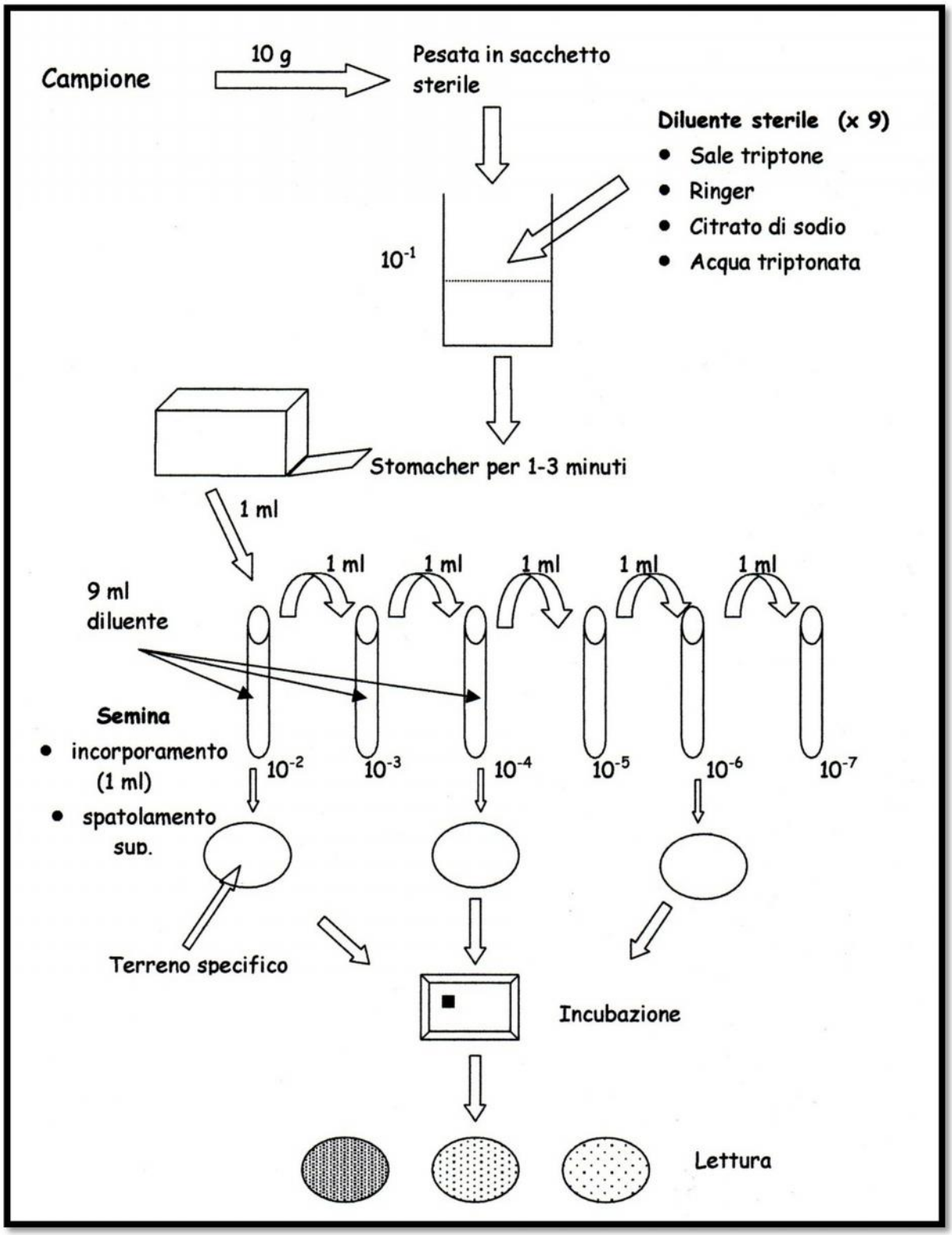


Fig. 11- Preparation of a solid sample for microbiological analysis Fonte**: Galli & Franzetti**

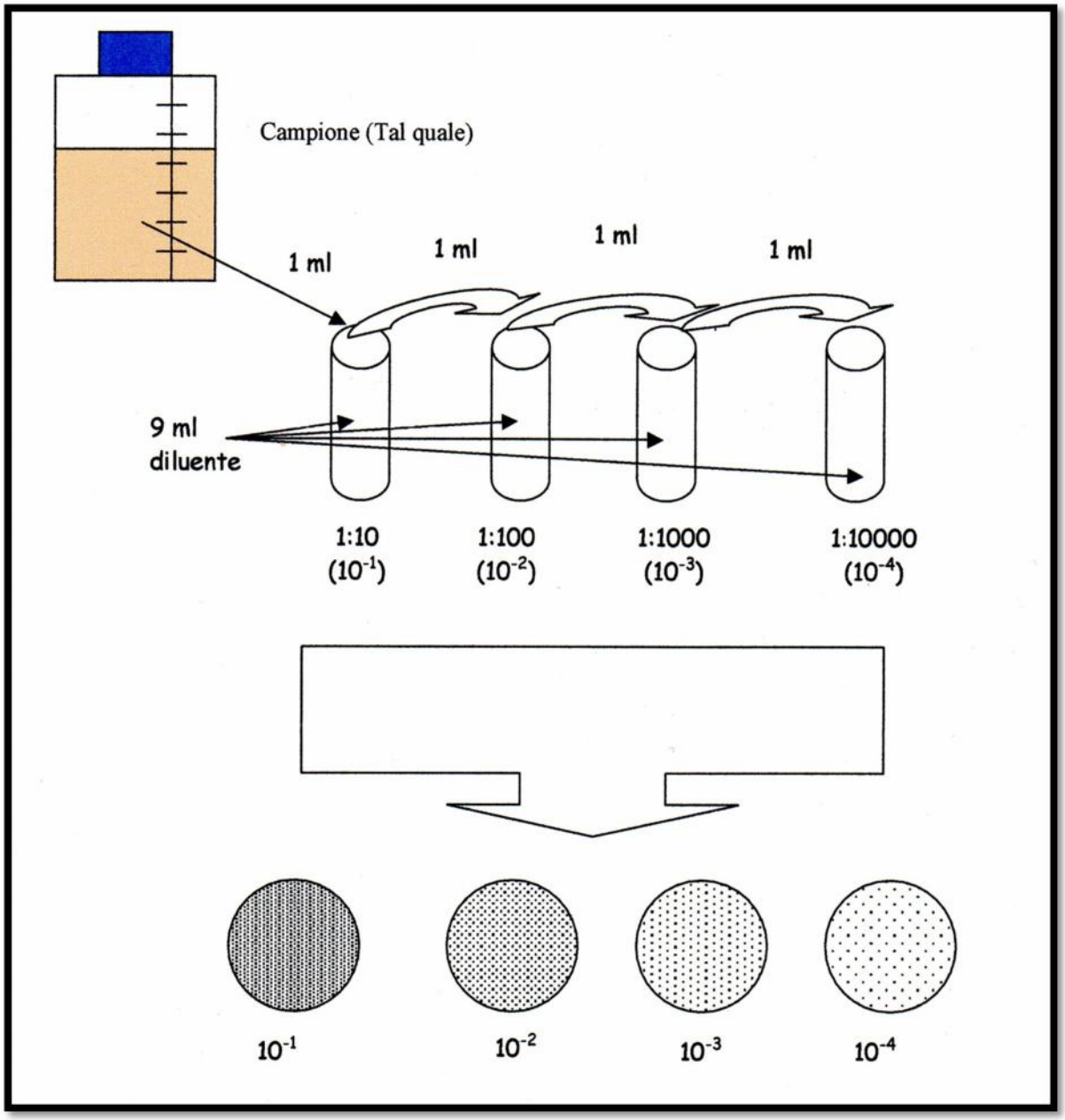


Fig. 12 – Pouring a liquid product Source: Galli & Franzetti 2003

- *Liquid products*
  No preparation is required, but they are used in an unaltered state (Figure 12).
  Different diluents for the preparation of the stock dilution of a food sample have been proposed. Choosing the diluent is a very important phase of the analysis, as unfit diluents such as water, can harm microbial cells, leading to a subsequent underestimation of the number of microorganisms. The most used diluent is the salt-peptone solution (0,85% Na Cl + 0,1% peptone). Special diluents might be necessary for particular analysis (solutions with a low redox potential for the numbering of anaerobe germs or with concentrations of 15-20% NaCl or sucrose for the analysis of food with a low Aw).

8.3.1 COUNT OF SPOILAGE AND PRO-TECHNOLOGICAL GERMS

The microbiological analysis of spoilage and pro-technological microorganisms requires a quantitative analysis, namely the count of microorganisms. For each sample, scale dilutions have been carried out, with base 10 in a Peptone-Saline Solution (PSS) to a $10^{-7}$for the count of alterating and pro-technological microorganisms, with the exception of the count of moulds and yeasts, where the Peptone Water Broth (PWB) diluent is used. The mother solution is prepared in accordance with the same procedure explained previously. The dilutions have been seeded in triple in the culture mediums. Here follow the procedures for microbiological analysis in accordance with ISO regulations.

Total bacterial count

The method for the plate count at 30°C with the technique of deep pour refers to the UNI EN ISO 4833-1:2013. This is a determination allowing an assessment of the global quality of the product (Quality Index) and provides general information on the times of food conservation. For the solid sample the Plate Agar Count (PCA) medium is used, while for the liquid one the Plate Agar Count (PCA) + skimmed milk (PCM). Pour plate method at 30°C for 72 hours. All colonies grown are counted, except for yeasts and moulds.
The legal limit in accordance with the Regulation no. 853/2004 of the Parliament and the Council of Ministers of 29/04/2004 expresses the value in germs at 30°C (per ml or g) ≤ 1.500.000 (rolling geometric average, calculated over a period of two months, with at least two samplings per month.

Enterobacteriaceae

The method used for the numbering of *Enterobacteriaceae* refers to the ISO 21528-2:2004. The Violet Red Bile Glucose Agar (VBRG) medium is used, which contains the glucose as source of carbon, used from all the *Enterobacteriaceae*. Pour plate method in a double layer, incubation at 37°C for 24 hours. All the colonies presenting a diameter greater than 0.5 mm (red-violet colour, with or without red-violet ring) are counted. Then one proceeds with the confirmatory test by carrying out subcoltres of the characteristic colonies on a Nutrient Agar (NA) medium and incubate at 37°C for 24 hours. After the incubation,collect a part of every isolated colony on aoxidase kit cardboard for the oxidase reaction and examine the heat development within 10 seconds.
The *Enterobacteriaceae*, being negative oxidases, must not develop any colouring of the colonies in 10 seconds; in this case, they are stab inoculated in tubes containing the Glucose Agar (GA) medium, and incubated at 37°C for 24 hours. The tubes that, after the incubation, show the development of a yellow colouringare considered positive to glucose fermentation.
According to law 2073/2005 modified in 15/11/2005 the limit is ≤ 10 cfu/ml on five sample units for milk and milk derivatives.

Coliforms

The method used for the count of *coliforms* refers to the ISO 4832:2006. The research of coliforms (quality and hygiene indexes) can be carried out using as medium the Agar Crystal Violet Neutral Red Bile Lactose (VRBL). The pour plate technique is carried out and the incubation happens at 30°C for 24 hours. The colonies recognized as coliforms are those growing between the two violet-red agar layers (fermentation of lactose and red-neutral tint), surrounded by a violet ring and a diameter no less than 2 mm. For the confirmatory test the atypical colonies are distributed in Lactose Brilliant Green Bile Brothtubes containing a Durham bell with an incubation happening with the same method as VRBL. The tubes considered as positive are those where production of gas occurs, which is highlighted by the raising of the bell, following the liquid leaking out and the gas entering (CO_2).

The legal limit in accordance with the Regulation 2073/2005 and Single Supervisory Mechanism (SSM) is 10 cfu/ml on 5 sample units for milk and dairy products.

Escherichia coli

This germ is used as indicator of the hygiene level. The method used for the count of glucuronidase positive- *Escherichia coli*β refers to the ISO 16649-2:2001. The medium used is the Agar Tryptone-bile-glucoronic medium (TBX). Pour plate method is used, with incubation at 44°C for 24 hours. The glucuronidase positive-*E. coli*β are the blue colonies on the plates where ther are no more than 150 typical colonies and no more than 300 total colonies (typical and atypical).

The legal limit in accordance with the Regulation 2073/2005 must be between 100 and 1000 cfu/ml or g on of milk and dairy products

Coagulase-positive staphylococci

The method used for the count of *Coagulase-positive staphylococci* refers to the ISO 6888-2:2004. The medium used is the the Agar Baird-Parker with Rabbit Plasma Fibrinogen (RPF). The resulting medium has the advantage of highlighting the presence of coagulase-positive staphylococci directly on the plate, consistently reducing the usually necessary confirmatory tests. This medium therefore allows the simultaneous count and confirmatory test in a single operation. When used, add 10 ml of RPF supplement to 500 ml of medium. Use the pour plate method and incubate at 37°C for 24-48 hours. The *Coagulase-positive staphylococci* are characterized by the formation of grey or black colonies; the colouring occurs due to the reduction of tellurite in metallic tellurite, surrounded by anopaque fibrin ring appearing clear, stable and evident. Atypical colonies are also grey or black but do not present a clear ring.
Legal limits in accordance with the Regulation 2073/2005 must be between 10 and 100 cfu/ml or g.

Pseudomonas spp.

The method for numbering and researching the *Pseudomonas spp.* refers to the ISO/TS 11059 IDF/RM 225 01-08-2009. The medium used is the Pseudomonas Penicillin Pimaricin Agar base (PPA), with a pimaricin supplement. Rebuild the contents of the pimaricin supplement tube with 5 ml of sterile distilled water, later sterilely added to 500 ml of PPA medium. Spread plate method is used, with incubation at 25°C for 48 hours. The colonies to be counted are those which appear in a greenish-blue or white colour. A confirmatory test is then carried out, by making subcultures of the characteristic coloines on a Nutrient Agar (NA) medium and incubated at 25°C for 24/48 hours. After the incubation a part of each isolated colony is taken on a oxidasekit cardboard and is examined for the development of colour. The test is considered positive if the colonies become reddish-violet within seconds. The colonies which have been considered positive at the oxidase test are stab inoculated in tubes containing the Glucose Agar medium (GA) with incubation at 25°C for 24 hours. The tubes that, after the incubation, have not fermented the glucose of the Glucose Agar are considered negative. The limit based on regional lines Guide is 10^6 cfu / ml or g of milk and dairy products

Moulds and Yeasts

The method employed for counting moulds and yeasts ($a_w > 0,95$) refers to the ISO 21527-1:2008. As already mentioned at the beginning of this chapter, the sample is homogenized in a Peptone Water Broth (PWB) diluent. The count of moulds and yeasts can be carried out using a Rose Bengal Agar (DRBC Agar). The Rose Bengal dye limits the development of certain mould colonies such as Neurospora and Rhizopus that, growing rapidly, might darken slow-growing moulds. Spread plate method and incubation of plates not turned upside-down at 25°C for 5-7 days. The plates containing from 10 to 150 colonies are selected, and all the different colony morphologies (fungal, yeast-like etc.) are selectively counted.
The limits are comprised between 100 and 1000 cfu/ml or g on milk and milk derivatives (WQA 2011 e FDC 2009).

Psychrotrophs

The method used for counting psychrophilic microorganisms refers to the ISO 17410:2001. The mediums used are the Plate Count Agar (PCA) for the solid sample and the PCA + skimmed milk (PCM) for the liquid sample. Spread plate method and incubation at 6,5°C for 10 days.
The plates taken into consideration are those containing 10-300 colonies.

Lactic bacteria

The method used for the count of *mesophilic lactic-acid bacteria*referso to the ISO 15214:1998. The medium used for the count of *mesophilic lactic-acid bacteria* is MRS Agar. MRS Agar ISO Formulation is prepared in accordance with a modification of the original formula by De Man, Rogosa and Sharpe and corresponds to the medium indicated by ISO 15214 for the count of the *mesophilic lactic-acid bacteria* in food. Pour plate method and incubation, in aerobiosis, at 30°C for 72 hours. The plates to be taken into consideration are those with a number between 10 and 300 characteristic colonies, that are lenticular and with a 1-3 mm diameter on MRS.

8.3.2.1.TECHNIQUES OF MICROBIAL COUNT

In this paragraph the methods of calculation employed by the laboratory for quantitative microbiological tests are explained, based on the count of plate colonies and the criteria of the results obtained. These principles constitute the main step to ensure the validity of microbiological examinations. The criterion employed is represented to the ISO regulation 7218:2007: “General requirements and guidance for microbiological examinations”.
An N number of microorganisms present if a sample is determined through a Petri plate count of colonies. The count is carried out when on at least a plate there colonies in the measure of 10-300 (total, typical or compliant with the identification criteria).
The N number of microorganisms in the sample is calculated with the arithmetic mean between two successive dilutions using the equation:

$$N = \frac{\Sigma C}{V \; x \; 1{,}1 \; x \; d}$$

- N = number of microorganisms,
- ΣC = sum of the colonies in the plates taken into consideration,
- V = volume, expressed in ml, of the inoculum poured on each plate,
- d = dilution factor corresponding to the first dilution taken into consideration.

The result is to be expressed in CFU/g for solid products or CFU/ml for liquid products.
When the method used requires an identification, an A number of presumptive colonies submitted to a confirmation (generally 5) are identified by each of the plates counted.
After the identification, calculate the a number of confirmed colonies for each plate via the following equation

$$a = \frac{b}{A} x\ C$$

- a = number of colonies after the identification,
- b = number of colonies confirmed with the identification criteria among the A colonies identified,
- A = number of presumptive positive colonies, submitted to a confirmatory test (usually 5),
- C = number of presumptive colonies counted on plates

In case there is a low count of colonies (<10 colonies) or absence thereof, it is necessary to report the results as follows, respectively:

-Pour plate method of 1 ml, for a presence of 1-3 colonies the result is expressed as <40 CFU/g or ml, in the case of absence of colonies express the result as <10 CFU/g or ml (where 40 and 10 come from the product of 4 and 1 times 1/d, that is the dilution factor of the initial suspension or of the first dilution taken into consideration.

-In case of spread plate method of 0,1 ml, in case of presence of 1-3 colonies, the result has to be expressed as < 400 CFU/g or ml, in the case of the absence of colonies express the result as < 100 CFU/g or ml.

8.3.3 RESEARCH OF PATHOGENIC MICROORGANISMS

The research of pathogenic microorganisms is never a count but a qualitative assessment, namely the presence/absence in a pre-determined quantity of food (25 g or ml).

RESEARCH OF SALMONELLA SPP.

The method used for the research of *Salmonella spp.* refers to the UNI EN ISO 6579:2009. For the analysis of *Salmonella* the following procedure is employed:

- *Pre-enrichment*
  Add 25 g or ml of sample to 225 ml of Buffered Peptone Water (BPW ISO).
  Incubation at 37°C for 18 hours.
- *Selective enrichment*
  From the cultures transfer:

a) 0,1 ml in 10 ml of Rappaport Vassiliadis Soy Broth (RVS) and incubate at 41,5°C for 24 hours,
b) 1 ml in 10 ml of Mueller Kauffmann Novobiocin Broth (MKTTn) and incubate at 37°C for 24 hours.

- *Isolation*
  From the RVS and MKTTncultures proceed with:
- pour on two plates of Agar Xilose Lysine Deoxycholate (XLD) and two plates of Brilliance Salmonella Agar Base (BSA) for every culture broth,

- Incubate upside-down at 37°C for 24 hours and observe the presence of typical colonies, of reddish-purple colour on the BSA and black on the XLD.
- *Confirmation*
  Consists of a bedding on a selective differential medium on Nutrient Agar (NA) plate, on order to obtain well isolated colonies. Incubation at 37°C for 24 hours.
  From the cultures in Nutrient Agar the following tests are undertaken:
- Biochemical confirmation API 20 E
- Biochemical confirmation: TSI Agar, Urea-fortified Agar, medium for the decarboxylation of L-lisine, reseach of β–galactosidase, medium for the Voges – Proskauer reaction, fortified medium for the reaction of the indole.
- Serological confirmation: it includes the determination of the presence of somatic, immobilization and capsular Salmonella antigens (O, Vi and H) with the agglutination test on a slide.
- *Expression of the results*

In the regulation CE no. 2073/2005 it is established that Salmonella spp. must be absent in 25 g of product.

RESEARCH OF YERSINIA ENTEROCOLITICA

The method used for the research of *Yersinia enterocolitica* refers to ISO 10273:2005. The procedure for the analysis of *Yersinia enterocolitica* is as follows:

- *Pre-enrichment*
- 10 g or ml of sample are sterilely put in 90 ml (1/100) of IrgasanTicarcillina Potassium Chlorate Bhoth (ITC broth) medium and incubated at 25°C for 48 hours;
- 1 g or ml of sampleare added to 9 ml (1/10) of Peptone Sorbitole Bile salts Broth (PSB broth) medium and incubated at a temperature of 22-25°C for 72 hourse with stirring and for 5 days without stirring.
- *Selective enrichment*
- ,Transfera loopfulfrom the culture in ITC brothon a plate of Salmonella/Shigella Agar with Sodium Desoxicholate and Calcium Chloride (SSDC agar) and incubate at 30°C for 24 hours.
- Transfer a loopful from the culture in the PSB broth on a Yersinia Selective Medium (CIN) plate. Then add 0,5 ml of PSB culture broth to 4,5 ml of potassium hydroxide in saline solution(KOH) and mix for 20 seconds, and transfer a loopful on CIN and incubate at 30°C for 24 hours.
  Examine the plates in order to locate the presence of *Yersinia enterocolitica*characteristic colonies, on the CIN shown as small, smooth and of a red colour in the center and a translucentedge with an appearance described as a "bull's eye", while on the SSDC they are shown as small, circular and grey/colourless. If there are no colonies, or they are not characteristic, carry on the incubation to a maximum of 48 h.
- *Isolation*
  Select at least 5 suspected/characteristic colonies, or all of them if in smaller number, from every CIN and SSDC plate. Streak the colonies into Nutrient Agar (NA) plates, in order to obtain well-isolated colonies. Incubate at 30°C for 24 h. Examine the purity of the colonies on the NA plate; if mixed culture are present, proceed to the subculture of each colony type in otherNA plates and incubate as above.
- *Confirmation*
  From the NA cultures proceed with the determination of the oxydase. Then proceed with the presumptive identification of pure cultures in NA, which resulted negative to oxydase. The following confirmatory tests are then undertaken: urea (positive), indole (positive or negative),

glucose fermentation (positive), formation of gas from glucose (negative), lactose fermentation (negative) and formation of H_2S (negative). The API 20E (BioMerièux) commercial kit for biochemical identification is used.

- *Expression of results*
  Indicate the presence or absence of alleged pathogenic*Y. enterocolotica* in 10 g or 1 ml of product. In milk and dairy products the *Y. Enterocolitica* must be absent.

RESEARCH OF LISTERIA MONOCYTOGENES

The procedure for the qualitative reseach of *Listeria monocytogenes*refers to the UNI EN ISO 11290-1:2005.

- *Selective pre-enrichment*
  25 g or ml of the sample are sterilely put in 225 ml of Half Fraser Broth (HFB) primary enrichment broth medium, incubation at 30°C for 24 hours.
- *Selective enrichment*
  From the HFB broth culture:

- Transfer a loopful on a Listera Agar (ALOA) plate and on a Listeria Selective Medium Oxford (OXFORD) plate;
- transfer 0,1 ml of HFB in 10 ml of Fraser Broth (FB) secondaryenrichment broth, incubation at 37°C for 48 hours. Later, after the FB incubation (with or without colour change in the broth) a similar pour on ALOA and OXFORD mediums is undertaken.
  Incubation at 37°C for 24h and in case of absence of growth, or growth, incubate at 37°C for 24 hours.

The *L. monocytogenes* colonies in ALOA are green-blue in appearance, surrounded by an opaque ring, while the suspected *L. monocytogenes*colonies in OXFORD are greenish-black in appearance, with a diameter of 2 mm, with a black centre forming a crater and a black ring around, sometimes called navel-shaped colonies.

- *Confirmation*
  Take 5 colonies from each plate on a selective medium and streak them on as many Agar Tryptone-Soy-Yeast Extract (TSYEA) plates and incubate at 37°C for 18/24 hours or until the growth is satisfactory.
  From the TSYEAcultures the following steps are undertaken:
- Gram stains technique;
- Catalase: a colony is diluted in a drop of hydrogen peroxide. The appearance of gas bubbles indicates the presence of the catalase enzyme.
- API Listeria: it is a standardized system for the identification of the different species of *Listeria*, using miniaturized tests together with a specific database. The identification of the Listeria species is obtained through a 4-digit numerical profile, that for the *L. monocytogenes* is 6150.
- CAMP test: it is a system for the identification of the *Listeria* using*Staphylococcus aureus*, *Rhodococcusequi*, *L. monocytogenes, L. ivanovii*and *L. innocua*as references strains. The positive reaction with *R. equi* appears with a huge ring (5-10mm) of "arrowhead" hemolysis. The positive reaction with *S. aureus* appears with a small ring of evident hemolysis inside the hemolytic ring of *Staphylococcus aureus*, extending about 2 mm from the strain to be tested.
- *Expression of results*

The legal limits regarding this pathogen are very restrictive due to its highly dangerous nature, as it can cause food-borne illnesses, abortions and meningitis, even causing death in the consumer. The qualitative analysis must show the absence of the pathogen in 25 g or ml of product, in accordance with the Regulation 2073/2005.

RESEARCH OF ESCHERICHIA COLI O157:H7

The research for this pathogen refers to the UNI EN ISO 16654:2003. For the analysis of the *Escherichia coli O157:H7*the procedure is as follows:

- weigh 25 g or ml of the sample under test and add 225 ml of Tryptone Soy Broth modified with Noviobiocin (TSB + N), incubation at 41,5°C for 6 hours;
- after the incubation, proceed with the magnetic immunoassay separation. This technique consists of the separation and concentration of microorganisms by magnetic immunoassay particles covered in anti-*E.coli O:157:H7* antibodies. It is carried out in this way:
- prepare, at room temperature, 20 µl of the immunoassay particles in an Eppendorf tube,
- take 1 ml from the stock dilution (25 g or ml sample + 225 ml of TSB+N) and trasnfer it in the tube,
- mix the suspension on the rotary shaker set at 12 r/min for 10 minutes,
- place each plastic Eppendorf tube on the magnetic support and allow the magnetic particles to gather against the magnet by gently oscillating the support by 180°,
- carefully open the cap without perturbating the particles on the walls of the tube,
- remove the liquid by slowly drawing from the lower part of the tube, then add 1 ml of washing buffer (PBS + Tween 20),
- remove the magnet from the support,
- mix the tube contents and put again the magnet in the support,
- repeat the washing procedure several times,
- remove from the magnetic extractor ad add 100 µl of PBS + Tween 20 to the tube and proceed to the suspension of magnetic particles,
- transfer 50 µl of the product on a Mac ConkeySorbitole Agar (SMAC) plate and 50 µl on a Cefixime-Tellurite (CT-SMAC) plate and streak accurately. Incubation at 37°C for 18-24 hours.
  The colonies appear 1 mm in diameter, transparent, colourless and with brownish-yellow nuances at the centre of the colony on CT-SMAC. From each plate at least five colonies are poured on Nutrient Agar (NA), whose plates are then incubated at 37°C for 18-24 hours.

- For confirmation biochemical tests are undertaken by agglutination commerical kits and by API 20E (BioMerièux).
- Indicate the presence or absence of *E. coli* O157:H7 in 25 g o ml of product, in accordance with Regulation 2073/2005 *E. coli O:157:H7* must be absent in 25 g or ml of product.

RESEARCH OF CAMPYLOBACTER SPP.

The method used for the research of *Campylobacter* spp. refers to the UNI UNI EN ISO 10272-1:2006.
10 g or ml of sample are taken; add to 90 ml of Bolton Broth in order to obtain a 1:10 ratio and homogenize, incubation at 37°C for 4 – 6 hours in microaerophilic conditions and then at 41,4°C for 44 hours in microaerophilic conditions. After the incubation, a loopfulis taken from the culture broth and strak on a Campylobacter Blood Free Medium Base Bolton (mCCD agar) plate and on a Karmali agar plate. The plates, kept in microaerophilic conditions, are put in a thermostat at 41,4°C for 44 hours.

Typical colonies appear grey-white in colour, with a metallic appearance, flat and wet with a tendency to spread.
To confirm, at least a suspected colony is taken from each plate (one must operate quickly as the colonies are air-sensitive), then streaked onto a Columbia Blood Agar plate in order to obtain well-isolated colonies. Incubate in microaerophilic conditions at 41,5°C for 24 h. From the Columbia Blood Agar cultures, tests are carried out to assess morphology, motility and growth in microaerophily at 25°C, growth in anaerobiosis at 41,5°C and presence of oxidase. For the results, indicate the presence or absence of *Campylobacter spp.* in 10 g or ml of product.

COUNT OF BACILLUS CEREUS

The methodused for the count of *Bacillus cereus* refers to the EN-ISO 7932:2005. 10 g or ml of the sample are added to 90 ml of Peptonate Saline Solution (PSS) and transfer 0,1 ml on the surface of ready Petri dishes containing Mannitol Egg Yolk Polymyxin Agar (MYP). Spread plate method, incubation at 30°C for 18/24 hours. In case colonies are absent or growth insufficient, incubate the plates for 24 more hours. Typical *B.cereus*colonies are counted in plates containing 15 – 150 colonies, considering as such thegrowth that presents the following characteristics:crenatedcolonies, 5 mm in diameter, pink with a clear opaque ring caused by the precipitation of the yolk, which presents the same colour of the colonies (mannitol -, lecithinase +). Since some strains of *B. cereus* produce a scarce amount of lecithinase, also the ringless colonies, even if having typical characteristics, have to undergo the following confirmatory tests:

- hemolysis test on Trypticasesoja agar + 5% mutton blood (AS)
- biochemical confirmation (optional).

For the expression of results we proceed with the calculation of the number of identified colonies per each plate and present in the test portion. The minimum infective dose is 100 CFU/g of food.

In accordance with the Regulation (CE) 2073/05 the legal limits are 50 – 500 cfu/g, between m and M with at least 1 sample on five sample units.

9 RESULTS AND DISCUSSION

9.1 CHEMICAL PARAMETERS

The analysis of the chemical parameters of the dairy matrices allowed the assessment of the pH and the Aw water activity. Here are reported the results relative to variations in pH during the freezing of single samples from each dairy (Figures 13).
All determination and experiments were performed in triplicate. Results are presented as mean value of three determinations. Data were statistically analyzed using ANOVA XLSTAT 2007 to evaluate the effect of freezing time on microbiological profile. Differences were considered significant for $p \leq 0.05$.

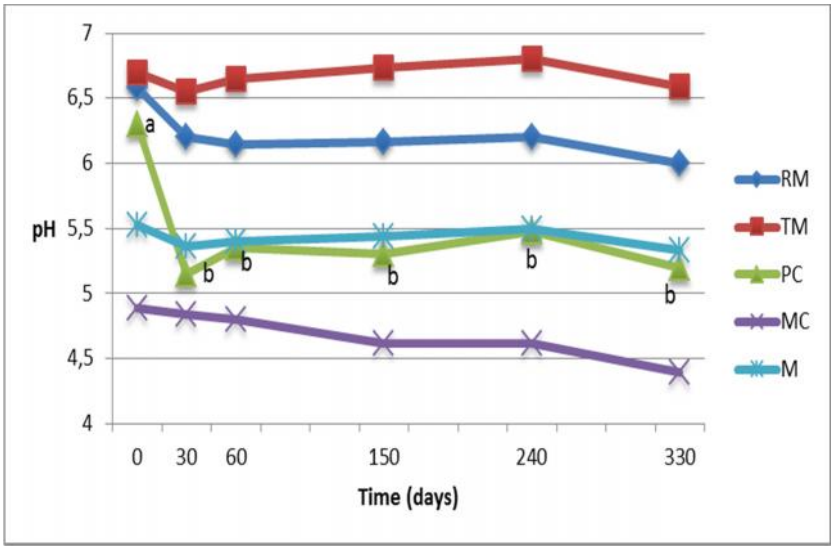


Fig. 13- pH values (mean and standard deviations) in raw milk (RM), thermized milk (TM), premature curd(PC), mature curd (MC) and Mozzarella cheese (M) during freezing of dairy A, B and C.
a-b different letters correspond to statistically significant differences ($\rho<0.05$).

It has not been possible to repeat all the chemical analysis in the months following the first, as the sample resulted unfit for analysis after thawing, while the five matricess have been analyzed for the entire analysis period only for pH and Aw free water.
Concerning the pH in raw and thermized milk, the value is between 6.61 and 6.78. In mature curd and mozzarella there are initial values from 5.94 to 5.52 and final values from 4.90 to 5.48 respectively. Therefore, during the conservation at -20°C the pH showed a steady trend without significant variations, anyway always with values above to pH 4,5, a value that allows the growth of all microorganisms, including pathogens.
Only premature curd showed a pH value of decrease during the first month of freezing. This trend can be attributed to a high content of sweet whey, still subjected to lactic bacteria fermentation (Guimãraes *et al.* 2010).
Here the results relative to the activity of water in the single samples of each dairy during the different times of analysis are provided(Fig. 14-15-16):

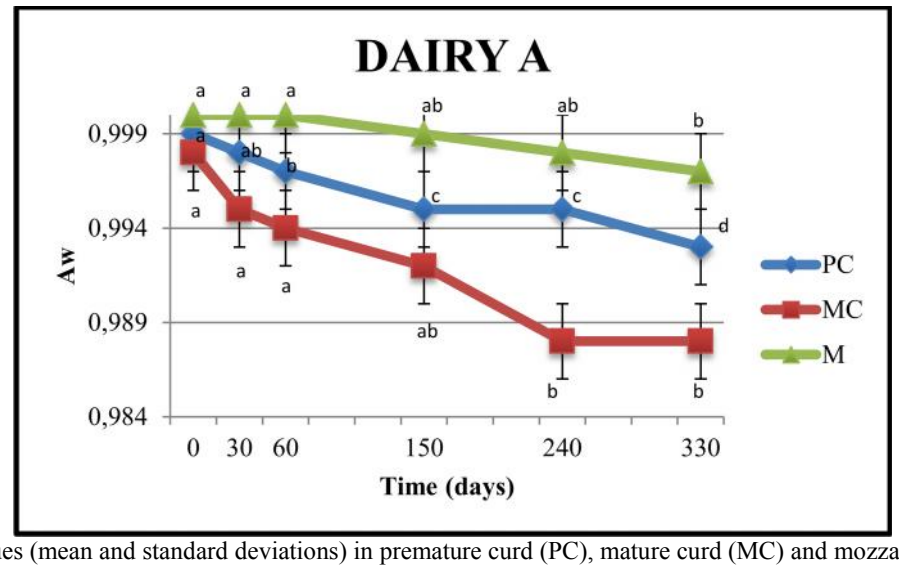


Fig. 14 - Aw values (mean and standard deviations) in premature curd (PC), mature curd (MC) and mozzarella (M) of the dairy A during freezing; different letters a-d correspond to statistically significant differences ($\rho<0.05$)

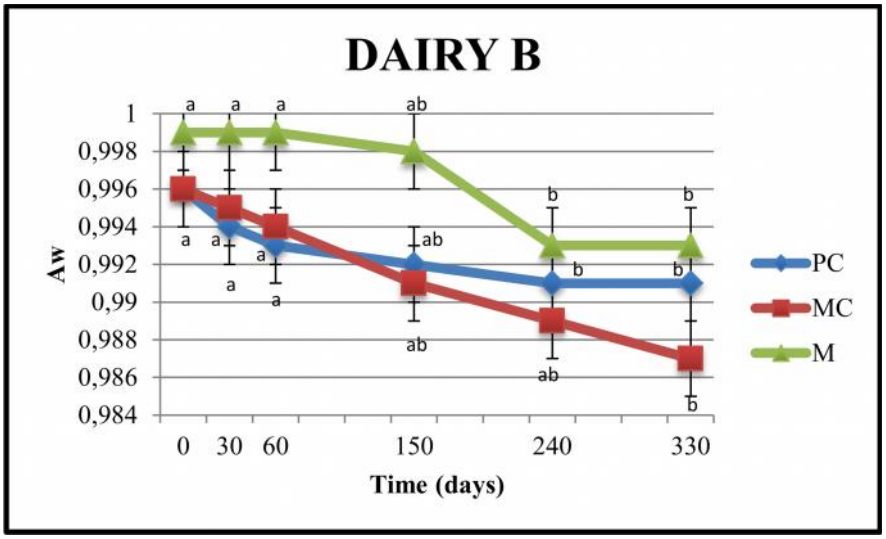


Fig. 15 - Aw values (mean and standard deviations) in premature curd (PC), mature curd (MC) and mozzarella (M) of the dairy B during freezing; different letters a-b correspond to statistically significant differences ($\rho<0.05$)

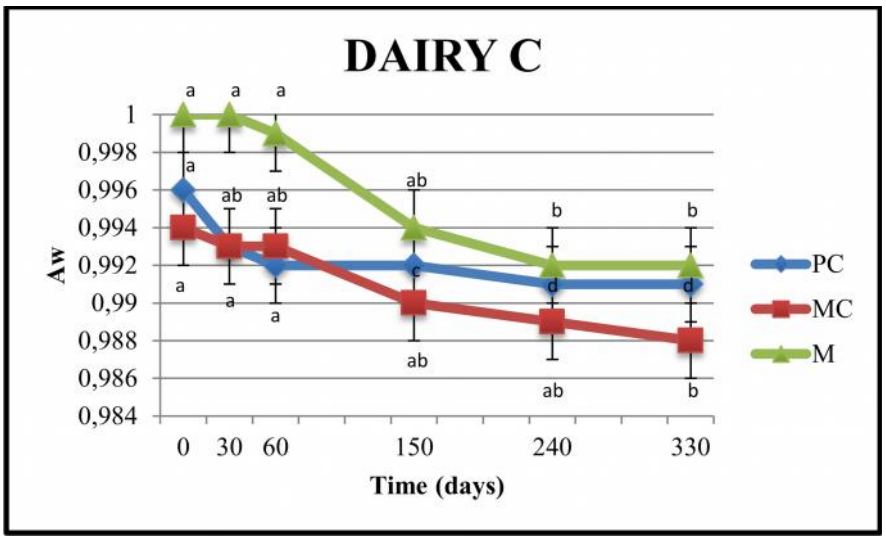


Fig. 16 - Aw values (mean and standard deviations) in premature curd (PC), mature curd (MC) and mozzarella (M) of the dairy C during freezing; different letters a-b correspond to statistically significant differences ($\rho<0.05$)

Water acticity (A_w) in the premature curd , mature curd and mozzarella initially showed similar values between 1,000 and 0,994 and final values between 0,988 and 0,987 . No significant differences ($p\leq 0.05$) were detected during freezing storage. The graphs show a general reduction of the values of a_w in the course of 11 months of freezing storage, that reduces available water for the metabolism of microorganisms (Jay *et al*., 2009).

FRESH MILK QUALITY

	Dairy A	Dairy B	Dairy C
Fat (%)	7.2 ± 0.1	8.0±0.2	8.5±0.1
Protein (%)	4.3 ±0.1	4.5±0.1	4.4±0.2
Lactose (%)	4.7±0.2	4.5±.01	4.6±0.1
Acidity (°SH)	8.9±0.1	9.0±0.1	8.9±0.1
Somatic cell count *	281,000	225,000	167,000

Tab. 8 - Fresh raw milk composition

Reg. (CE) n° 853/2004 del Parlamento e del Consiglio 29.04.2004: maximum somatic cell (/ml) ≤ 400. 000

The content of fat and protein falls within the limits laid down by the Production Specifications, i.e. minimum 7,2 % fat and 4,2 % minimum of proteins. There is no limit on other parameters except for somatic cells. By law the somatic cells must fall within the limits indicated by Reg. EC n° 853/2004 of the European Parliament and of the Council of 29.04.2004 and their value must be ≤ 400,000 cells/ml.

9.2 MICROBIOLOGICAL PARAMETERS

From the quantitative research on matrices such as milk, semi-processed, finished product, it emerged that pathogenic microorganisms were absent in all samples taken from the dairies. From the point of view of the contamination of pathogens, the samples examined have all presented a good quality, probably obtained thanks to the strict compliance with hygiene measures.
For the quantitative microbiological analysis, it has been necessary to transform in basic logarithmic terms 10 of all microbiological parameters. For simplicity, we include the graphs referring to each microorganism for each matrix sampled of dairies A, B and C, examined individually.

PROTECHNOLOGICAL BACTERIA

Lactic bacteria (LB) were 6.6 and 6.5 log/ml in raw and thermized milk.
In normal conditions, some strains of Lactobacillus are present in buffalo milk in higher concentrations than those contained in bovine milk. By metabolic activity of these bacilli the flavor and typical aroma of buffalo mozzarella cheese originates.
Values of 6.8-6.9 log/g were found in premature and mature curd, respectively, while 4.0 log/g were found in mozzarella cheese. A decreasing of LB was observed during freezing storage (fig.17): 13% in

raw milk, 21% in thermized milk, 30% in premature curd and 47-48% in mature curd and mozzarella cheese.

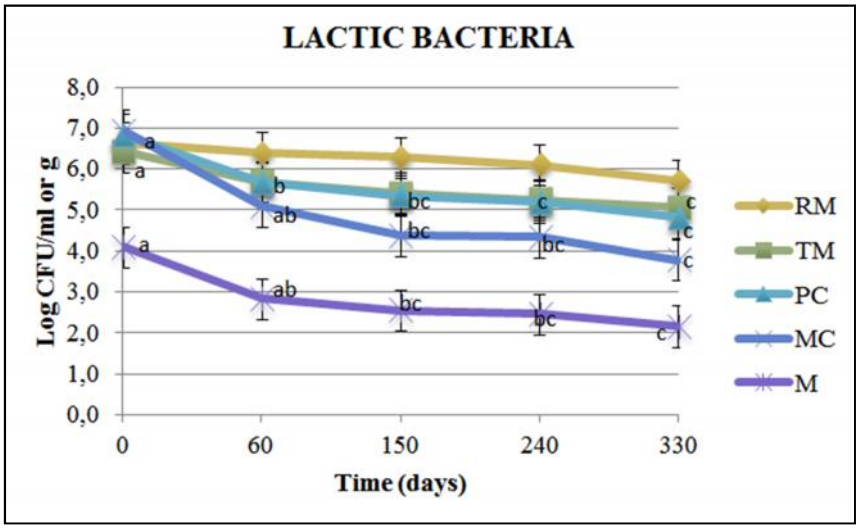


Fig. 17 – Mean values and standard deviations for Lactic Bacteria detected in dairy A, B and C. a-c different letters correspond to statistically significant differences ($\rho<0.05$)

SPOILAGE MICROORGANISMS

In raw milk, premature curd and mature curd, the initial Total bacterial count (TBC) value ranged between 5.5 – 6.3 log CFU/ml or g. In mozzarella this value was less than 4 log CFU/g and this value was constant in11 months of analysis, ensuring healthy of the product. For the other matrix a decreasing of TBC values was observed during frozen storage.

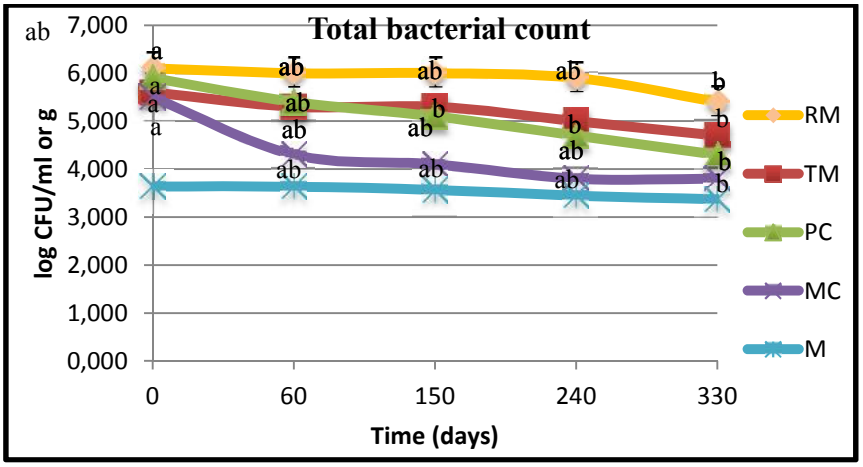


Fig. 18- Mean values and standard deviations for Total Bacterial Count in dairy A, B and C a-b different letters correspond to statistically significant differences ($\rho<0.05$)

Coagulase-positive Staphylococci were present in concentration of 3.2 and 2.4 log/ml in fresh raw milk and thermized milk, respectively, 1.5 and 1.3 log/g in premature and mature curds. A significant decreasing was observed in thermized milk. (fig.19).

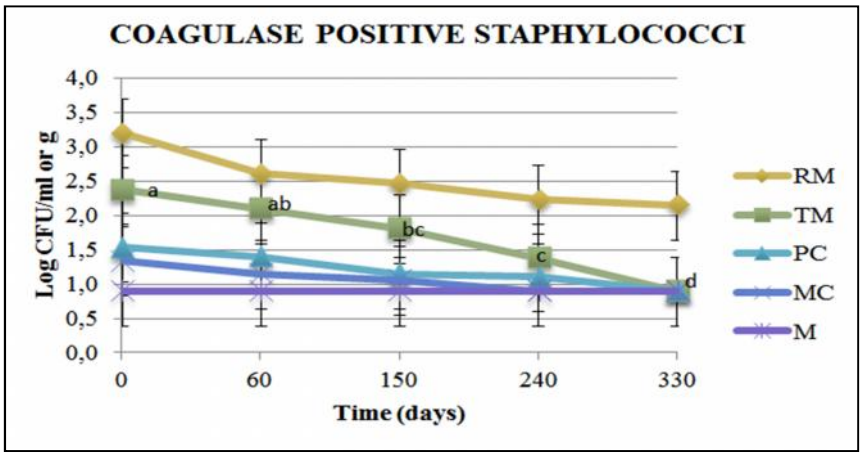


Fig. 19- Mean values and standard deviations for Coagulase positive Staphylococci detected in dairy A, B and C. a-d different letters correspond to statistically significant differences ($\rho<0.05$)

Enterobacteriaceae, in not stored samples, were present in concentration between 5.4 and 4.6 log/ml or g in raw milk, thermized milk, premature curd and mature curd, while in mozzarella cheese a lower amount 1.7 log/g was present. During freezing storage, the greatest decreasing of *Enterobacteriaceae* (80%) was observed in mature curd. In the other samples, decreasing of 40-60% were observed during frozen storage (fig. 20).

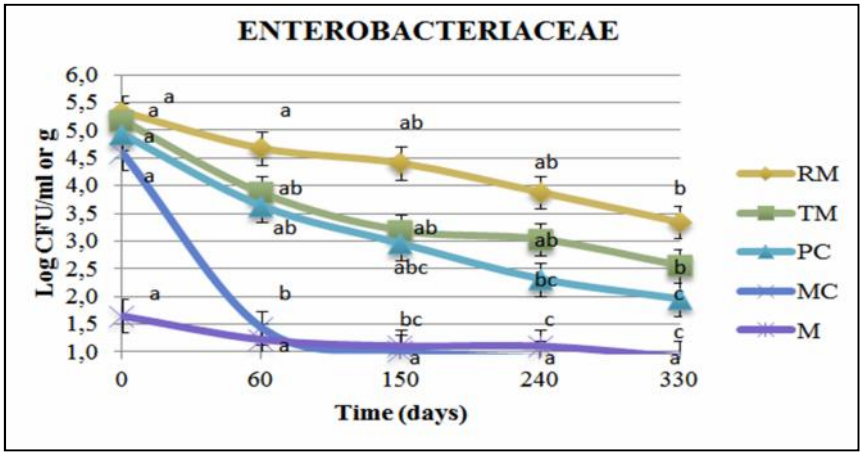


Fig. 20- Mean values and standard deviations for Enterobacteriaceae detected in dairy A, B and C. a-c different letters correspond to statistically significant differences ($\rho<0.05$)

Coliforms had an initial value of 5-5.5 log/ml, in thermized milk and raw milk, 4-4.7 log/g in mature and premature curds. Mozzarella cheese showed the lowest *Coliforms* concentration, with value of 1.7 log/g. During freezing, *Coliforms* decreased in all samples as shown in fig. 21.

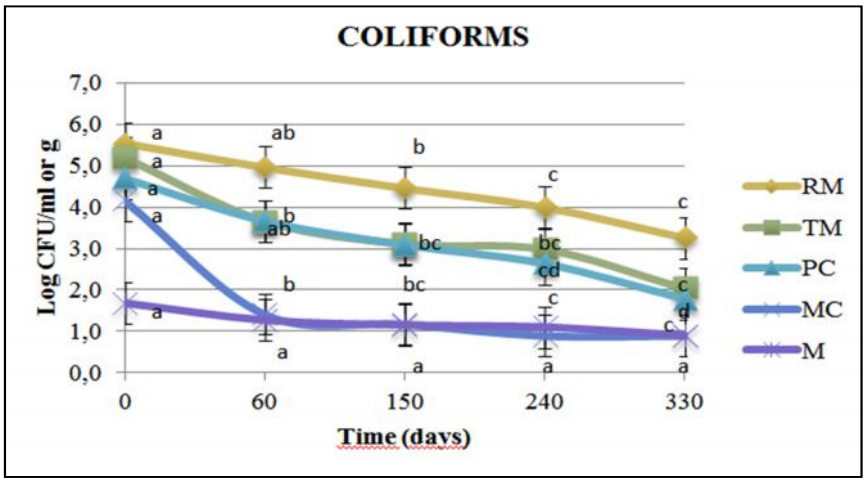


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Fig. 21- Mean values and standard deviations for Enterobacteriacea detected in dairy A, B and C. a-c different letters correspond to statistically significant differences ($\rho<0.05$)

Similar results were obtained for sheep milk cheese (Tejada et al., 2006) during a frozen storage for 9 months where decreasing of *Enterobacteriaceae, Coliforms and Staphylococci* counts was observed. *Escherichia coli β-glucuronidase positive* decreased of 53% in raw milk, 66% in mature curd and 70% in thermized milk and in premature curd (Fig.22).

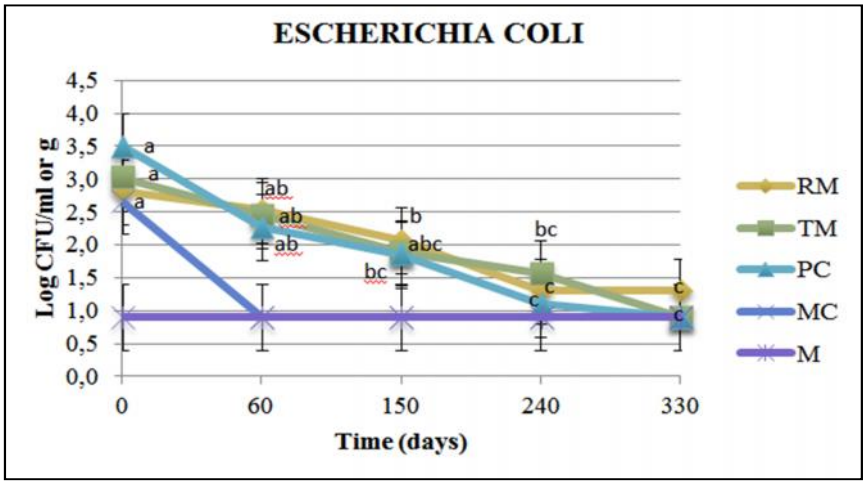

Fig. 22 - Mean values and standard deviations for Escherichia coli β-glucuronidase positive detected in dairy A, B and C. a-c different letters correspond to statistically significant differences ($\rho<0.05$).

Pseudomonas was present in initial values of 7.0-3.8 log/ml in raw and thermized milks, 2.7-2.4 log/g in premature and mature curds, respectively (Fig. 23).

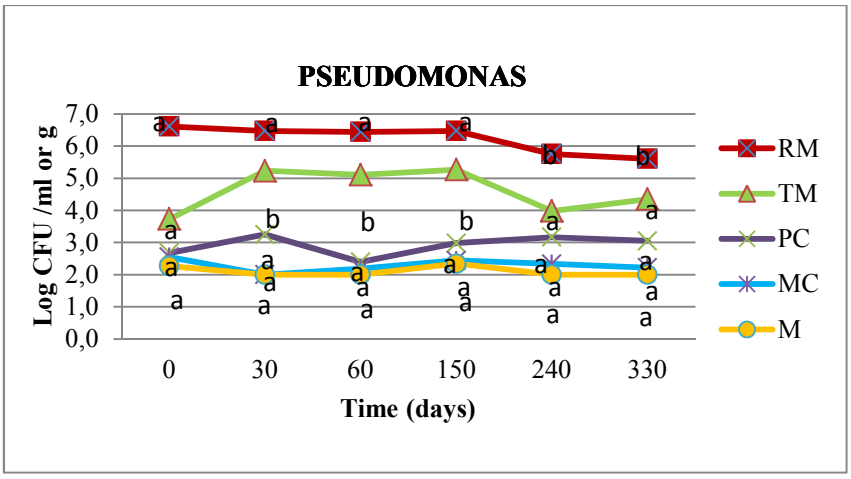

Fig. 23 - Values and standard deviations for Pseudomonas detected in dairy A, B and C. a-c different letters correspond to statistically significant differences ($\rho<0.05$).

Psychrotrophs, in the fresh state, reported concentrations of 7.0 log/ml in raw milk, 6.5 log/ml in thermized milk, 5.1 log/g in premature curd, 3.4 log/g in mature curd and 2.6 log/g in mozzarella cheese (fig. 24).

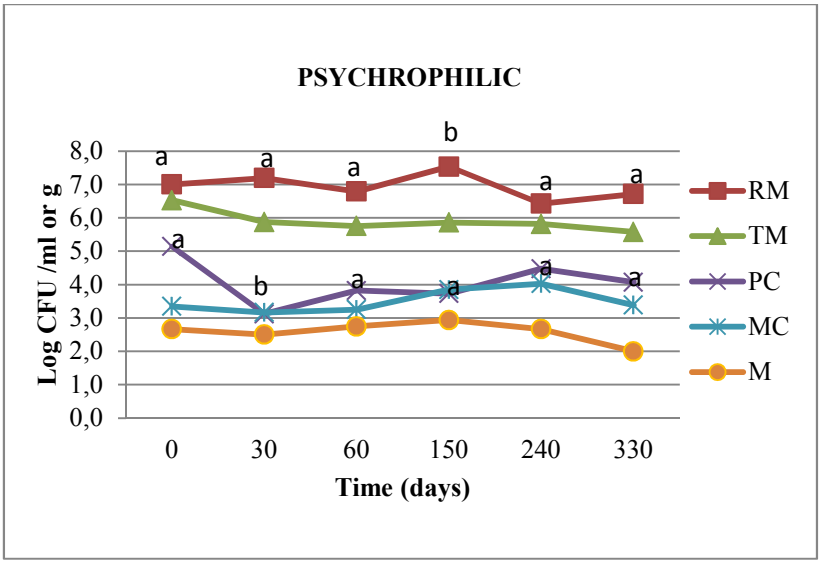

Fig. 24- Values and standard deviations for Pseudomonas detected in dairy A, B and C. a-c different letters correspond to statistically significant differences ($\rho<0.05$)

Yeasts were 3.8 log/ml in fresh raw milk, 2.4 log/ml in fresh thermized milk, 3.7 log/g in fresh premature, mature curds and mozzarella cheese; Mouldswere 3.0 log/ml in raw milk, 2.2 log/ml or g in thermized milk and premature curd, 1.2-1.5 log/g in mature curd and mozzarella cheese.

MOULDS AND YEASTS

The graphs of tree dairy A,B and C, for the group Yeasts and Moulds are here provided (Figures: 25-27 RM; 28-30 TM; 31-33 PC; 34-36 MC; 37-39 M):

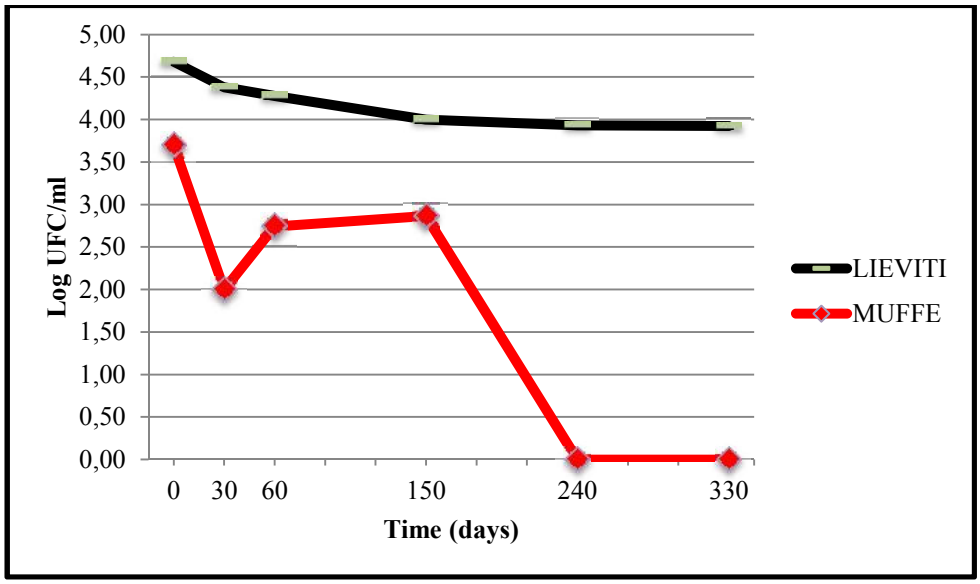

Fig. 25 - **Trend of Moulds and Yeasts in dairy A raw milk**

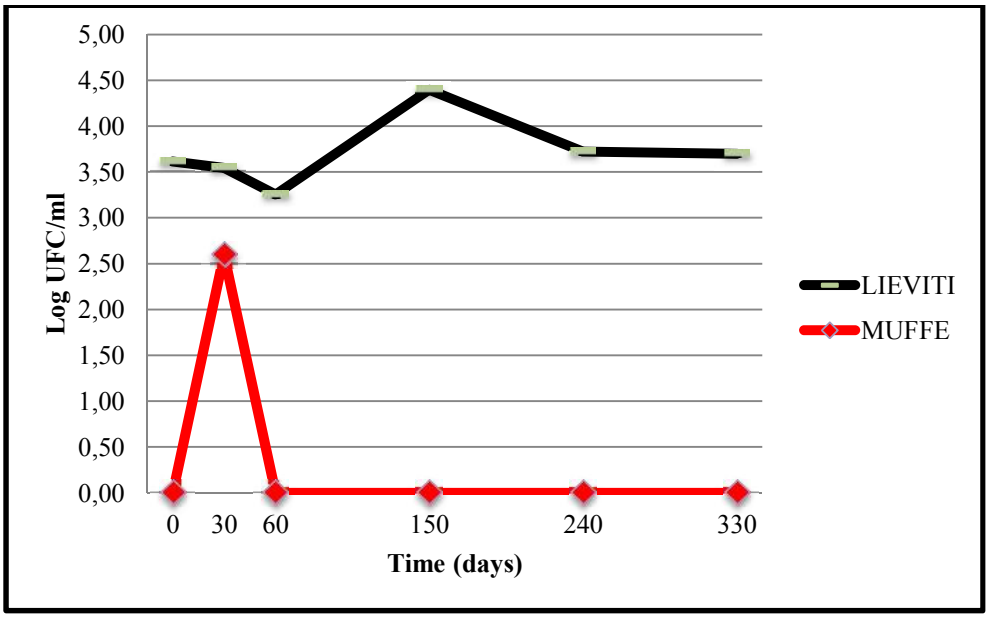

Fig. 26 - **Trend of Moulds and Yeasts in dairy B raw milk**

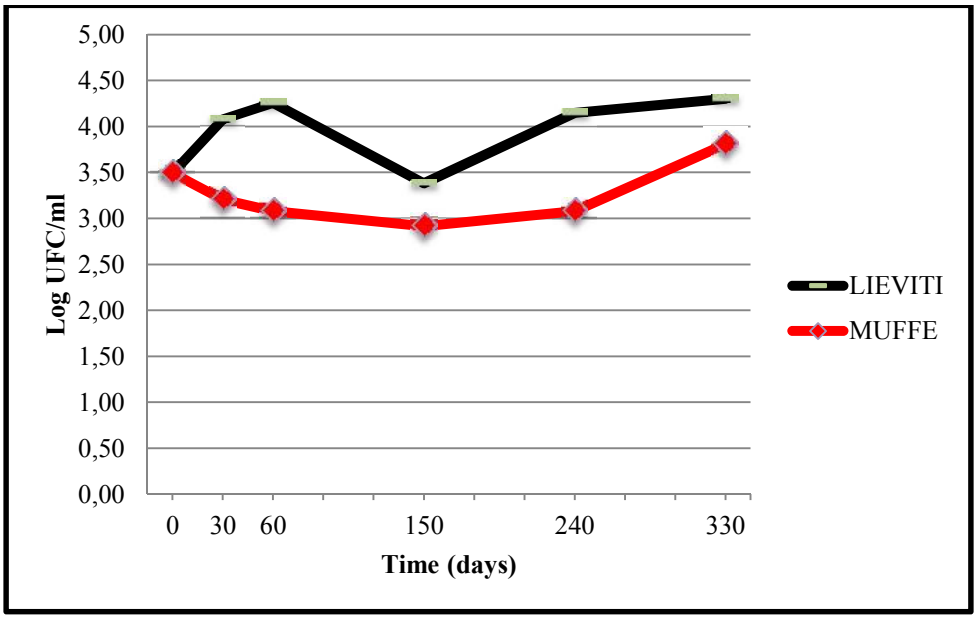

Fig. 27 - **Trend of Moulds and Yeasts in dairy C raw milk**

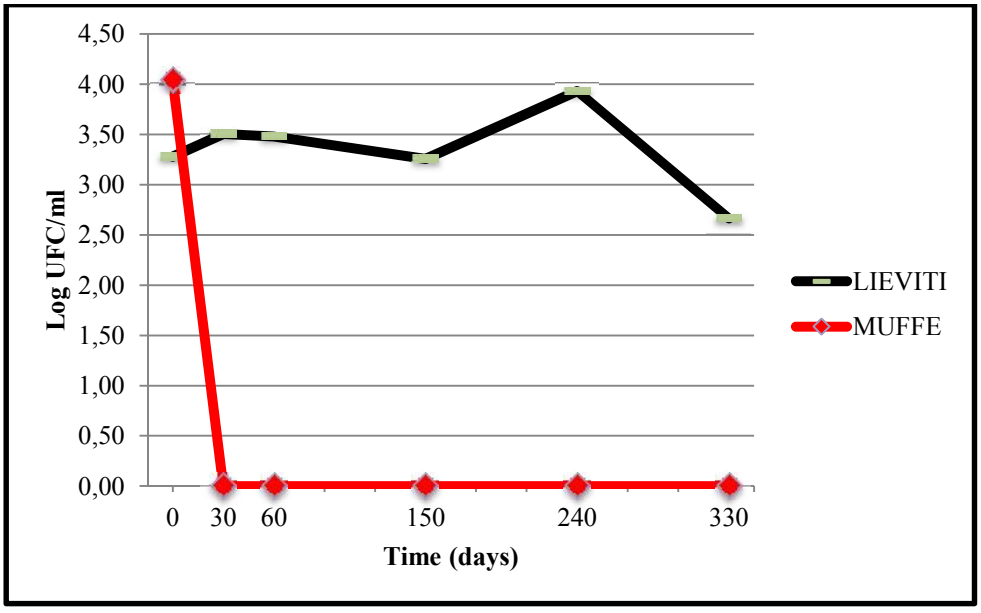

Fig. 28 - **Trend of Moulds and Yeasts in dairy A thermized milk**

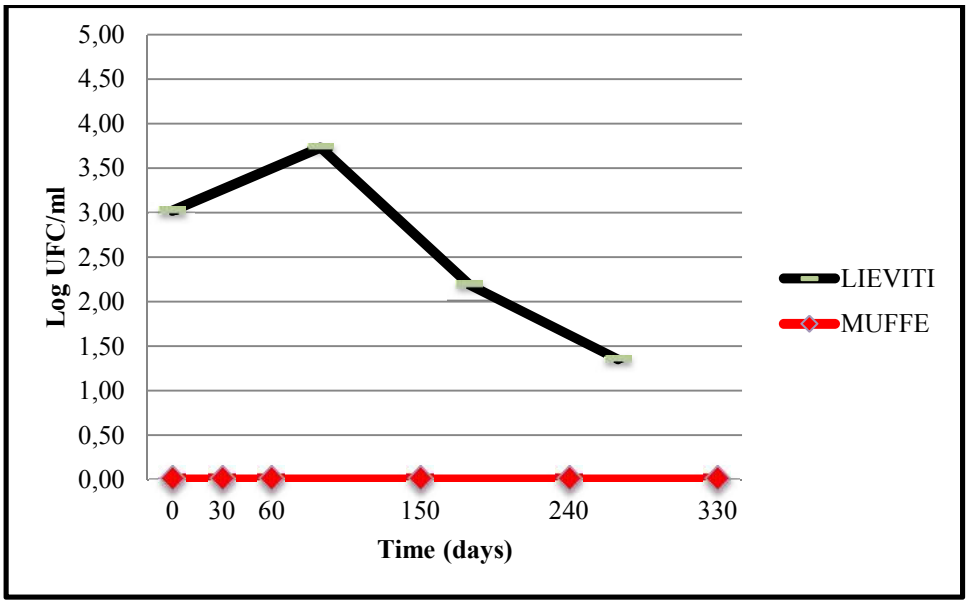

Fig. 29 - **Trend of Moulds and Yeasts in dairy B thermized milk**

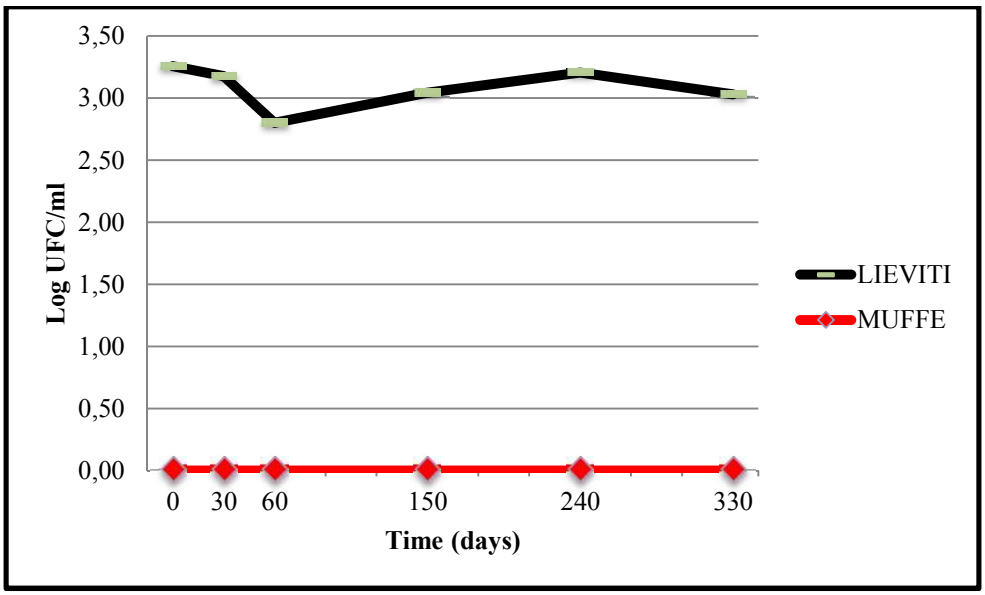

Fig. 30 - **Trend of Moulds and Yeasts in dairy C thermized milk**

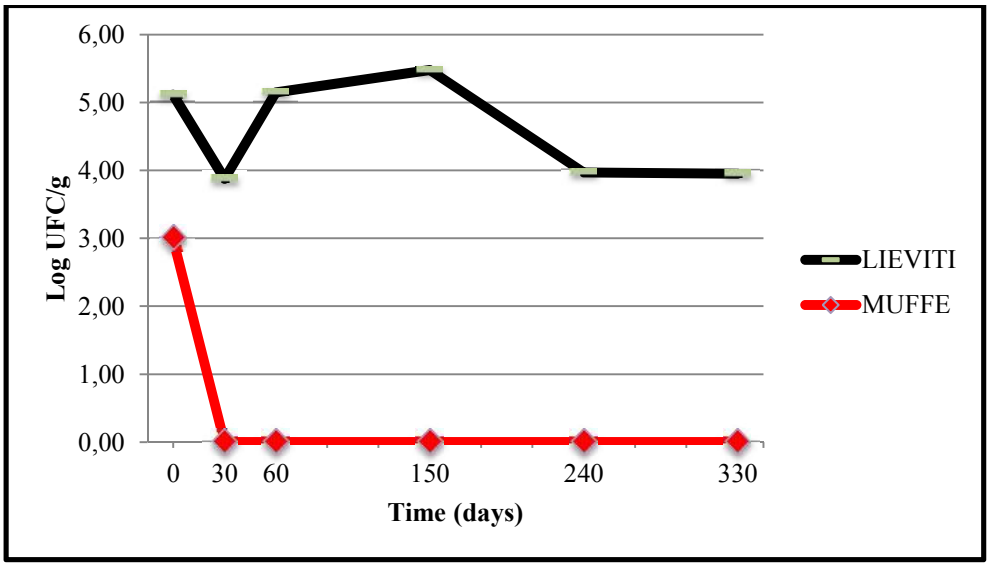

Fig. 31 - **Trend of Moulds and Yeasts in dairy A premature curd**

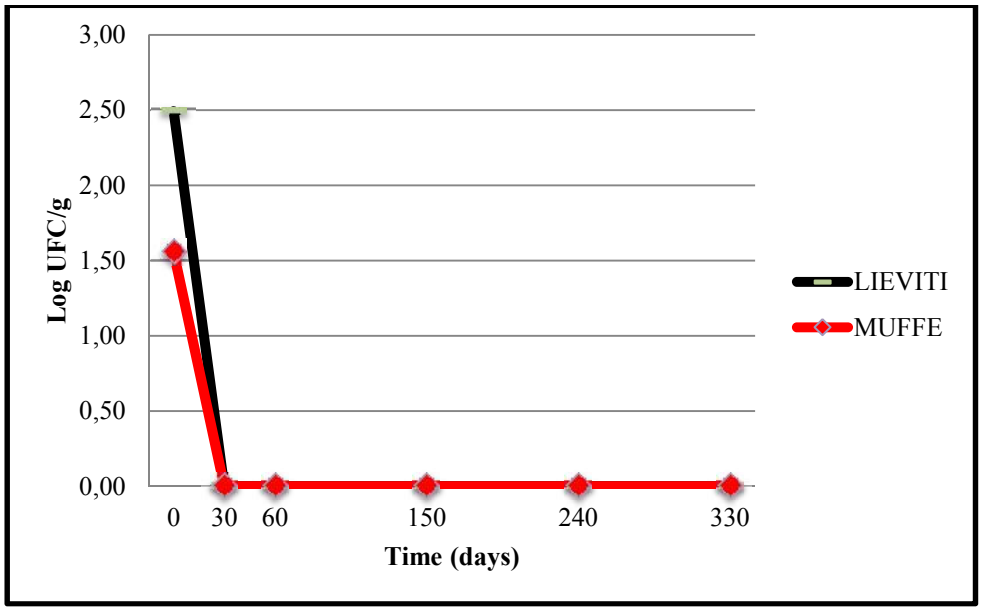

Fig. 32- **Trend of Moulds and Yeasts in dairy B premature curd**

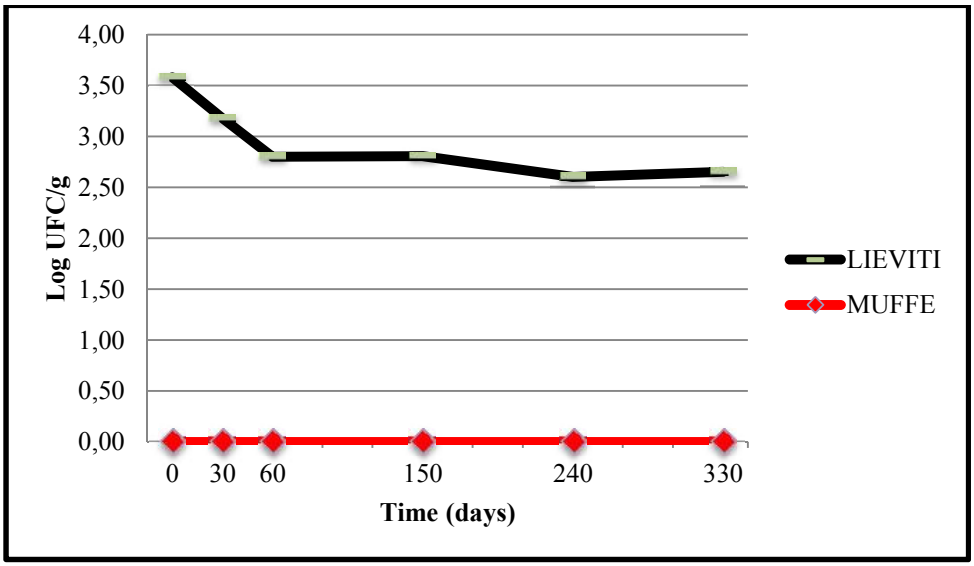

Fig 33 - **Trend of Moulds and Yeasts in dairy C premature curd**

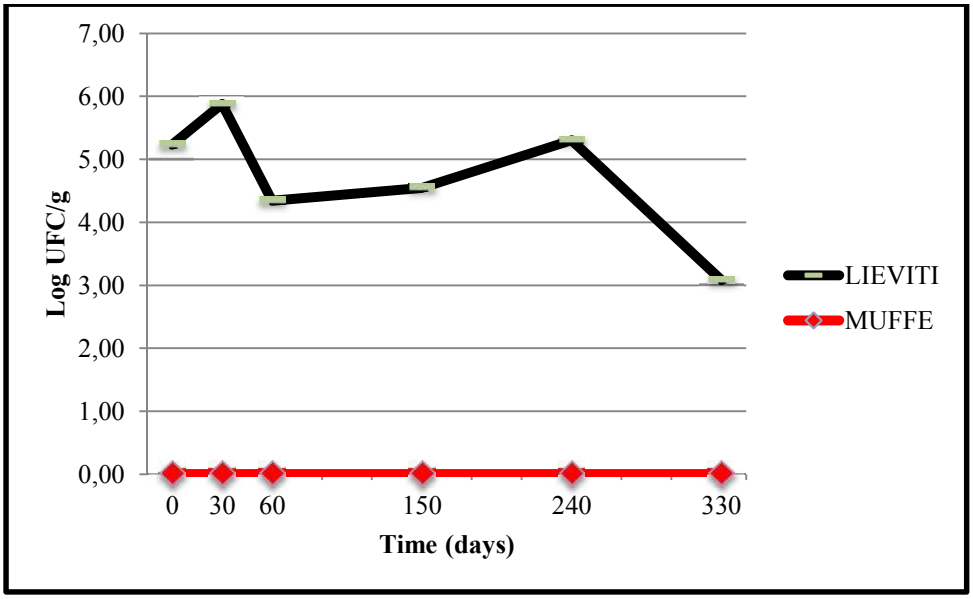

Fig. 34 - **Trend of Moulds and Yeasts in dairy A mature curd**

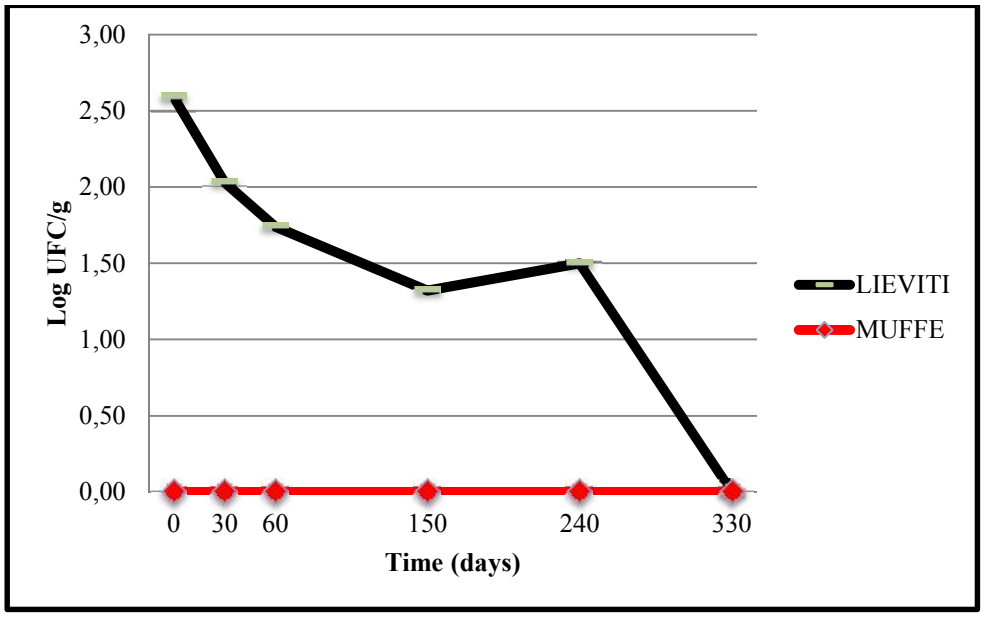

Fig. 35 - **Trend of Moulds and Yeasts in dairy B mature curd**

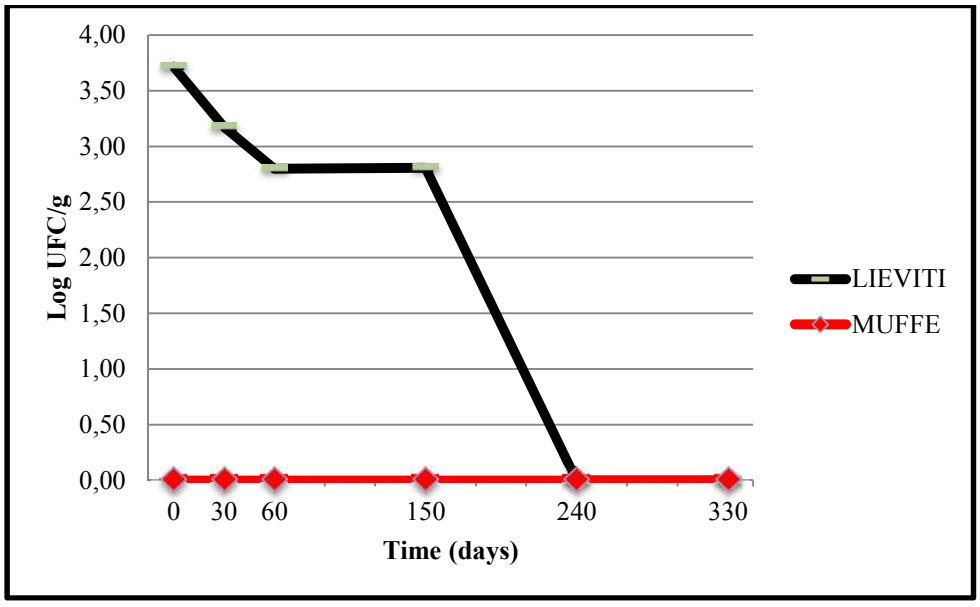

Fig. 36 - **Trend of Moulds and Yeasts in dairy C mature curd**

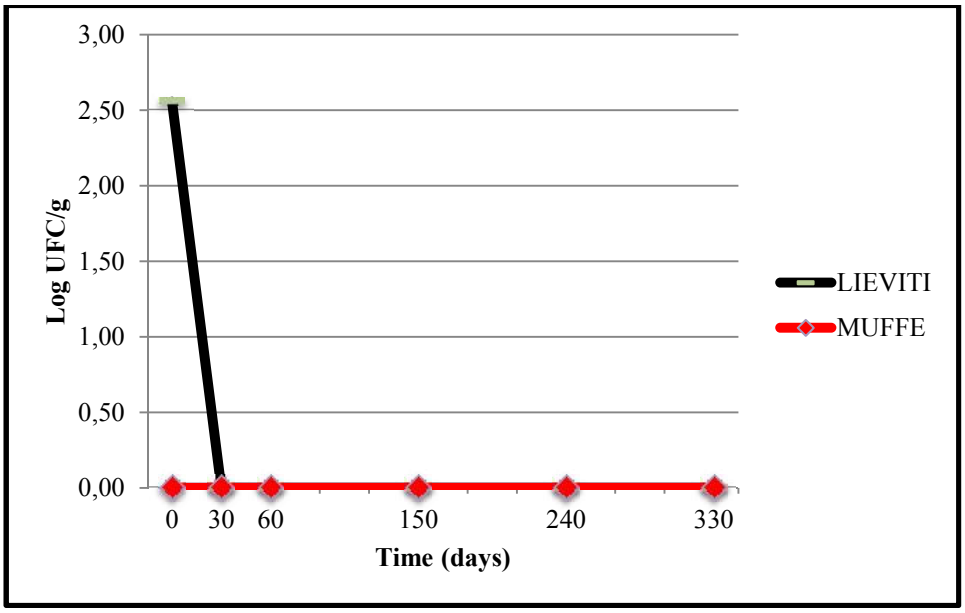

Fig. 37 - **Trend of Moulds and Yeasts in dairy A mozzarella**

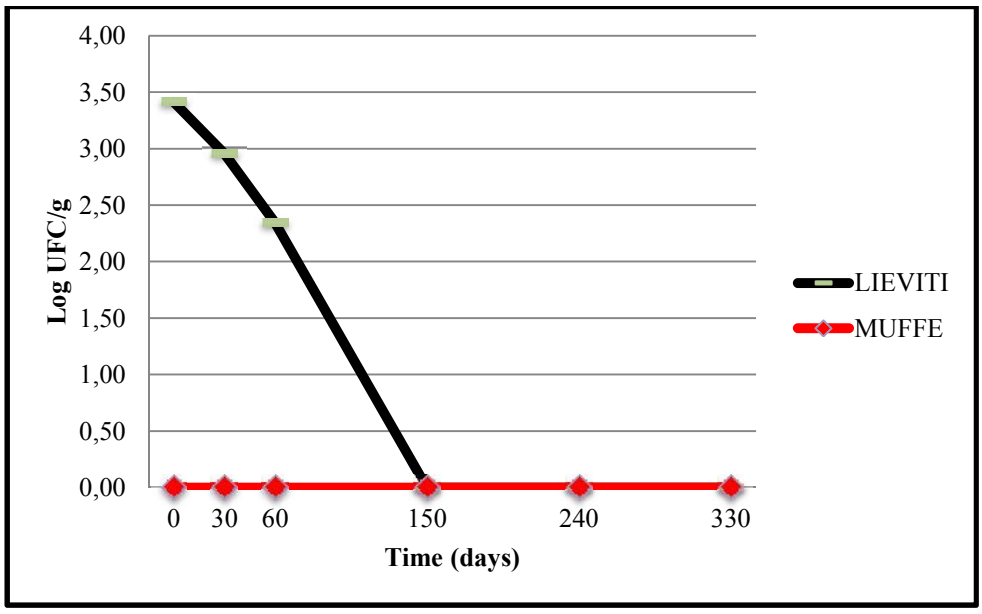

Fig. 38- **Trend of Moulds and Yeasts in dairy B mozzarella**

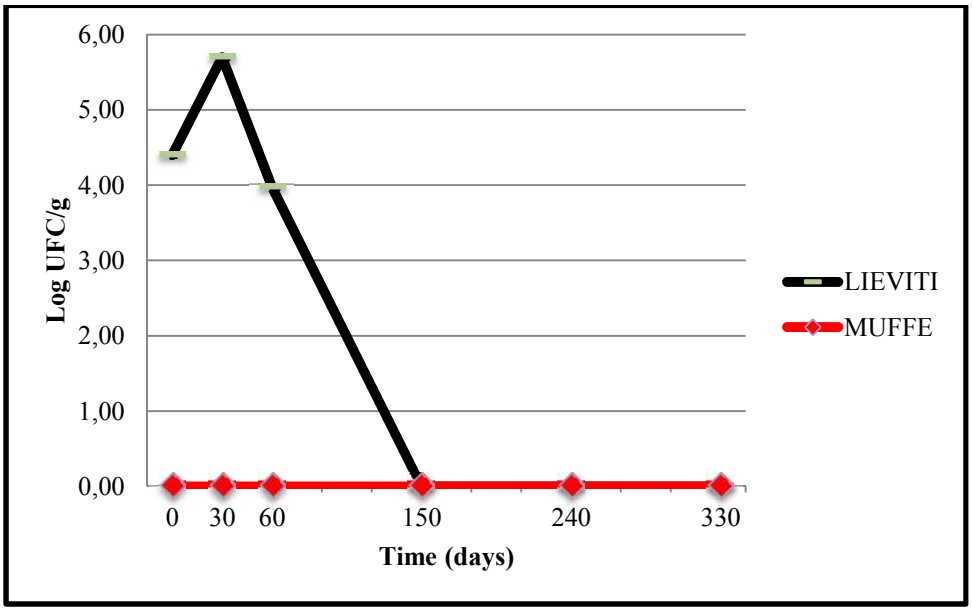

Fig. 39 - **Trend of Moulds and Yeasts in dairy C mozzarella**

What emerges from these graphs is the decrease or absence of Moulds, which are present in a fresh state in the samples of raw and thermized milk of dairies A and C with values around 4,0 log CFU/ml, and in the premature curd of dairy A with a value of 1,60 log CFU/g. In the course of freezing the moulds are inferior or absent in all samples. Yeasts have been found, in a fresh state, with initial value of 3,8 CFU/ml in raw milk, of 3,2 CFU/ml in thermized milk and of 3,7 -3,8-3,4 log CFU/g in premature, mature curd and mozzarella cheese respectively in a fresh state. In the course of time is highlighted a slight decrease between 4,0 -2,4 in raw and termized milk and 3,3-3 and 0 in premature mature curd and mozzarella, until to be absent.

10 CONCLUSIONS

Milk and buffalo semi-processed product freezing is done to guarantee a prolonged storage of the product. The risk is represented by thawing, where a proliferation of microorganisms might occur, with the development of pathogens dangerous for the consumer's health.

The work here undertaken has aimed at verifying first and foremost the quality of milk, semi—processed products and buffalo mozzarella, and has traced the microbiological profile during the freezing of such products.

From the obtained results no positive results have emerged, except for moulds and yeasts that have showed a randomized trend. No positivity has emerged for pathogenic microorganisms. Such results might represent a reference for future producers who will employ this method, where eligible.

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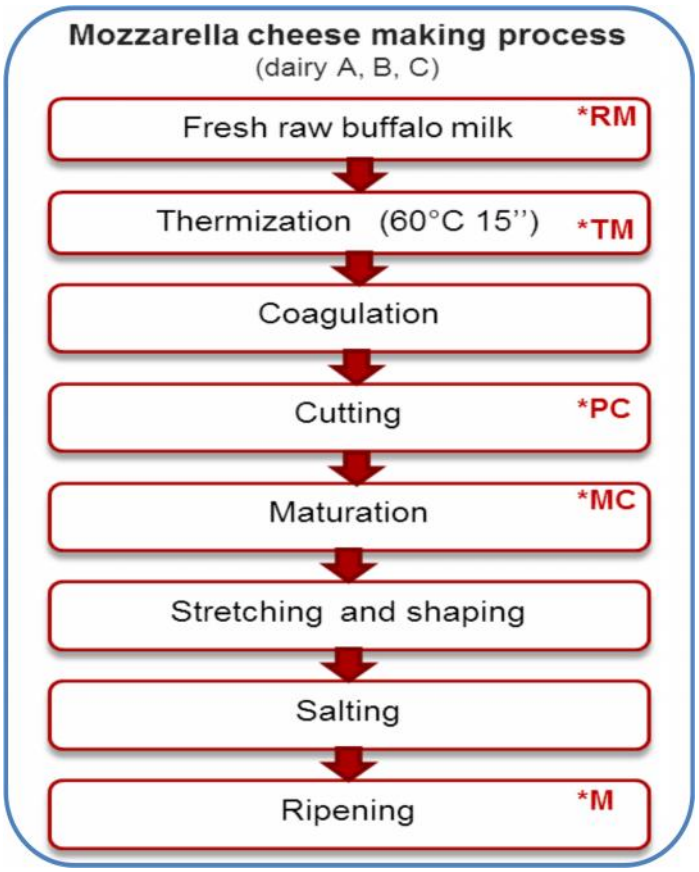
EXPERIMENTAL PART_Phase II:

"STUDY OF MARKERS OF FRESHNESS IN BUFFALO MOZZARELLA CHEESE, THROUGH A PROTEOMIC APPROACH".

12 OBJECTIVE PHASE II

The work presented here is to objectively evaluate the protein-based profile of matricess from a whole dairy-milk supply chain when it is subjected to freezing temperatures for 11 months, (buffalo milk, curd and mozzarella). The aim is to identify through a proteomic study, the presence of a molecular marker which can differentiate between the frozen matrix and that fresh so that it can become a useful monitoring system on the misuse of milk or frozen curd production of BCM, (Buffalo Mozzarella Campana), a product of excellence and synonymous of " freshness".
The practise of using raw or semi-processed, non-fresh materials has become customary in the last decade by some less virtuous dairies. The following are examples of some of the causes of these practises:
- during the winter months the demand for BCM slows while the production of buffalo milk is very high.
-the accumulation of raw materials during the winter months when the prices are lower compared to the stronger demand of BCM in the summer months.
-the economic crisis which has greatly affected the costs of raw material, staff and equipment.
-a flood in the market, from Eastern Europe for example, of relatively cheaper milk or frozen curd products
All of this does not help the image of a highly respected product and one of many made in Italy which occupies, the (ISMEA of 2014) data, the eighth place among the many Italian products PDO and PGI preceded by
Parmesan Reggiano,
Grana Padano Cheese,
Apple of Trentino,
Parma Ham,
Pecorino Romano Cheese,
Balsamic Vinegar,
Gorgonzola
to mention some of them.

13. EXPERIMENTAL PLAN



LEGEND *:

RM: raw milk

TM: thermized milk (60°C pe 15'')

PC: premature curd (pH 6,3 - 6,2)

MC: mature curd (pH 5,0 - 4,8)

M: mozzarella

Sampled matrices were subjected to:

- **CASEIN EXTRACTION**
- **PREPARATION OF SAMPLES FOR SDS-PAGE ANALYSIS**
- **PREPARATION OF SAMPLES FOR UREAPAGE**
- **DENSITOMETRIC ANALYSIS**

14. MATERIALS AND METHODS

14.1 SAMPLING

Samples were collected in three dairies located in Campania Region in Caserta, Salerno and Napoli, named respectively as A, B, and C in April, May, July 2014.
From each dairy eleven portions were collected during cheese making process. Each portion consisting of five sample units - raw milk, termized milk (65-68°C), premature curd (pH6.2 to 6.3), mature curd (pH 5.0 to 4.8), and mozzarella. Samples were transferred in laboratory, analyzed in the fresh state and then frozen at -20 °C for eleven months. Monthly, frozen samples were thawed at 20°C and subjected to analysis.
Proteomic analyses were performed at one month intervals in a period of eleven months toInstitute Zooprofilattico Sperimantale del Mezzogiorno, Department Foods Inspection, Milk Laboratory.

14.2. CASEIN EXTRACTION FROM MILK

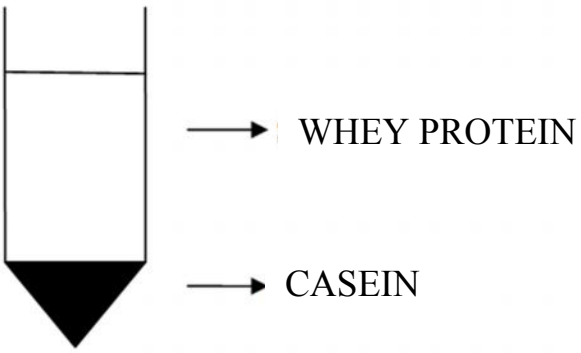
The preparation is performed according to the ASHAFFENBURG & DREWRY method.

Reagents:

- ACETIC ACID TO 10%
- SODIUM ACETATE TO 1 N
- CLEANSER (50 ml of 10%, glacial acetic acid plus 50 ml of sodium acetate 1N makeup
- 1 liter deionized water pH 4.6)
- DICHLOROMETHANE
- ACETONE

Procedure:

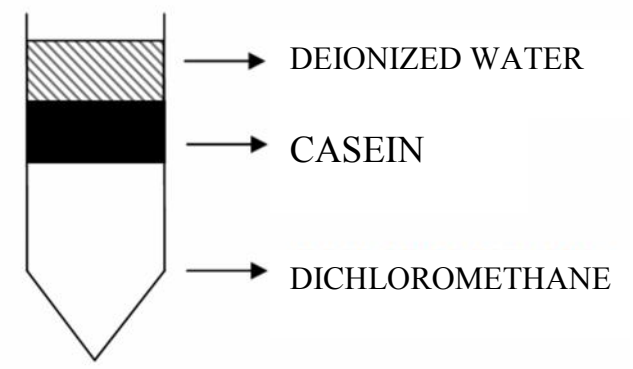
- Pour 50ml of milk into a matrices test tube
- centrifuge at 4000 rpm for 10 minutes
- then refrigerate for 5-10 minutes
- move away the fat which has solidified on the surface with a spatula and check that the volume is not in excess, in which case bring the milk plasma to a volume of 40 ml
- divide into two test tubes and add 20ml of milk to 20 ml of deionized H_2O for each test tube
- heat the vials for 10 minutes in a thermostatic bath at 40°C
- when the tubes are hot add 5 ml of 10% of acetic acid and shake. You will notice the onset of flocculation
- then add 5 ml of sodium acetate and 1N before
- proceeding with another centrifuge of 4000 rpm for 10 minutes after which let the tubes cool. You will find the following result:



- The serum proteins are removed while the left over caseins are subjected to further washing as they may contain other serum proteins within.

Casein washing:

- Add 20 ml of the cleansing solution (50 ml of 10% acetic acid plus 50 ml of 1N sodium acetate brought to an amount of one liter using deionized water at a pH of 4.6) and crush the caseins with a spatula
- centrifuged at 4000 rpm for 10 minutes
- discard the wash solution and wash twice more until the solution runs clear
- add 30 ml of deionized water and mix with Turrax attempting to break up the casein on the bottom of the tube
- check that the pH corresponds to 4.6 using a Litmus test and if not adjust the pH with glacial acedic acid 25% and test the pH again
- add 20 ml of dichloromethane
- centrifuge at 4000 rpm for 10 minutes. You will find the following result:



- Remove the deionized water and the dichloromethane taking care not to break or release a casein buttom from the tube
- repeat this proceding with dichloromethane until both phases of extraction become clear, approximately 2 or 3 times.
- To the purified caseins add 50 ml of acetone
- then centrifuge for a final time at 4000 rpm for 10 minutes
- remove the acetone from the tube and mash the protein extract to form a thin film on the sides.

- Leave in a fume filtration cabinet overnight and the next day mash into a fine powder. Finally, seal the tube with parafilm and keep in a freezer at -20°C.

14.3. CASEIN EXTRACTION FROM CURD AND MOZZARELLA

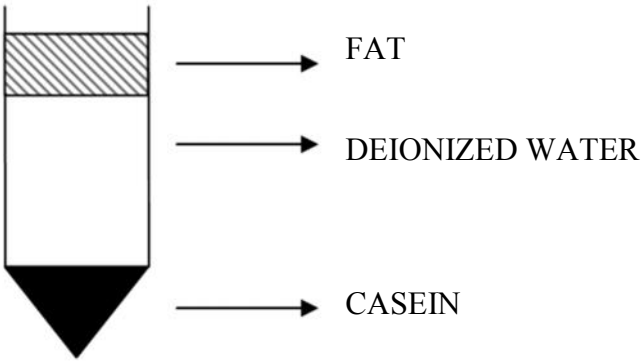
(EC REGULATION 273/2008 ALL 9 par. 6,1)

Reagents:

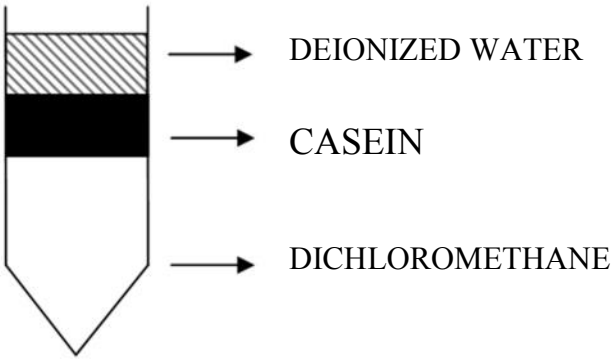
- ACEDIC ACID ICE AGE to 25%
- DICHLOROMETHANE
- ACETONE

Procedure:

- Weigh the equivalent of 5 g of curd and mozzarella in test tubes
- broken into small pieces into two Falcon
- add 30 ml of deionized water with an homogenizing rod (Turrax 8000-10000 rpm)
- add 20 ml of deionized water to reach a volume of 50ml
- check that the pH is 4.6: if it does not correspond with regular glacial acetic acid 25%, add just a few drops
- centrifuge at 4000 rpm for 10 minutes. You will reach the following result:



- Remove the fat with a spatula and the serum
- add 30 ml of deionized water and mix with Turrax at 20000 rpm attempting to break up the casein in the bottom of the tube
- check that the pH corresponds to 4.6 using a Universal Indicator Paper : to adjust use glacial acetic acid 25%,
- add 20 ml of dichloromethane
- centrifuge at 4000 rpm for 10 minutes. The result is the following:



- Remove the deionized water and the dichloromethane, taking care not to break or to release a casein button from the tube
- repeat this procedure with the dichloromethane until the two phases of extraction become clear. (Two or three times)
- to the purified caseins add 50 ml of acetone and mash
- centrifuge for a final time at 4000 rpm for 10 minutes.
- Remove the acetone from the tube and mash the protien extract to form a thin film on the sides.
- Leave in a fume filtration cabinet overnight and the next day mash into a fine powder. Finally, seal the tube with parafilm and keep in a freezer at -20°C.

14.4 ELECTROPHORESIS SDS-PAGE

Preparation of samples

SDS-PAGE means (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis)

- Weigh 10 mg of precipitated casein (dried, as indicated above) with an analytical scale using 5 ml tubes and dissolve the following reagents:
- 10 ml of 9M ureaprepared by dissolving 54.05 g of urea in 100 ml ofdeionized water
- 10 µl of β-mercaptoethanol ready to use Bio-Rad
- Carefully shake to the vortex and leave it for the necessary time to allow the solubilization of casein to act, a few hours-
- One can then proceed by adding:
- 1 ml of Fullington solution (750 mg TRIS, 2 g of SDS and 5 ml of β-mercaptoethanol at pH 6.8 in 100 ml)
- a sucrose spatula
- 10 µl of phenol blue bromine (10 x Blue Juice Invidrogen)

The samples obtained were subjected to denaturation by boiling at 100° C for 10 minutes and subsequently cooled prior to loading on the gel.

Setting electrophoresis (preparation: glass, gel, running gel)

Use of the instruments*:*

- A POWER BIO-RAD manufactuarer, (modello:POWER-PAK ™Universal BASIC POWER Supply, 100-120 / 220-240), provides constant current for all electrophoresis.
- A COOLING SYSTEM manufacturer MPM INSTRUMENTS srl (Bath Criostatico model: M408-BCV), exchanging heat with the system keeps the temperature low;
- A ELETROPHORETIC ROOMmanufacturer BIO-RAD (PROTEAN® ll Xi cell);
- GLASS two rectangular (27x21 cm);
- Four plastic SPACERS, 0.5 cm thick, for spacing the pair of glasses;
- A pair of COMBS in thick Teflon as spacers;
- Two pairs of SCREWS for assembling theglasses;
- A SUPPORT for the glass;
- A pair of SEALS
- EPSON PERFECTION 4490 PHOTO

Glass Assembly

It is necessary that the glasses be washed and degreased with denatured ethyl alcohol at least in the two inner faces of the glazing. Similarly the spacers. With the pairs of glass coverings, the lower of the pair should always be positioned in the front, the spacers placed laterally and then the higher glass coverings are placed behind. On the outside close the two screws and place the whole 'sandwich' on the base of the horizontal strips which will serve as a seal so that the gel, once poured, won't leak out. Before pouring the gel test with water to ensure that the glass has been assembled well and is aligned at the bottom. If, after the water is poured, there is no leakage soak up the water with some blotting paper and proceed to pour the gel. Remember to pour the first RUNNING GEL up to about 1 cm away from the bottom of the wells and then the STACKING GEL. Insert the comb and mark with a glass marking pen the lower level of the cockpit; this will serve as a reference for the stacking gel.

Preparation of gel and running buffer

- RUNNING GEL 12% COMPOSITION (amount for two gels)
- Water (16.5ml)
- 30% Bis-Acrylamide Acrylamide (20.0 ml)
- 1.5 M Tris pH 8.8 (45,43 g tris in 250 ml of water, pH 8.8) (12.5 ml)
- 10% SDS (500 ul)
- 10% ammonium persulfate (APS)* (500 ul)
- TEMED (tetramethylethylenediamine) (20 ul)

*The APS should be freshly prepared (100 mg of ammonium persulfate in 1000 ml of distilled water in an eppendorf) and stored at 4° C.
Note: the acrylamide polymerization is inhibited by the presence of oxygen.

Procedure:

- Prepare the gel under a fume cabinet and when you get to the SDS stage continue as follows
- Mix with an armature (mechanical mixer) and refrigerate.
- When the solution is cold add the temed and then the APS.
- Pour the gel in between the panes of glass until 1 cm from the previously marked measures.
- Once prepared, the gel is poured by placing the dropper on one of the corners of the glass, taking care not to form air bubbles.
- delicately pour the isopropanol keeping it to a large, flat, even surface.
- The gel is left to polymerize for about an hour at room temperature. (Make sure the gel sets with a slight incline with respect to the base).
- The upper surface of each gel is then rinsed with distilled water 2 or 3 times to remove the isopropanol and any residue of acrylamide. Dry with some blotting paper or paper towel.

➢ STACKING GEL 5% COMPOSITION (Amount for two gels)

- Water (6,8 ml)
- 30% Acrylamide Bis-Acrylamide (1.7 ml)
- 1 M Tris pH 6,8 (1,25ml)
- 10% SDS (100 µl)
- 10% APS (100 µl)
- Temed (20 µl)

Procedure:

- Add the components above, under a fume cabinet, until the SDS stage then mix and refrigerate
- To the cold solution add the temed and APS
- Insert the combs in the centre
- Pour the gel taking care to avoid air bubbles
- Wait for the gel to polymerize (about 30 minutes to an hour)
- After the gel sets remove the combs and delicately dry the 15 wells with blotting paper

Note: In the case of limited time the gel can be preserved overnight by wrapping it in damp paper towel.

➢ RUNNING BUFFER (for an electrophoretic run)

- Tris (15.1 g)
- Glycine (72 g)
- SDS (5 g)
- All dissolved in 5 litres of deionized water and brought to pH of 8.6.

Loading of the sample and start of the run

- Loading of the Standard (Precision Plus Protein Standard of a molecular weight ranging from 10 kDa to 250 kDa.) 20 µl
- Load the hydrolyzate with the plasmin 10µl
- 20 µl samples using a 100 µl Gilson dropper
- The feeder is also connected to the electrophoretic chamber as well as a cooling system set to 10 °C to neutralize the heat generated.
- Pour a small amount of running buffer into each of the 15 wells.
- At this point mount the two pairs of glass onto the suitable supports of the electrophoretic chamber holder
- Then attach the running buffer, first in the space between the two pairs of glass, making sure there is a perfect seal. Next do the same to the outside of the container using about half the gel (12 cm from the bottom). The gel between the two panes of glass must be flush with the buffer.
- Cover
- You turn on the feeder in order to supply the electrophoretic chamber , to which it is connected, a constant source of high voltage (220 V).
- The time of the course varies but is no less than 5 hours.
- As soon as the Bromophenol Blue dye (BB) reaches the bottom of the glass turn off the current.
- Now the current is off, disassemble the glass being careful not to damage the gel.

Preparation of Buffalo milk casein hydrolyzed with plasmin

- Add 700mg of casein extracted from 100ml of buffalo milk
- 10 ml of ammonium bicarbonate 0.1 M pH 8.0
- Take 100 ul of plasmin ready to use [Plasmin 5 U (1 ml) from bovine plasma]
- Pour in the stock solution composed of 700 mg of casein and 10ml of ammonium bicarbonate 0.1 M pH 8.0
- Before putting the enzyme take 2 ml of the stock and add to the first only of the 5 wells prepared
- At T0 (zero time) add 2ml of the stock , more 2 ml of TCA acid at 24% to arrive at a final concentration of 12% . This acid serves to stop the reaction between the casiens and the plasmin.
- Add the plasmin, as is, to the stock and place in a thermostat at 37°C.
- When the temperature reaches 37°C take out add 2 ml to the remaining 4 wells one at a time, always keeping the stock in the thermostat at 37°C T1 = 30'; T2= 1 h; T3= 2h; T4= 4h
- At every addition of stock add the TCA acid to stop the reaction between the casein and the plasmin.
- The experiment completed, keep the wells in the fridge or freezer if they are not to be used in the near future.

Staining - destaining and the preservation of the gels:

- STAINING SOLUTION

- Colour the gels and place in trays with Coomassie Blue R250 for at least 2 hours.
- To destain the gels place in bowl with a destaining solution
- Put on a rocking platform on a slow setting and check every 15 minutes during the process for about 1 hour.
- Change the destaining solution often to optimize the time. Take care not to destain the gel too much.

- DESTAINING SOLUTION

- 150 ml methanol
- 50 ml glacial acetic acid
- 300 ml water

After the staining - destaining the gel is placed in the stop solution for 10 minutes

- PRESERVATIVES

- Acetic acid 10%

Image capture

Remove the gel from the destaining solution and lay inside a sheet of Ercole plastic open on three sides. Cover with another layer of the plastic. Press evenly on a flat surface to eliminate any air bubbles. Insert the scanner and acquire the image (EPSON PERFECTION 4490 PHOTO).

14.5. PAGE-ALKALINE (pH8.6)

The PAGE-ALKALINE consists in preparation of a urea-based gel to separate proteins on the basis of the net negative charge, of urea with a basic pH of 8.6, by using a vertical one-dimensional tecnique.

Preparation of the samples

- Weigh 10 mg of casein in a 1.5 ml Eppendorf.
- 1 ml of 9 M urea.
- Add 10 ml of β -mercaptoethanol
- stir and disolve well
- then take 500 µl and pour into a 1.5 ml Eppendorf.
- To add weight to the sample add 500 µl of glycerol at 75 % (P/V)
- add 10 µl of Blue phenol bromine (BB) and mix
- weigh 220 mg dissolved in 1000 lt of distilled water in a 1.5 ml Eppendorf.

PAGE-ALKALINE (pH 8.6) stage
See the procedure for SDS-PAGE.

Gel preparationand running buffer
See the procedure for the SDS-PAGE

- RUNNING GEL COMPOSITION
- UREA 9M (15,54 g)
- Tris-HCI 1.5 M pH 8.8 (7.5 ml)
- Distilled Water (10.5 ml)
- Acrylamide-Bis-Acrylamide 30% (10.5 ml)
- TEMED (20ul)
- *ammonium persulfate (APS) 22% (200 ul)

*The ammonium persulphate must be freshly prepared and stored at 4°C. Note: The acrylamide will set spontaneously in the absence of oxygen.

- STACKING GEL COMPOSITION
- UREA 9M (2,2 g)
- Tris-HCI 0.5 M pH 6.8 (2 ml)
- 30% Bis-Acrylamide Acrylamide (1 ml)
- Glycerin 75 % (w/v) (1 ml)
- Distilled Water (4 ml)
- TEMED (4µl)
- Ammonium Persulphate (APS) 22% (30µl)
- Load 10 µl of the sample into the gel.

- RUNNING BUFFER:
- Glycine (72 g)
- Tris (15.1 g)

Make up a volume of 5 lt of deionized water with a pH of 8.6.

Loading of the samples and start of the run

- Load 20 ul of sample in to the gel (10ul hydrolized with chymosin). In this case we don't use standard but only hydrolyzed with chymosin.
- See the procedure for the SDS-PAGE

Preparation of Buffalo milk casein hydrolyzed with chymosin

- Add 700mg of casein extracted from 100ml of buffalo milk
- 10 ml of Citrate salts 0.1 M pH 6.0
- Prepare a solution A consisting of 100 µl of curd and 5 ml of Citrate salts
- Take 300 ul from stock solution A
- Pour in the stock solution, composed of 700 mg of casein and 10ml of Citrate salt 0.1 M pH 6.0 (PM 25,812 gr/lt 1 M)
- Before putting the enzyme take 2 ml of the stock and add to the first only of the 5 wells prepared
- At T^0 (zero time) add 2ml of the stock , more 2 ml of TCA acid at 24% to arrive at a final concentration of 12% . This acid serves to stop the reaction between the caseins and the chymosin.
- Add the solution A to the stock and place in a thermostat at 37°C.
- When the temperature reaches 37°C take out add 2 ml to the remaining 4 wells one at a time, always keeping the stock in the thermostat at 37°C T1 = 30'; T2= 1 h; T3= 2h; T4= 4h
- At every addition of stock add the TCA acid to stop the reaction between the casein and the chymosin.
- The experiment completed, keep the wells in the fridge or freezer if they are not to be used in the near future.

Staining / destaining and the preservation of the gel.

See the procedure for the SDS-PAGE
After the step of straining / discolouration, using the same colourant and decolourant, the gel is left in the stopping solution made up of 10% acetic acid for about 10 minutes.

Image capture

This gel image was taken using a scanner (EPSOM PERFECTION 4490 PHOTO).

15 RESULTS AND DISCUSSION

15.1 SDS-PAGE

The samples of raw milk, heated milk, premature and mature curd and mozzarella stored for twelve months at -20°C were analyzed by SDS_PAGE electrophoresis for the study of protein portion.
Several studies published in literature have examined the effects arising from the use of milk and curd frozen for the production of cheeses from different species such as bovine and

ovine.(Alonso et al., 2013; Zhang et al., 2006; Picon et al.,2013). Few data are available for the buffalo milk.

Di Luccia *et al*., 2009 have study in particular the fragment β-CN f(69-209) called γ4-CN with a molecular weight of 15.748 kDa. This proteic fragment, originating from the early hydrolysis of Lys_{68}-Ser_{69} by plasmin, has no counterpart in bovine milk . In fact previous studies have affirmed and subsequently confirmed that plasmin cleave preferentially Lys-X peptide bond, primarily hydrolyzing β-CN to γ-CN. These β-CN fragments (f) are a heterogeneous fraction (Eigel 1977) composed of f(29.209), f(106-209), f(108-209) peptides designated as γ1-CN, γ2-CN and γ3-CN, respectively.

The fragment named γ4-CN, is present in the fresh buffalo milk as a faint electrophoresis band, which drastically increases in intensity in refrigerated and frozen milk as well as in frozen buffalo curds. Since it was also detected in curd it has been proposed as a possible freshness marker

To assess the γ4-CN in various samples of buffalo milk ,curd and mozzarella one uses densitometric analysis, to determine the percentage of the protein in the analysis as compared to other proteins with increasing freezing time.

The samples loaded on SDS-PAGE gels are in sequence respectively: raw milk, thermized milk, premature curd, mature curd and mozzarella. After each month of storage starting from fresh matrices for both dairy.

The results obtained by electrophoresis SDS-PAGE are shown in figures 40-45 for dairy A. And in figures 46-51 for dairy B.

We have omitted the findings from the gels of dairy C because they were found to show a behavior very similar to those from dairy A and dairy B over the same period of investigation.

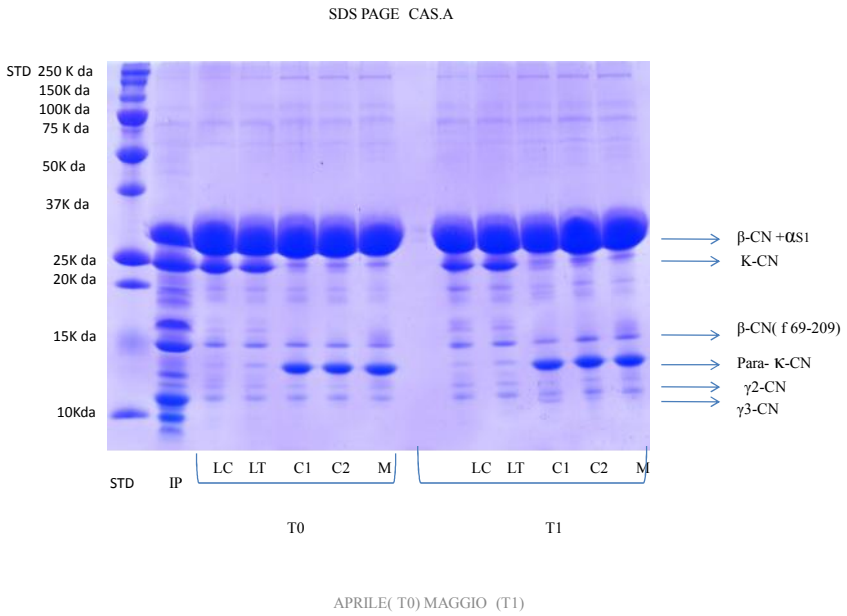
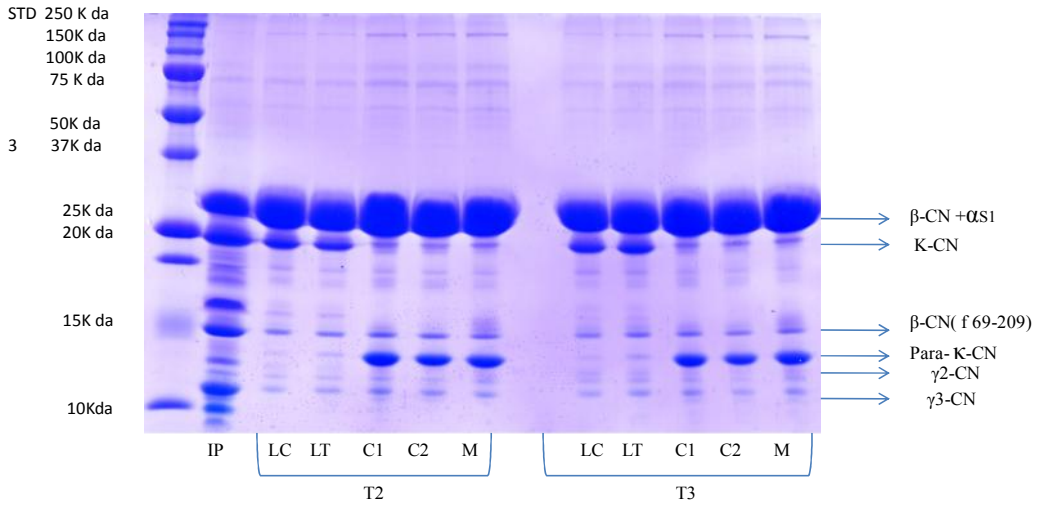


Fig.40

LEGENDA:
STD: standard
IP: idrolized with plasmin
LC: raw milk
LT: termized milk
C1: premature curd
C2: mature curd
M: mozzarella

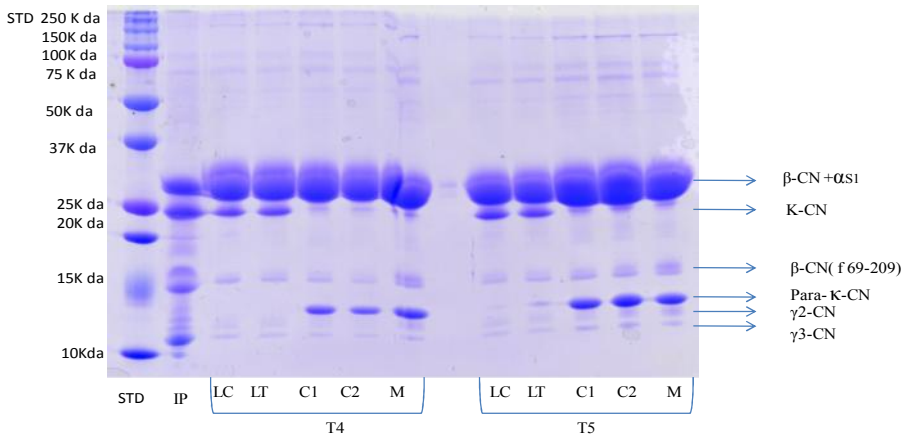
SDS PAGE CAS.A



- GIUGNO(T2) LUGLIO (T3)

fig.41

SDS PAGE CAS.A

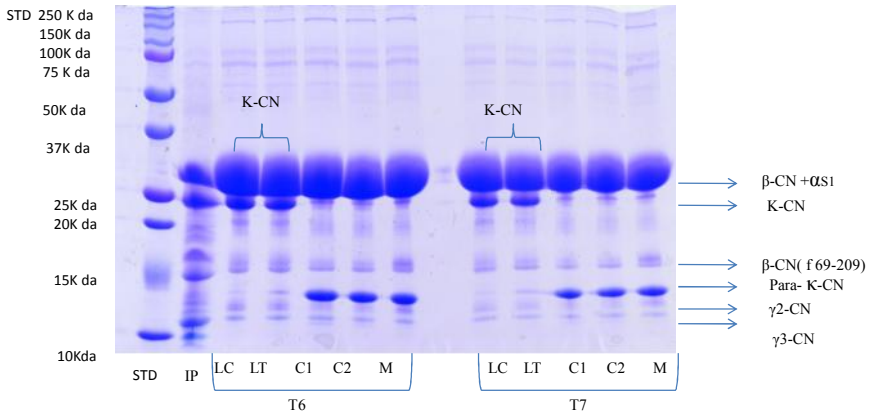


AGOSTO (T4) SETTEMBRE (T5)

fig.42

LEGENDA:
STD: standard
IP: idrolized with plasmin
LC: raw milk
LT: termized milk
C1: premature curd
C2: mature curd
M: mozzarella

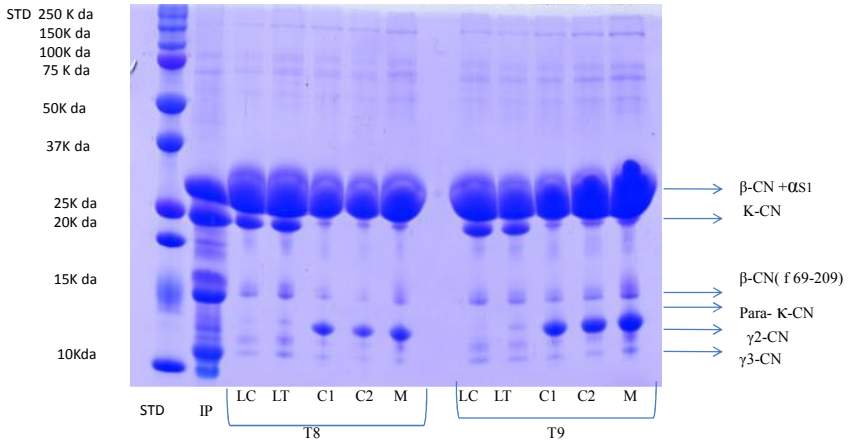
SDS PAGE CAS.A OTTOBRE



OTTOBRE (T6) NOVEMBRE (T17)

fig.43

SDS PAGE CAS.A

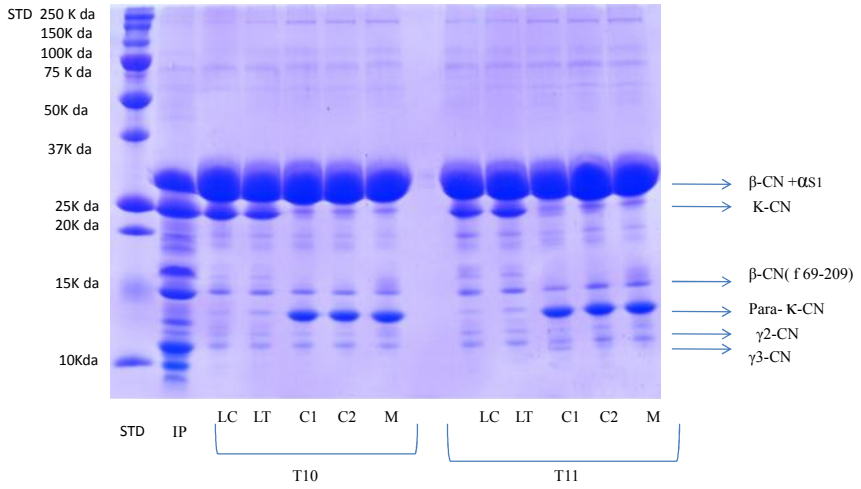


DICEMBRE(T8) E GENNAIO (T9)

fig.44

LEGENDA:
STD: standard
IP: idrolized with plasmin
LC: raw milk
LT: termized milk
C1: premature curd
C2: mature curd
M: mozzarella

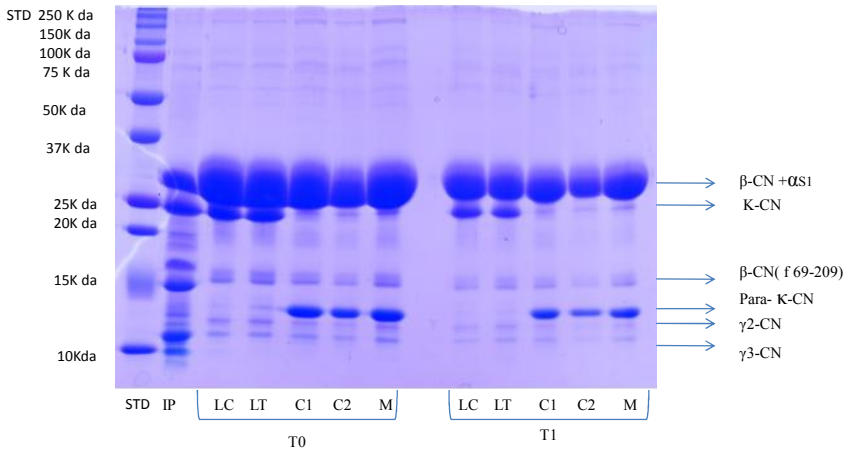
SDS PAGE CAS.A



FEBBRAIO (T10) MARZO (T11)

fig. 45

SDS – PAGE CASEIFICIO B

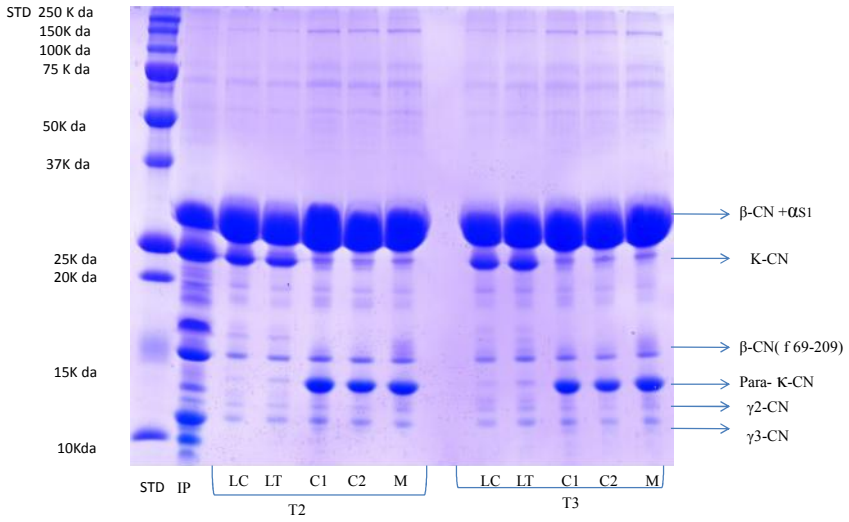


MAGGIO (T0) E GIUGNO (T1)

fig. 46

LEGENDA:
STD: standard
IP: idrolized with plasmin
LC: raw milk
LT: termized milk
C1: prematurecurd
C2: mature curd
M: mozzarella

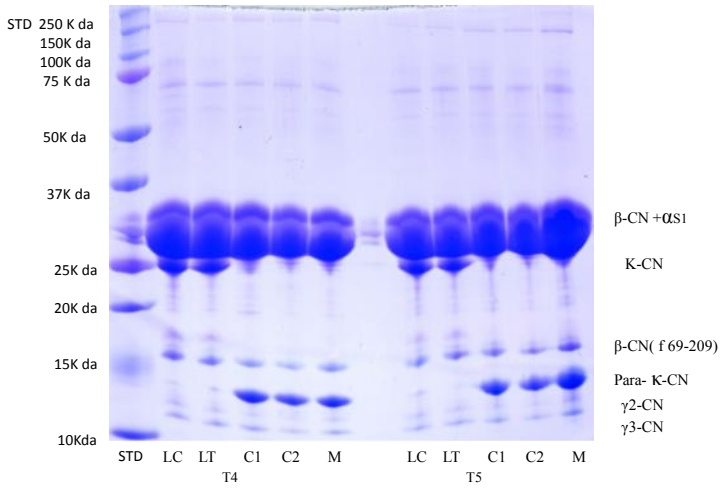
SDS – PAGE CASEIFICIO B



LUGLIO (T2) E AGOSTO (T3)

fig. 47

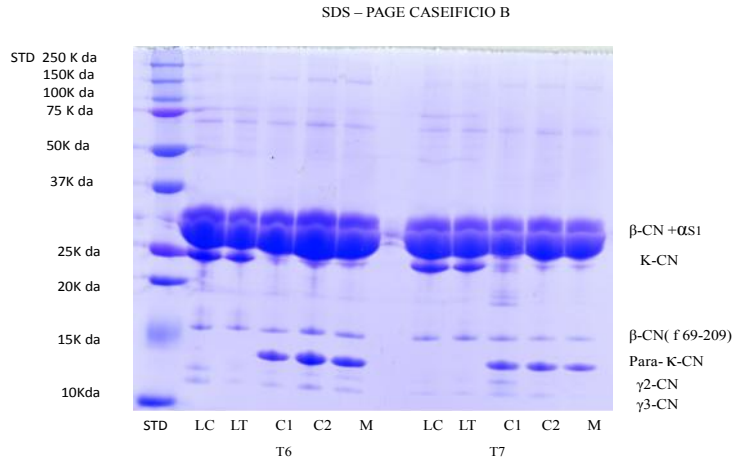
SDS – PAGE CASEIFICIO B



SETTEMBRE (T4) E OTTOBRE (T5)

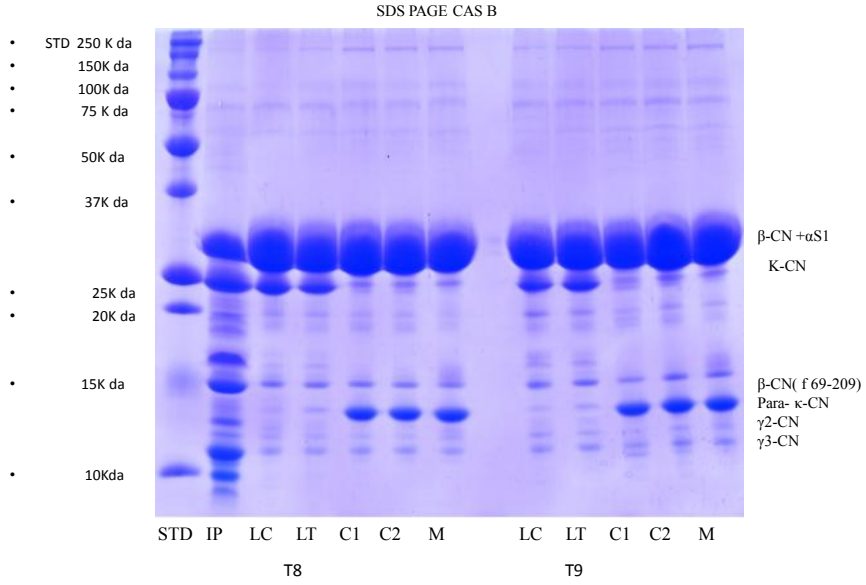
fig.48

LEGENDA:
STD: standard
IP: idrolized with plasmin
LC: raw milk
LT: termized milk
C1: prematur ecurd
C2: mature curd
M: mozzarella



NOVEMBRE (T6) E DICEMBRE (T7)

fig. 49



GENNAIO (T8) E FEBBRAIO (T9)

fig. 50

LEGENDA:
STD: standard
IP: idrolized with plasmin
LC: raw milk
LT: termized milk
C1: prematur ecurd
C2: mature curd
M: mozzarella

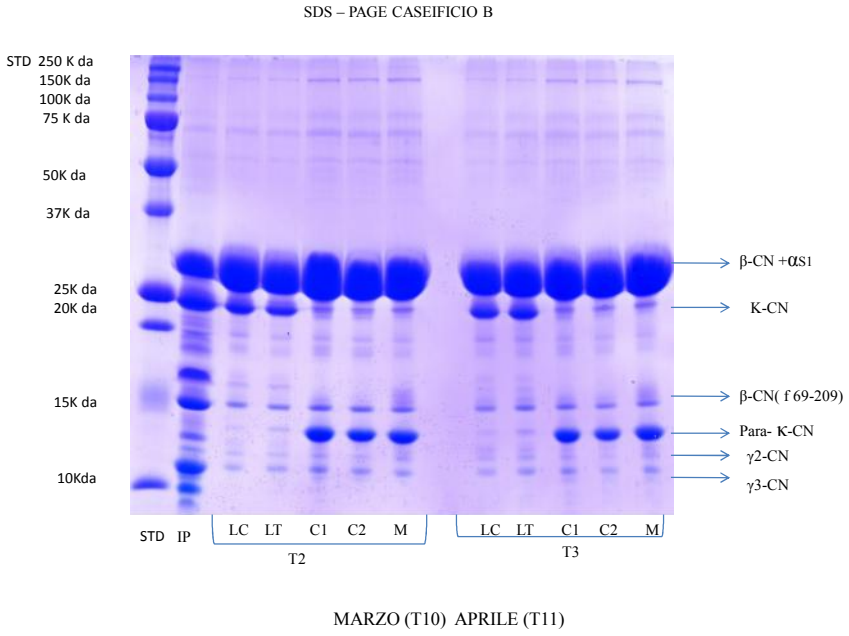


fig. 51

The first well of each plate is loaded with a marker kit of a molecular weight of between 10 to 250 kDa, in the second hydrolyzed plasma as is standard for assessing protein profiles obtained from the action of the plasma on β-CN and the matricess in sequence.
From the gels it is possible to observe various protein fragments with a molecular weight ranging from 25,200 to 11,500 kDa such as:

- β-CN f(69-209);
- para-k-CN;
- γ_2-CN;
- γ_3-CN.

Although our attention was focused on the fragment β-CN f(69-209).
Loading the samples on the gel with this sequence makes it quite difficult to evaluate the performance of each single matrices over the course of time. For this reason it was decided then to load only one matrices from each stage starting with the fresh and each successive one until the last months of the freezing. Analyses were conducted on **raw milk, premature and mature curd** also in accordance with the aim of the thesis, namely the search for possible indicators of the presence of milk and frozen curd in the processing process of PDO buffalo mozzarella using electrophoresis FAST SDS-PAGE.
Used precast gels Mini-PROTEAN® TGX™ BIO-RAD the percentage of polyacrylamide gels is 4-15%.

15.1.1 ASSESSMENT γ4-CN IN RAW MILK

The electrophoretic patterns relating to samples of raw milk starting from time t_0 to time t_{11}, are shown in Figure 23 for Dairy A and in Figure 24 for Dairy B.

Dairy A

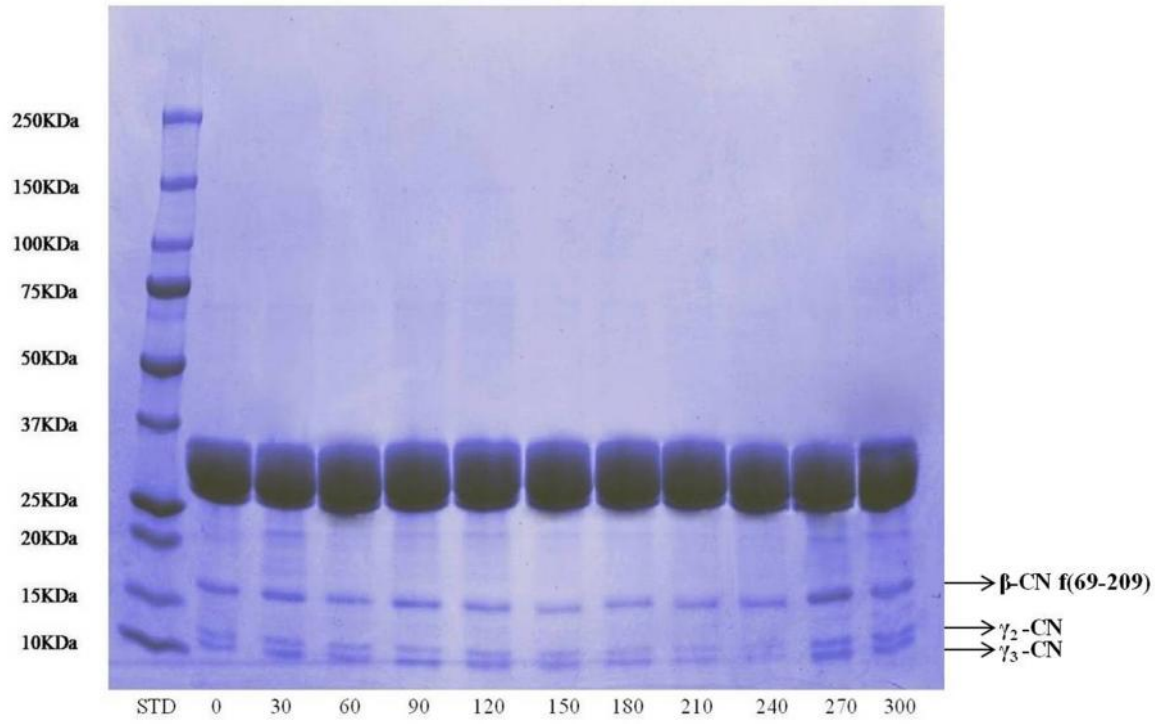


Figure 52: Electrophoresis on polyacrylamide gel (SDS-PAGE 4-15%) of the raw milk caseins.

Dairy B

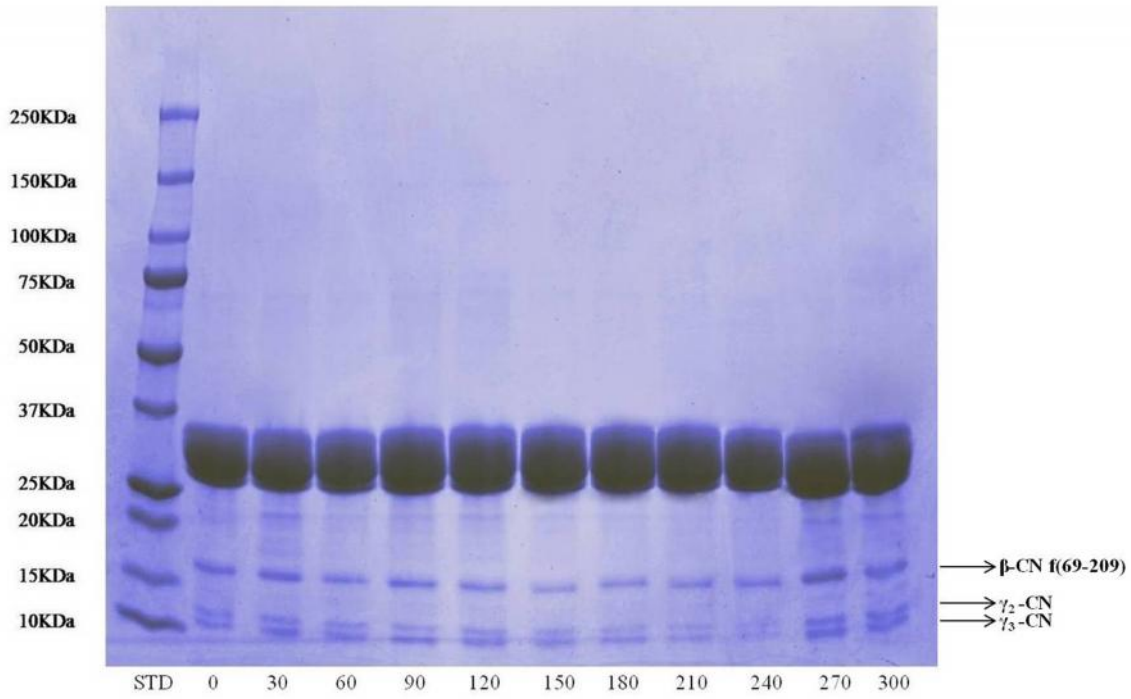


Figure 53: Electrophoresis on polyacrylamide gel (SDS-PAGE 4-15%) of the raw milk caseins.

Considering the trend of the y4-CN in both dairies we can see that there is a constant trend over time. To verify that what transpired from the gels was true we evaluated the y4-CN by the % of the normalized amount compared to the β -CN . The densitometric analysis results are reported in Table 9 and 10.

Tab. 9: Densitometric results relative to % of the normalized amount of y4-CN compared to αs1 + αs2 + β-CN from Dairy A.

DAIRY A		
LANE NUMBER	% NORMALIZED QUANTITY $\alpha_{s1} + \alpha_{s2} + \beta$-CN	% NORMALIZED QUANTITY γ4-CN
2	100	11,783
3	100	11,186
4	100	11,374
5	100	11,4
6	100	11,276
7	100	11,767
8	100	11,138
9	100	12,105
10	100	11,241
11	100	12,204
12	100	12,281

Tab. 10: Densitometric results relative to % of the normalized amount of y4-CN compared to αs1 + αs2 + β -CN from Dairy B.

DAIRY B		
LANE NUMBER	% NORMALIZEDQUANTITY $\alpha_{s1} + \alpha_{s2} + \beta$-CN	% NORMALIZED QUANTITY γ4-CN
2	100	10,14
3	100	10,52
4	100	9,403
5	100	9,976
6	100	9,4
7	100	10,2
8	100	10,062
9	100	9,805
10	100	10,096
11	100	9,759
12	100	10,245

These results enabled us to assess the trend of y4-CN over a year of experimentation (Figure 54 and 55).

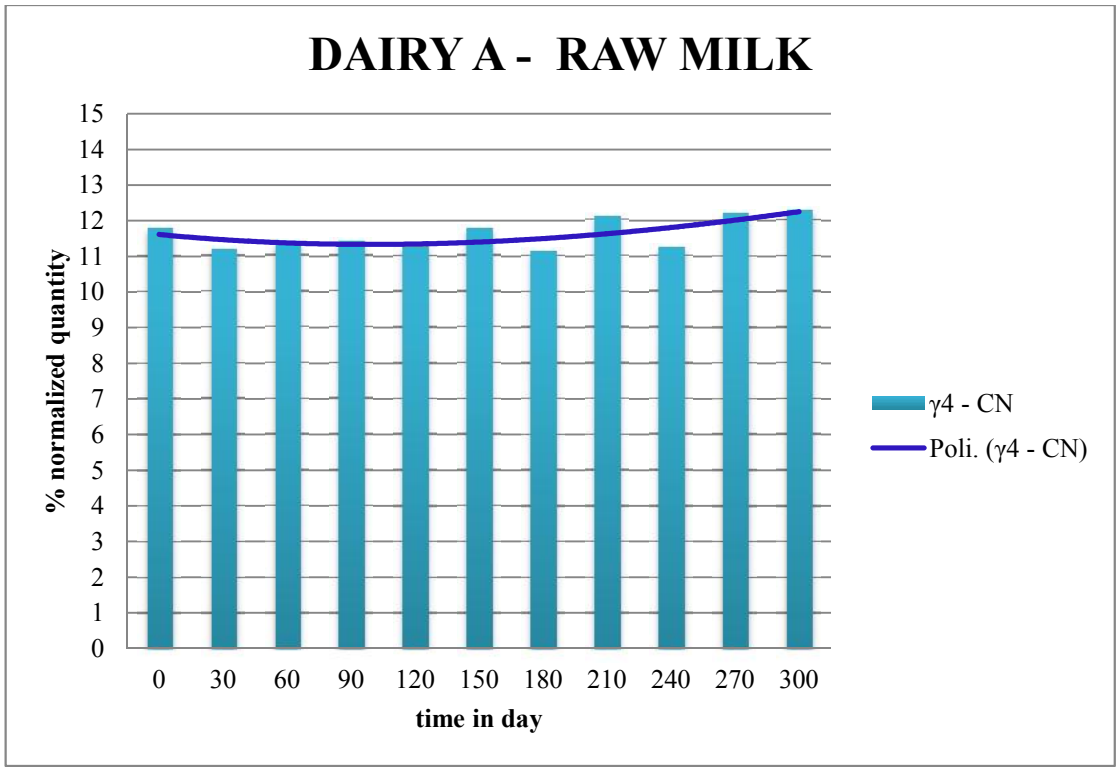


Figure 54: Trends in y4-NC **Dairy A**

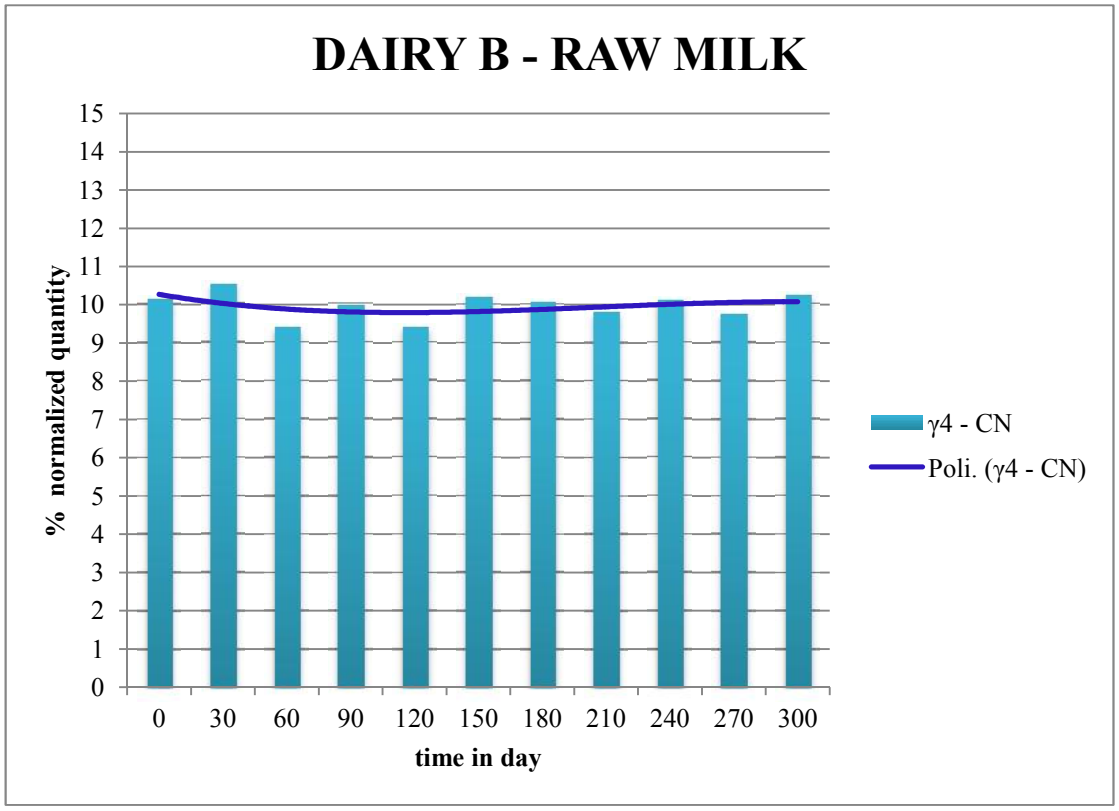


Figure 55: Trends in y4-NC **Dairy B**

From the two graphs we can confirm that there is a steady trend of y4-CN between fresh milk and milk stored at -20°C for 11 months.

15.1.2 ASSESSMENT γ4-CN IN PREMATURE CURD

The electrophoretic patterns related to premature curd samples from the time t_0 to time t_{11}, are shown in Figure 56 for Dairy A, and in Figure 57 for Dairy B.

DAIRY A

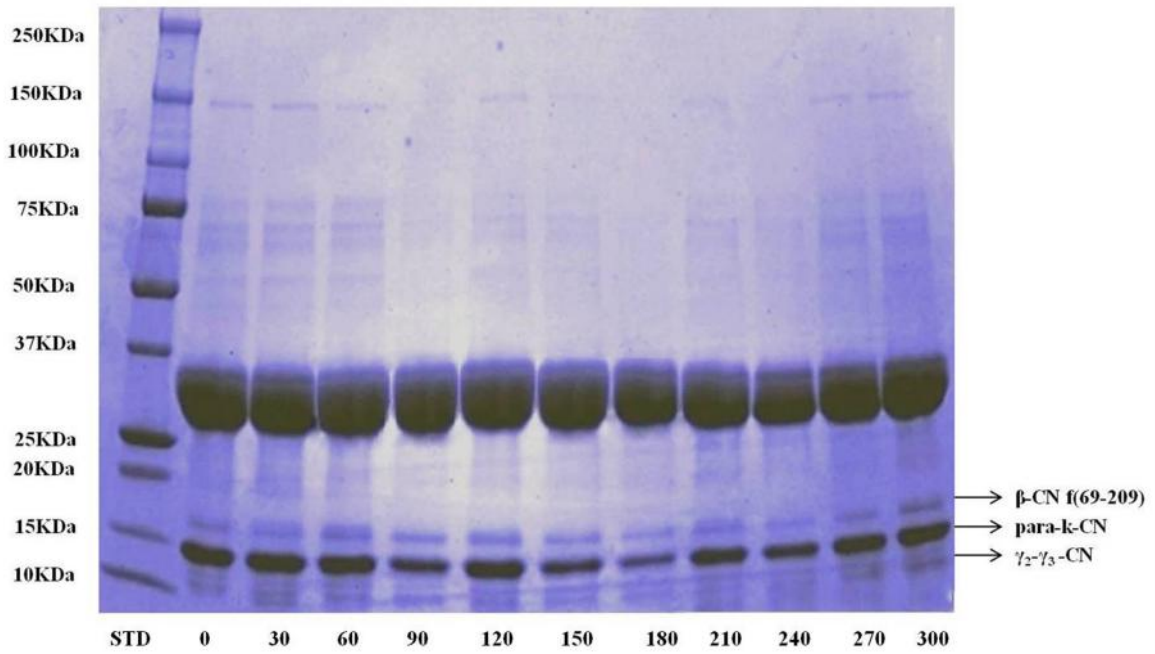


Figure: 56 Electrophoresis on polyacrylamide gel (SDS-Page 4-15%) of the casein of premature curd.

DAIRY B

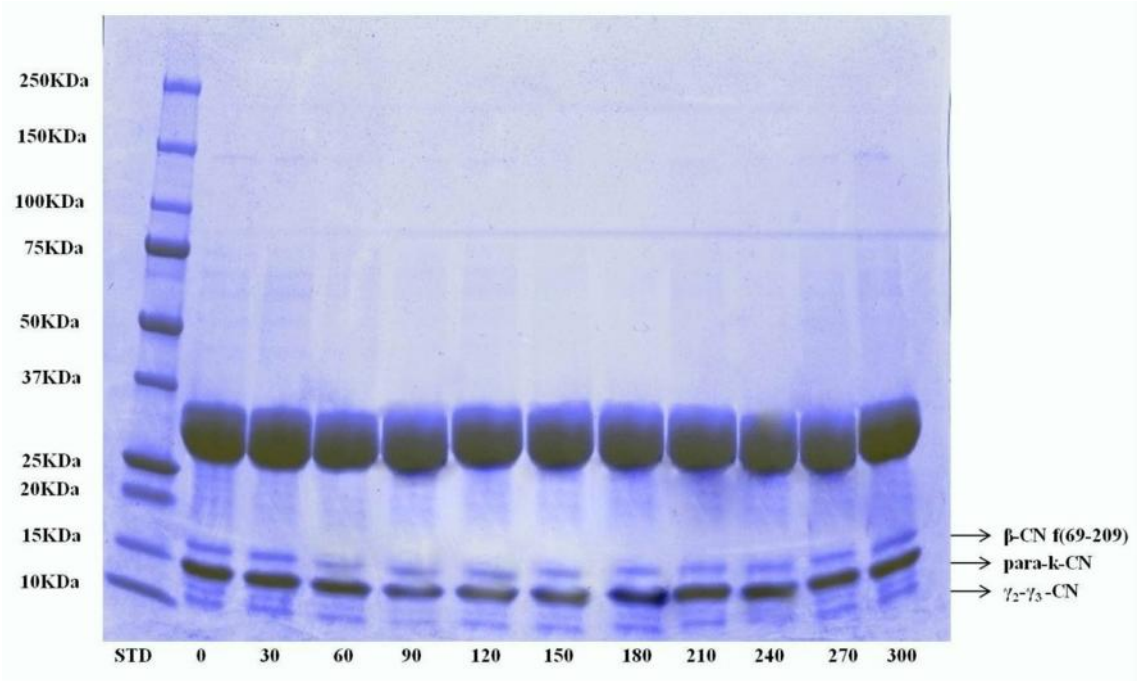


Figure 57 Electrophoresis on polyacrylamide gel (SDS-PAGE 4- 15%) of the casein of premature curd.

Considering the trend of the y4-CN in both dairies we see a constant trend over time. Unlike raw milk, in the case of premature curd , y4-CN was used to quantify the average % of the nomalized amount compared to the para-k-CN. The densitometric analysis results are shown in table 11 and 12.

Tab. 11: Densitometric results realative to the % of the normalized amount of y4-CN with respect to para-k-CN of Dairy A.

CASEIFICIO A		
LANE NUMBER	% QUANTITY NORMALIZED para-k-CN	% QUANTITY NORMALIZED γ4-CN
2	100	27,949
3	100	28,103
4	100	28,128
5	100	28,169
6	100	27,758
7	100	28,715
8	100	28,712
9	100	28,205
10	100	28,136
11	100	28,055
12	100	28,446

Tab. 12: Densitometric results relative to the % of the normalized amount of y4-CN with respect to para-k-CN of Dairy B.

CASEIFICIO B		
LANE NUMBER	% QUANTITY NORMALIZED para-k-CN	% QUANTITY NORMALIZED γ4-CN
2	100	40,476
3	100	38,17
4	100	40,013
5	100	38,671
6	100	39,365
7	100	38,291
8	100	38,531
9	100	39,096
10	100	38,379
11	100	38,265
12	100	40,486

These results enabled us to assess the trend of y4-CN over a year of experimentation (Figure 58 and 59).

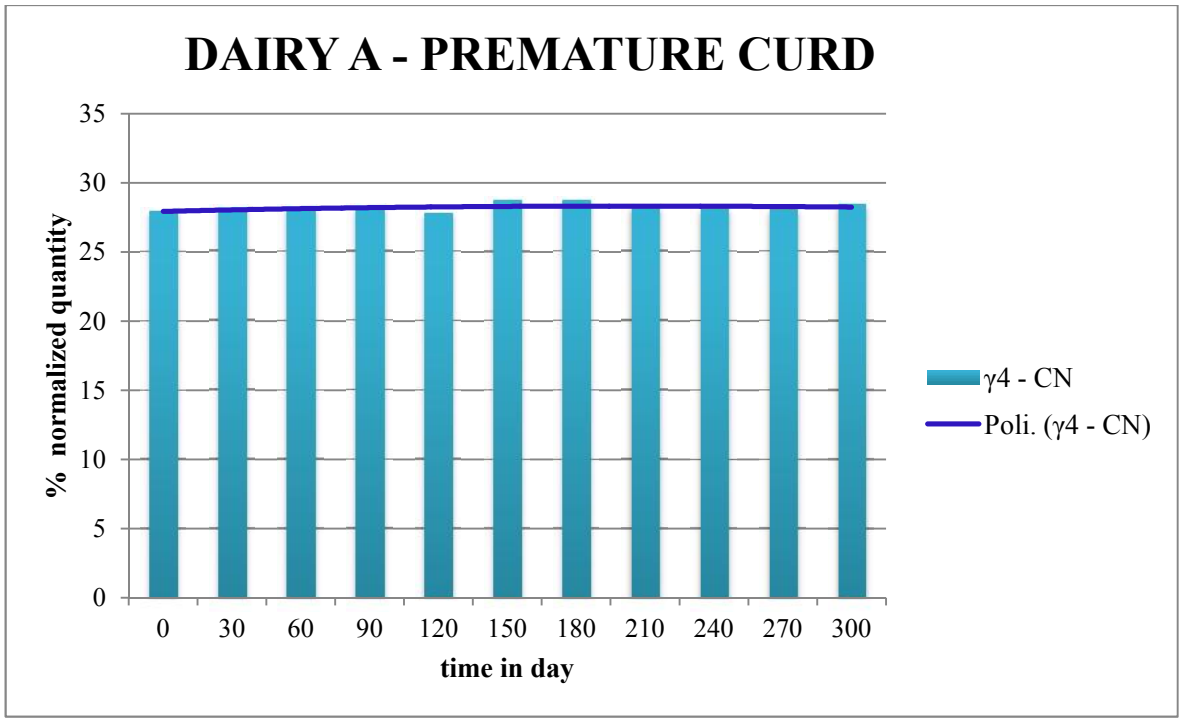


Figure 58: Trend of y4-NC from Dairy A

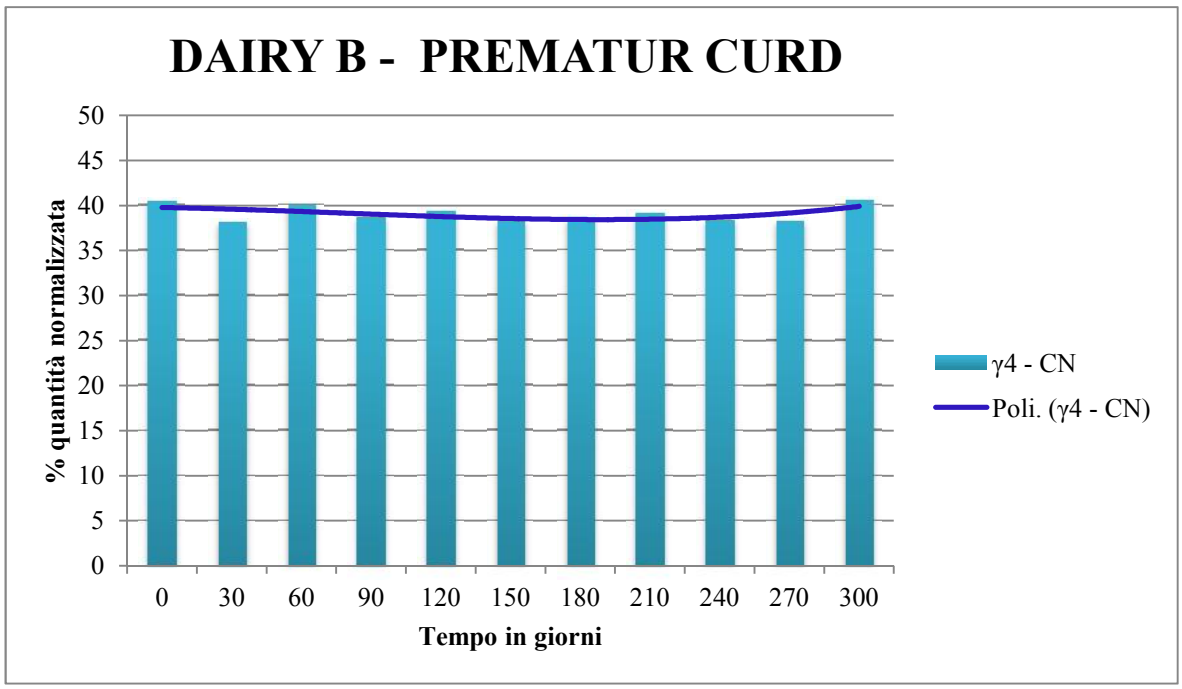


Figure 59: Trend of y4-NC from Dairy B

These graphs confirm a constant trend of the y4-CN between fresh premature curd (t_0) and premature curd kept at -20°C for 11 months.

15.1.3 ASSESSMENT y4-CN IN MATURE CURD

The electrophoretic patterns relative to the mature curd samples starting from time t_0 to time t_{11}, are shown in Figure 60 for Dairy A and in Figure 61 for Dairy B.

DAIRY A

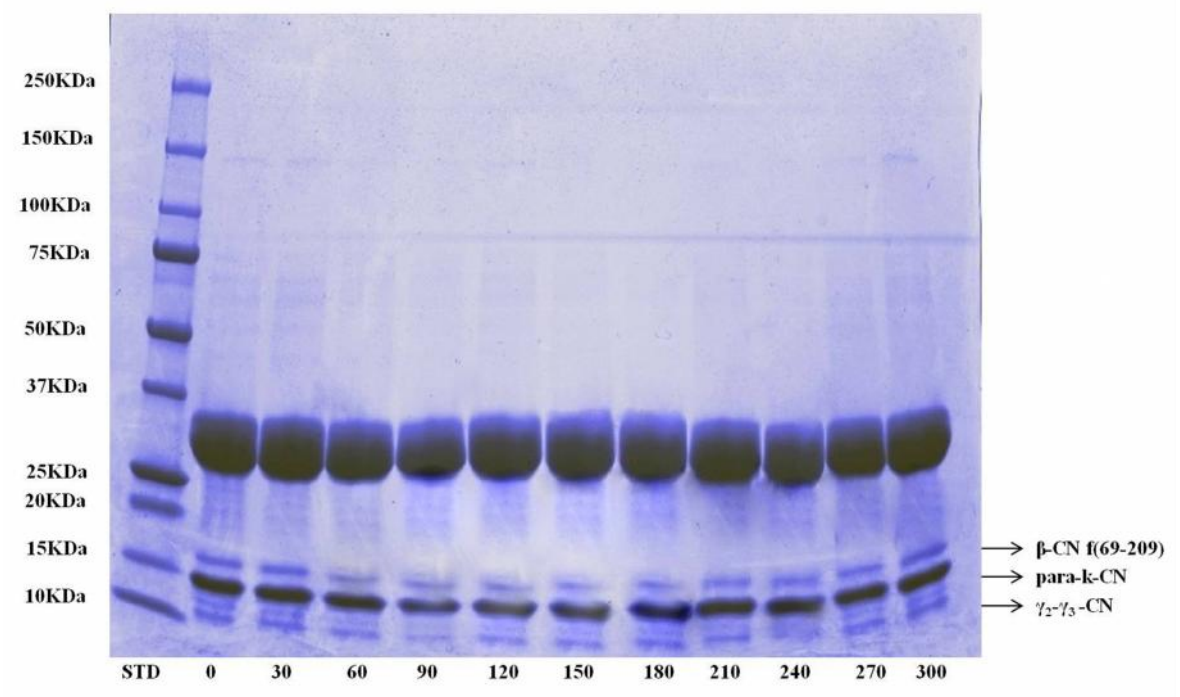


Figure 60: Electrophoresis on polyacrylamide gel (SDS-PAGE 4- 15%) of the casein of mature curd.

DAIRY B

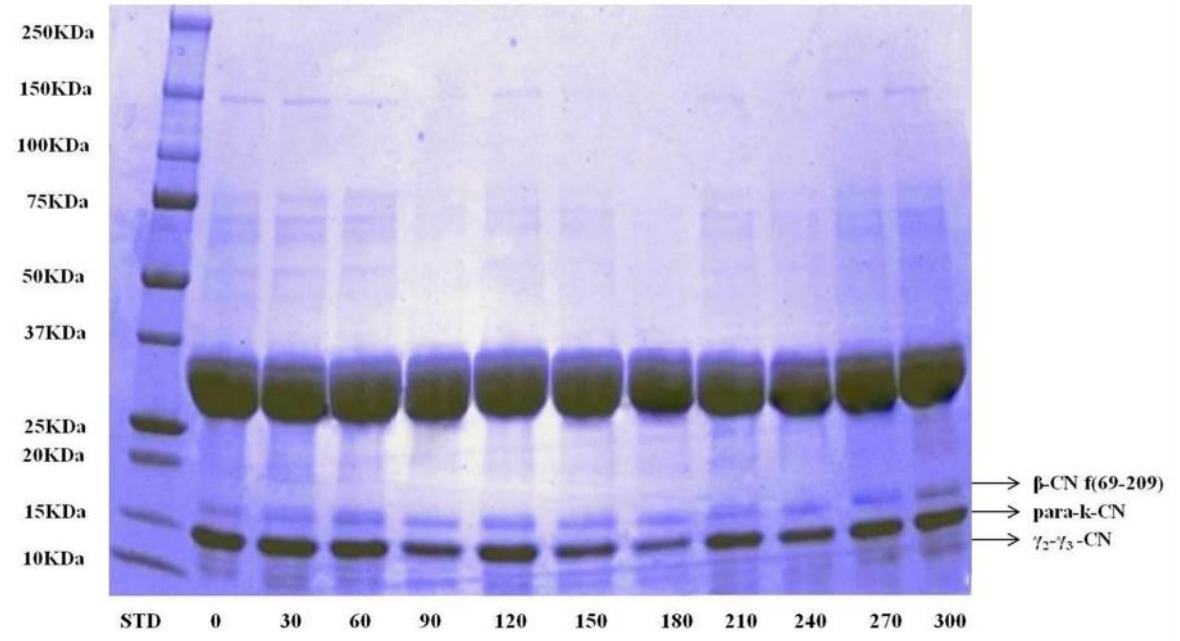


Figure 61: Electrophoresis on polyacrylamide gel (SDS-PAGE 4-15%) of the casein of mature curd.

Evaluating the evolution of the y4-CN over the course of time, also in mature curd, it is evident that there is a constant trend in both dairies (Figures 60 and 61). Also in the this case we see the application of evaluation of y4-CN by means of the % of the normalized amount compared to the para-k-CN. The densitometric analysis results are reported in Table 13 and 14.

Tab. 13Densitometric % results relative to the normalized amount of y4-CN compared to the para-k-NC from DairyA.

DAIRY A		
LANE NUMBER	NORMALIZED AMOUNT % para-k-CN	NORMALIZED AMOUNT % γ4-CN
2	100	30,987
3	100	31,389
4	100	29,617
5	100	30,69
6	100	28,761
7	100	30,8
8	100	29,232
9	100	31,17
10	100	30,856
11	100	31,014
12	100	31,006

Tab. 14: Densitometric % results relative to the normalized amount of y4-CN compared to the para-k-NC of Dairy B.

DAIRY B		
LANE NUMBER	NORMALIZED AMOUNT % para-k-CN	NORMALIZED AMOUNT % γ4-CN
2	100	50,168
3	100	48,213
4	100	48,825
5	100	50,304
6	100	48,875
7	100	48,306
8	100	50,483
9	100	50,838
10	100	48,421
11	100	49,652
12	100	50,698

These results made it possible to assess the trend of y4 CN over a year long experiment (Figure 62 and 63).

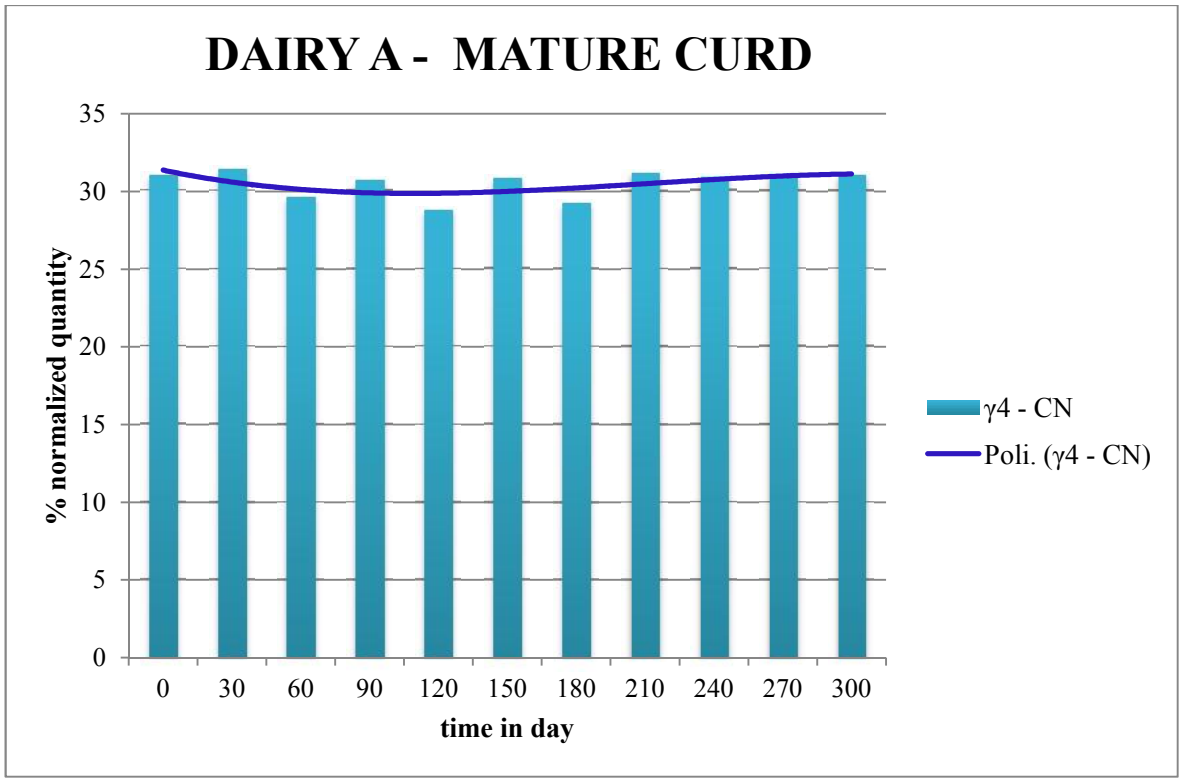


Figure 62: Trend of y4-NC from Dairy A

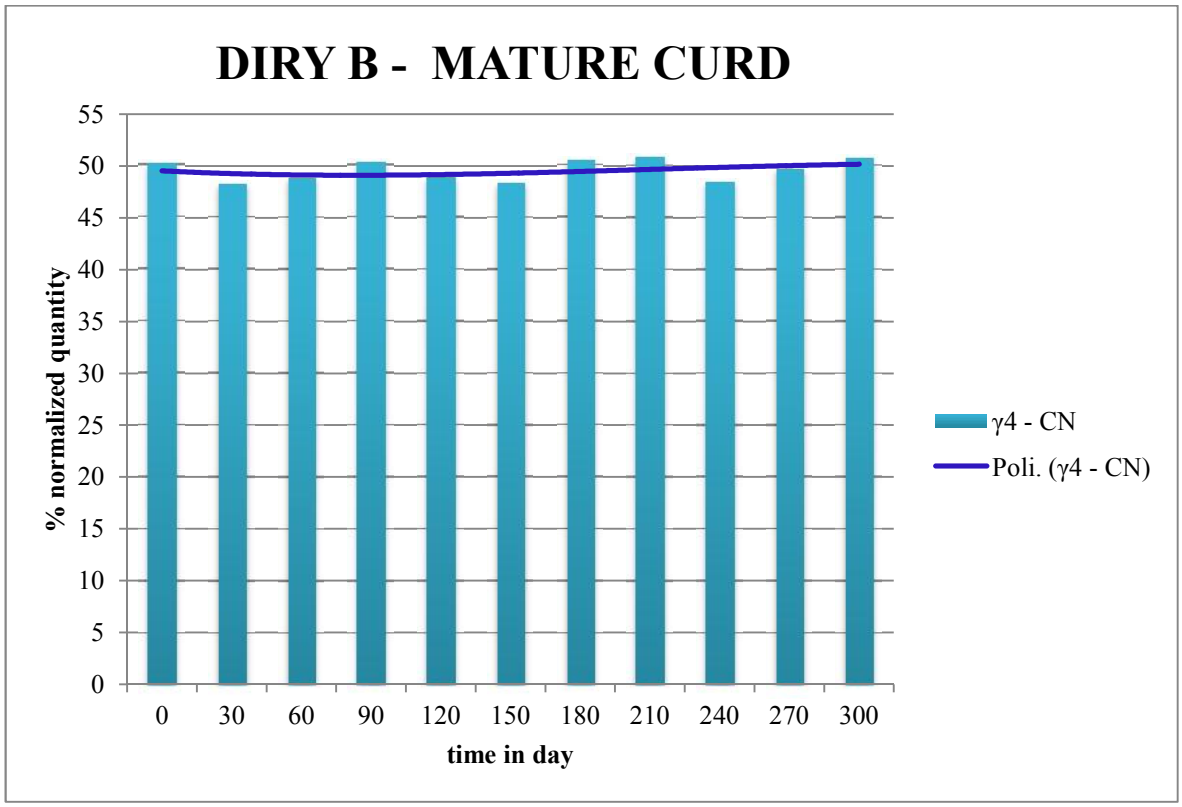


Figure 63: Trend of y4-NC Dairy B

Again in this last case the graphs confirm a steady trend of y4-CN between fresh mature curd and that which is stored at -20°C for 11 months.

Comparing the results obtained from the samples of both Dairy A and Dairy B, it must be noted that the latter has higher values of y4-CN. This can be attributed to increased proteolytic activity of plasmin in raw milk which occurs, for example, in mastitis milk or late lactation. Of the analyses performed in the laboratory, the contents of somatic cells in milk was higher in Dairy B compared to that found in Dairy A, however both have values fall within the legal limits.

16 RISULTS AND DISCUSSION UREA-PAGE

All five of the sampled matrices which have been preserved for eleven months at -20°C were analyzed and monthly analized by electrophoresis UREA-PAGE for the aim of study the protein fraction.
The samples loaded on the UREA-PAGE gel are in sequence respectively: raw milk, termized milk, premature curd, mature curd and mozzarella. Loaded after each month of storage starting from the matrices in a fresh state for the samples of all three dairies. The results from the elecrophoresis UREA-PAGE are shown in figures 64-67 for Dairy A,in figures 68-71 for Dairy B and in figures 72-75 for Dairy C.

UREA PAGE

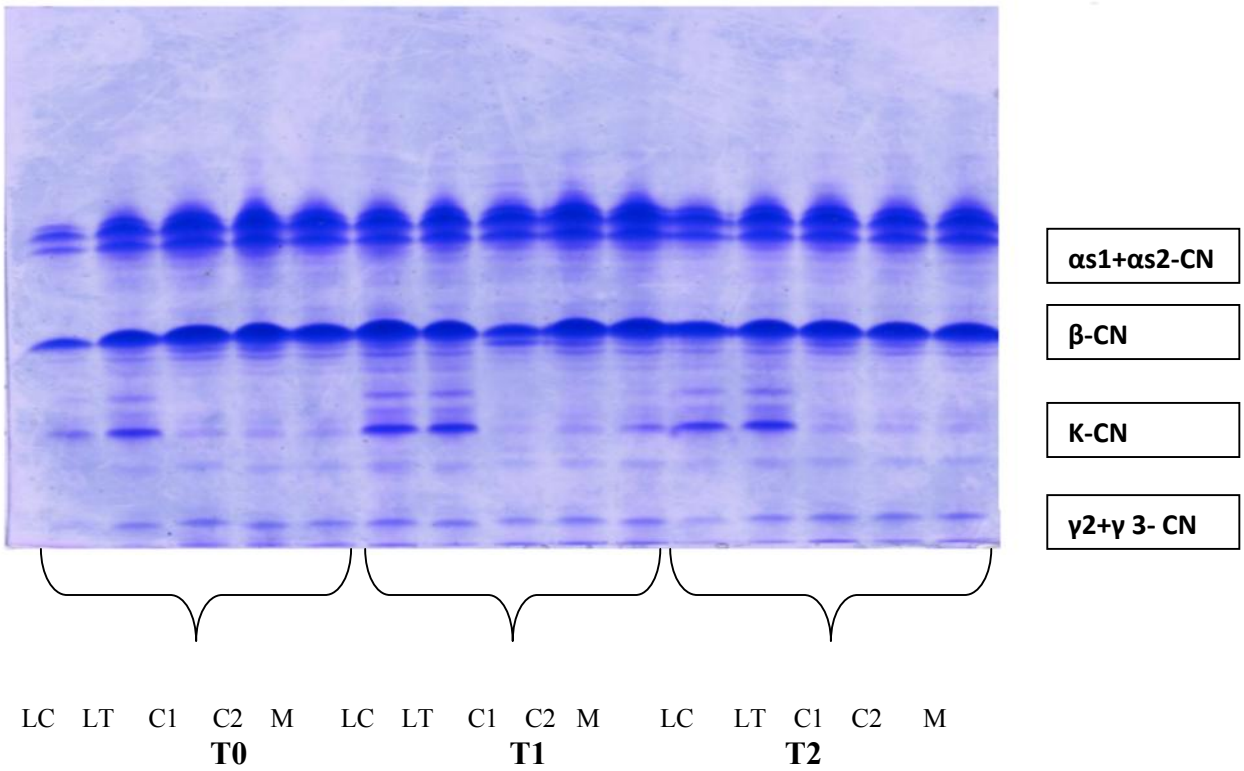


Fig. 64 Dairy A T0 April, T1 May, T2 June 2014

Legend:	LC	raw milk
	LT	termized milk
	C1	premature curd pH 6.8
	C2	mature curd 4,8
	M	mozzarella

UREA PAGE

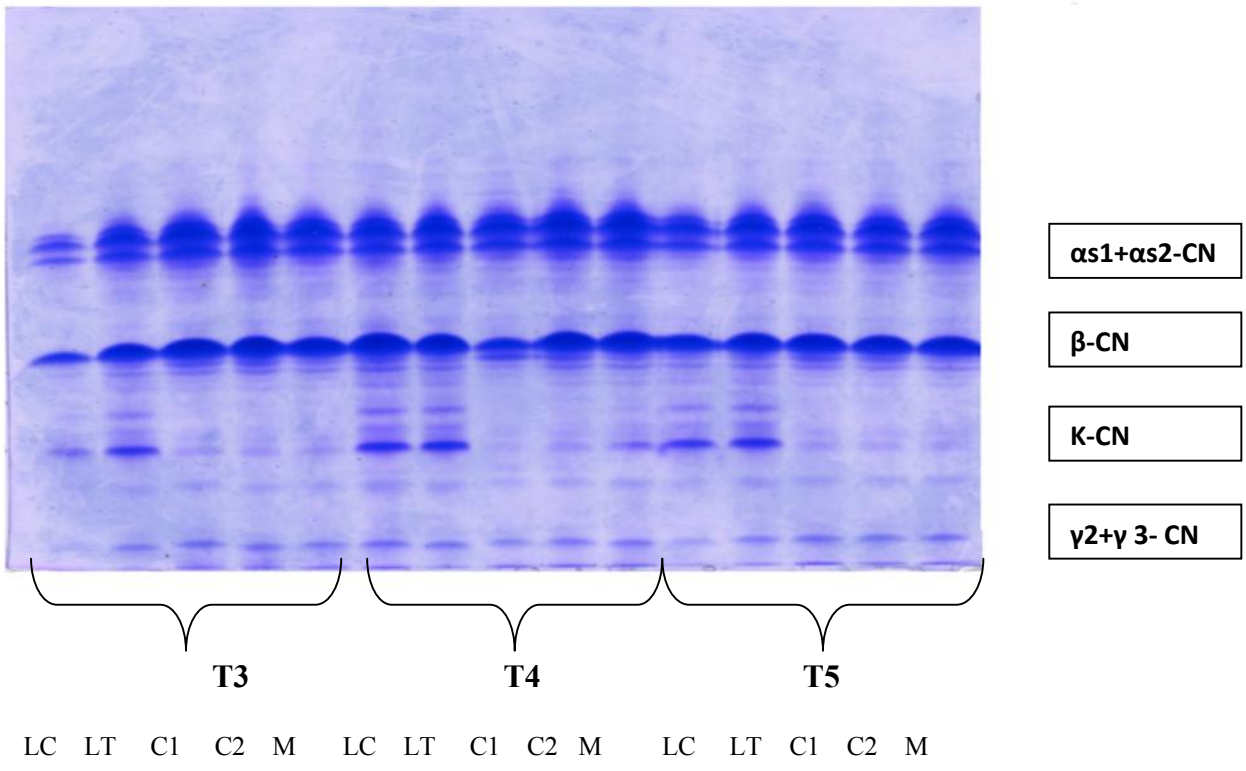


Fig. 65 Dairy A T3 July, T4 August, T5 September 2014

Legend:	LC	raw milk
	LT	termized milk
	C1	premature curd pH 6.8
	C2	mature curd 4,8
	M	mozzarella

UREA PAGE

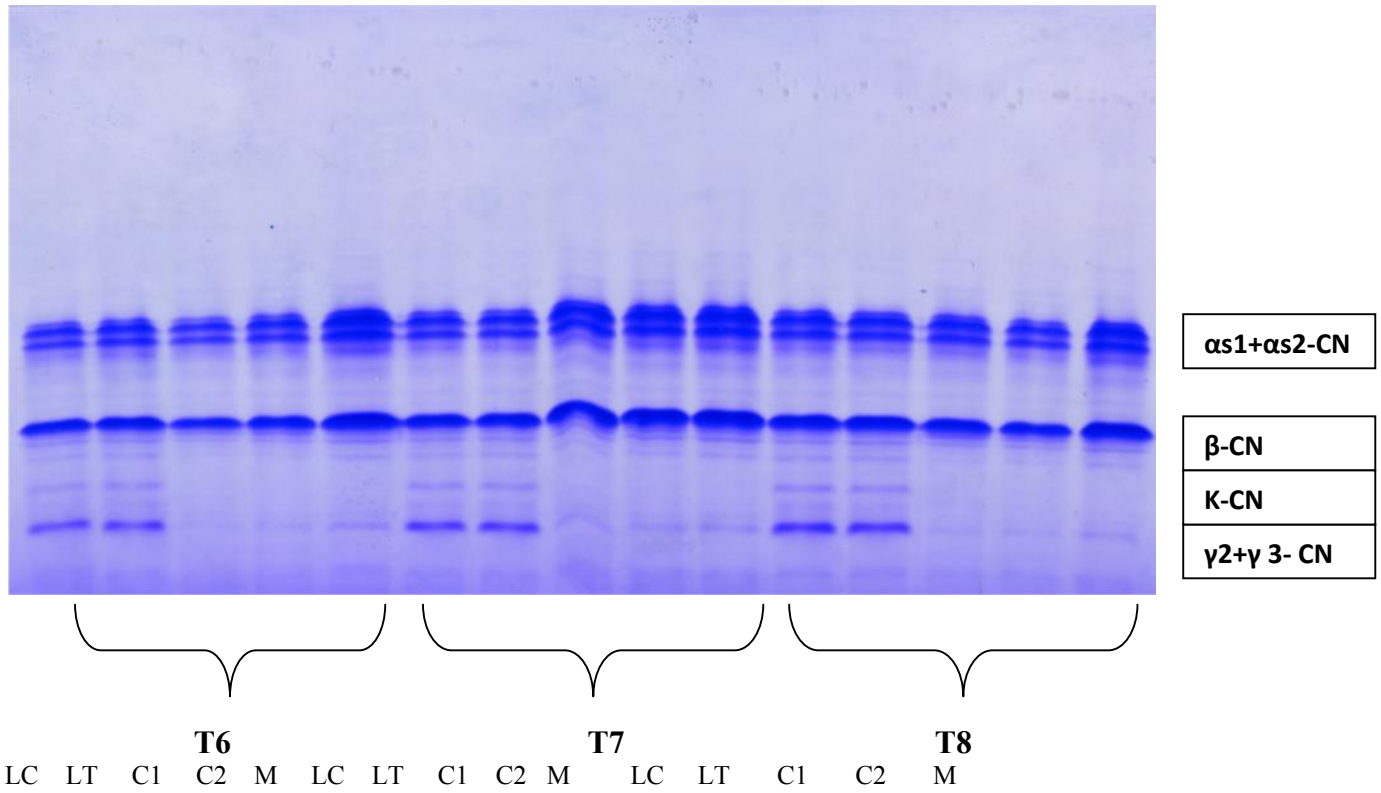


Fig. 66 Dairy A T6 October, T7 November, T8 Dicember 2014

Legend:	LC	raw milk
	LT	termized milk
	C1	premature curd pH 6.8
	C2	mature curd 4,8
	M	mozzarella

UREA PAGE

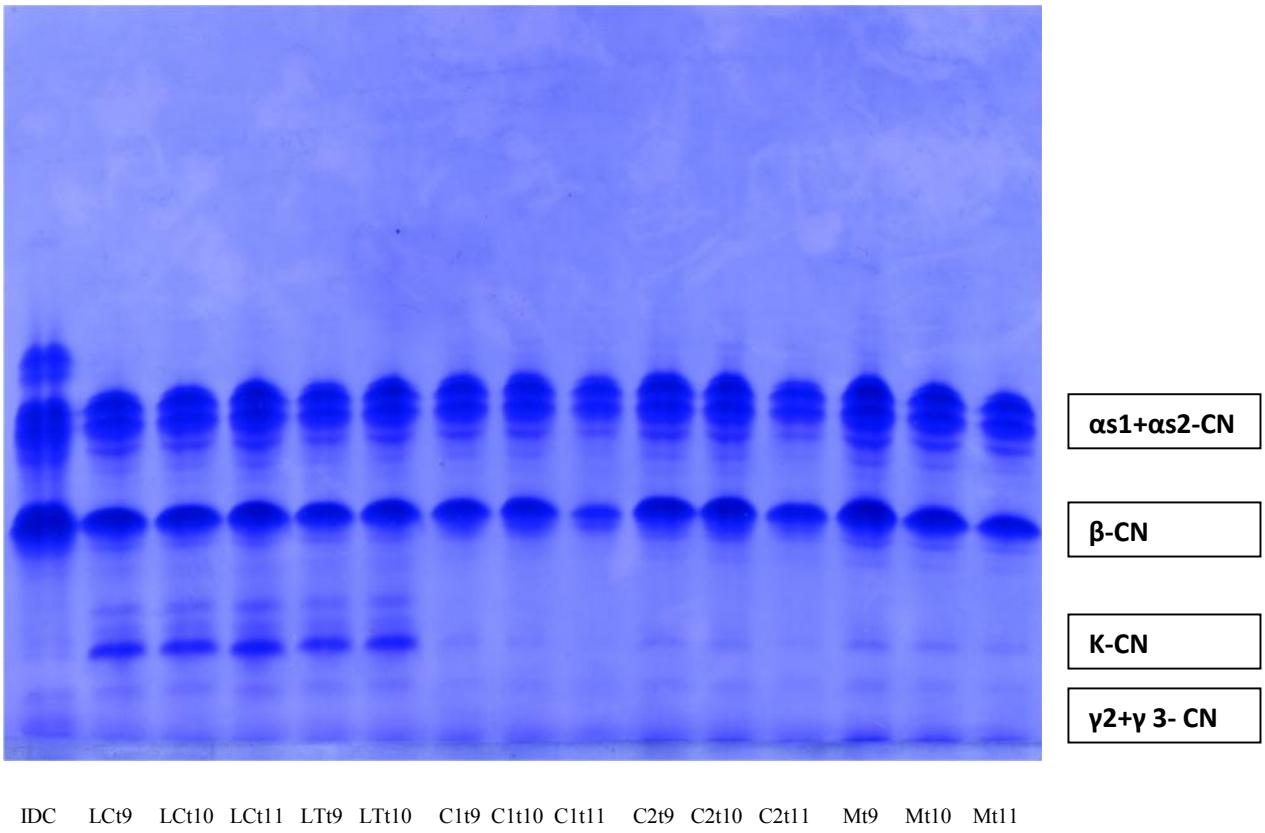


Fig. 67 Dairy A T9 Jenuary, T10 February, T11 March 2015

Legend:	IDC	idrolized with chimosin
	LC	raw milk
	LT	termized milk
	C1	premature curd pH 6.8
	C2	mature curd 4,8
	M	mozzarella

UREA PAGE

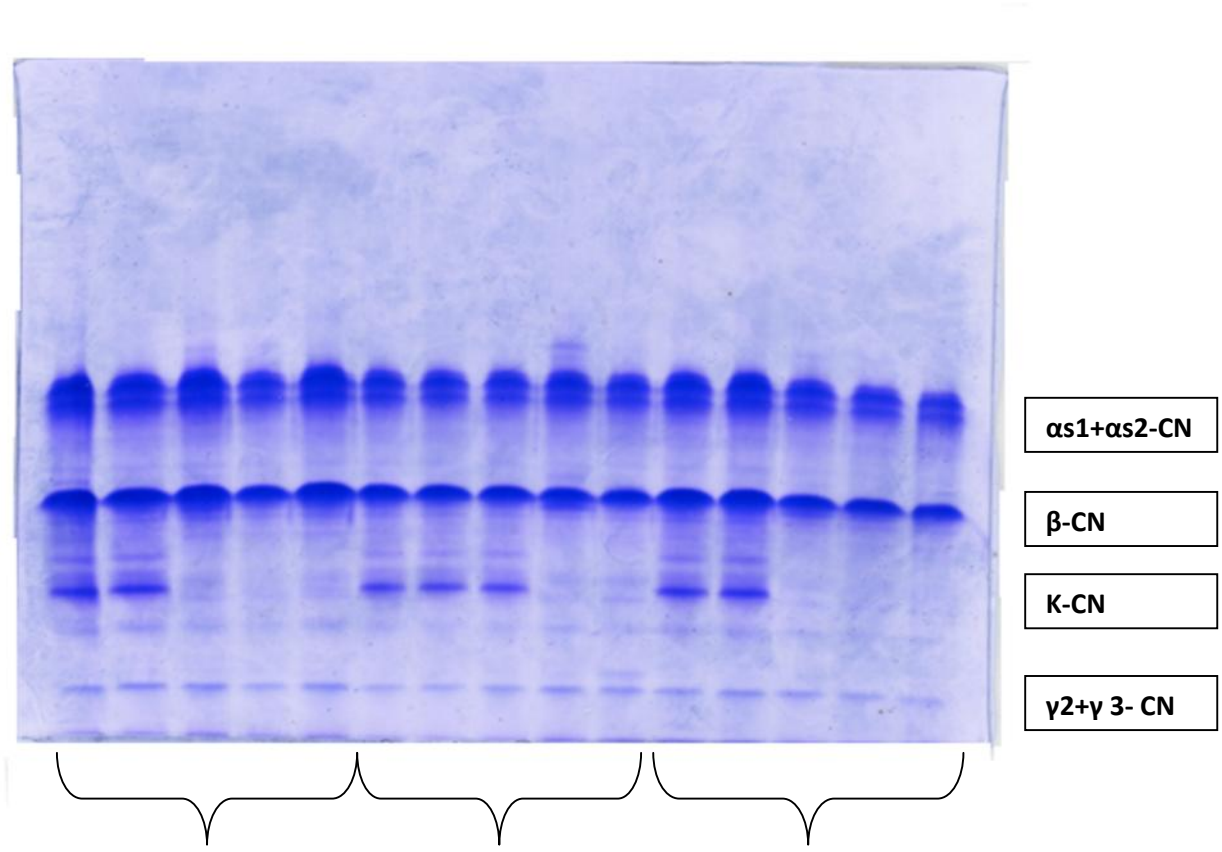


Fig. 68 Dairy B T0 May, T1 June, T2 July 2014

Legend:	LC	raw milk
	LT	termized milk
	C1	premature curd pH 6.8
	C2	mature curd 4,8
	M	mozzarella

UREA PAGE

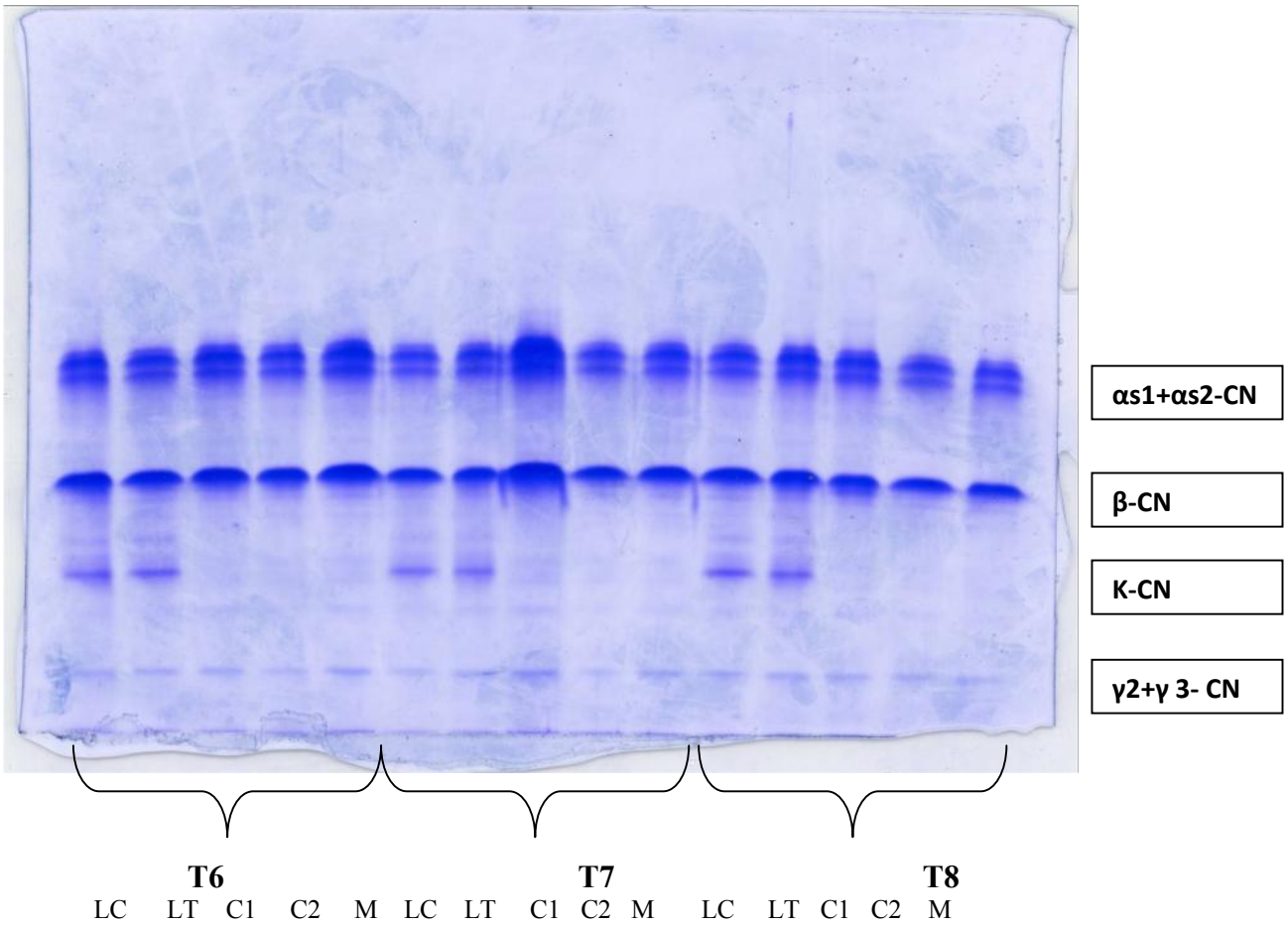


Fig. 69 Dairy B T3 August, T4 September, T5 October 2014

Legend:	LC	raw milk
	LT	termized milk
	C1	premature curd pH 6.8
	C2	mature curd 4,8
	M	mozzarella

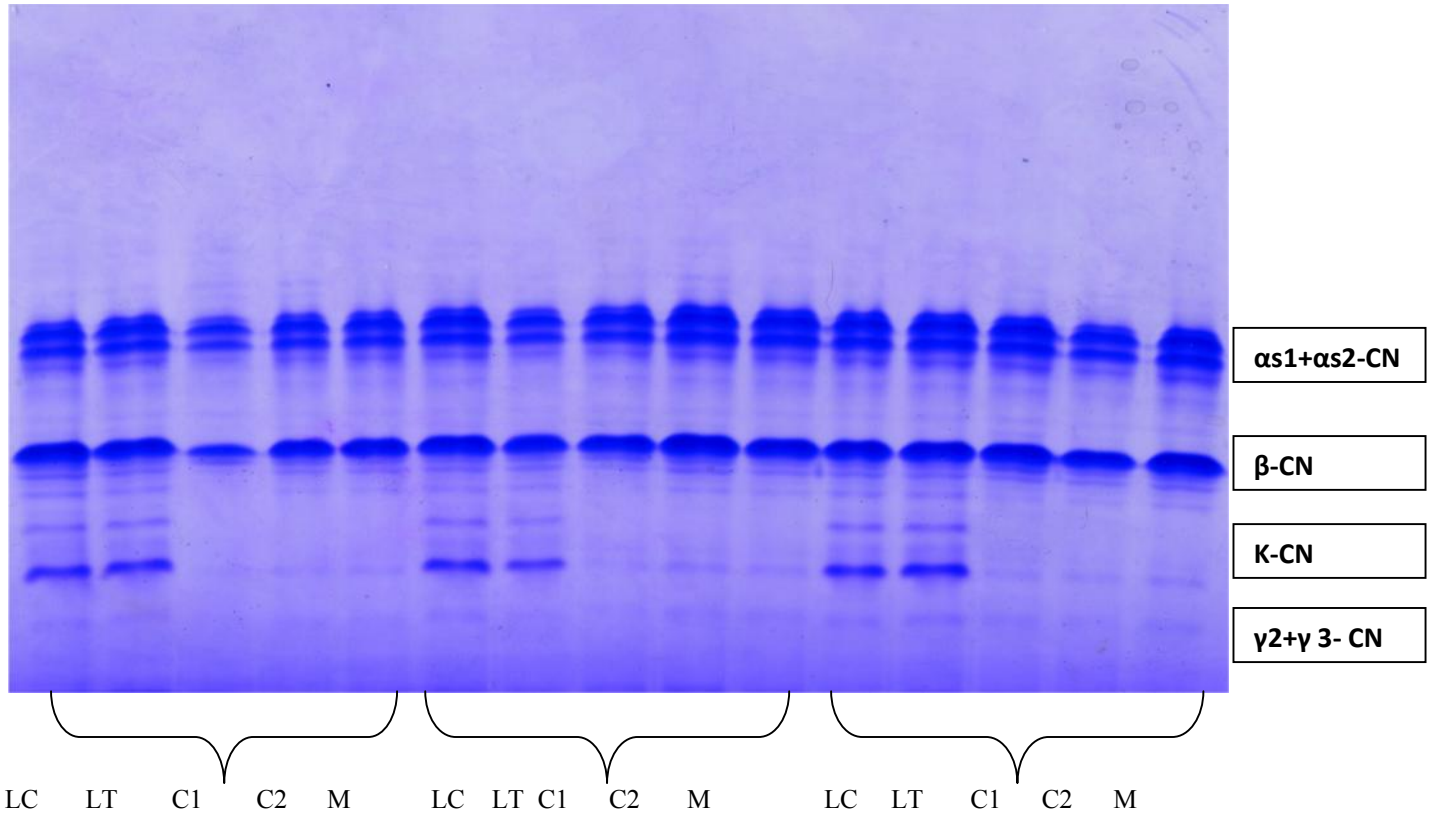
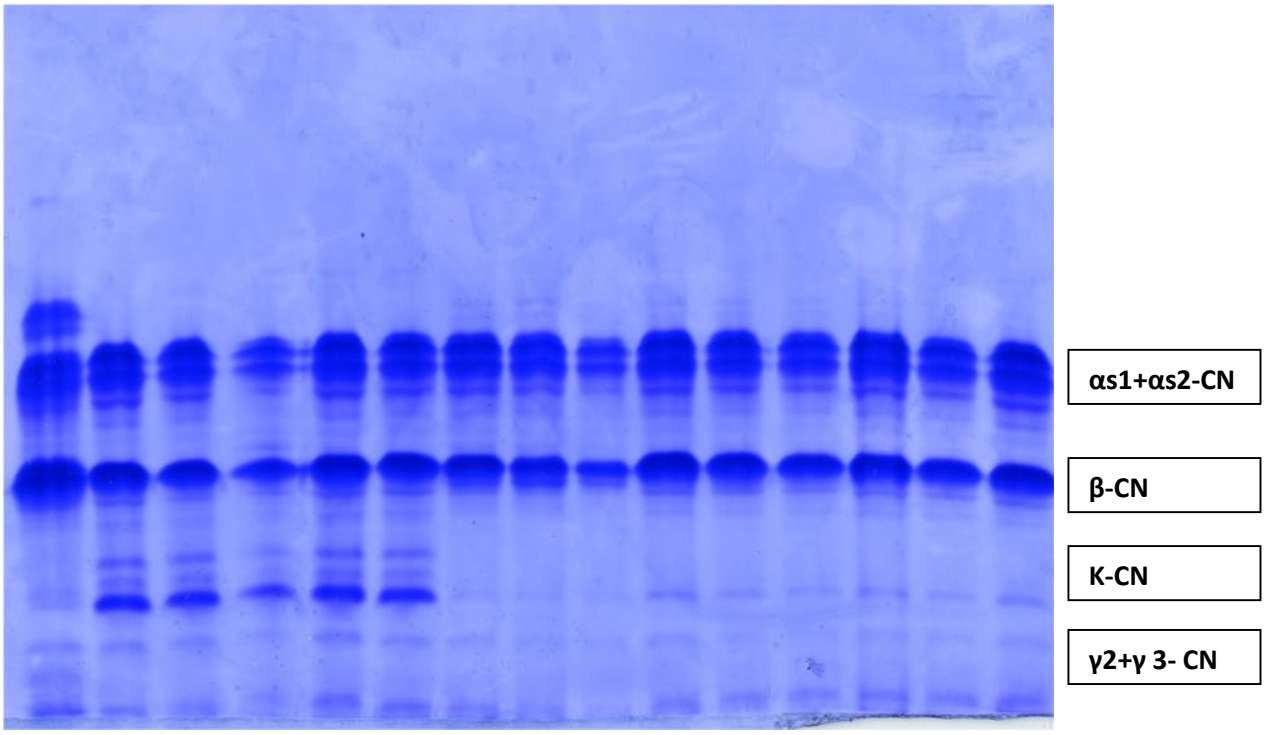


Fig. 70 Dairy B T6 November, T7 December 2014, T8 January 2015

Legend:	LC	raw milk
	LT	termized milk
	C1	premature curd pH 6.8
	C2	mature curd 4,8
	M	mozzarella

UREA PAGE



IDC LCt9 LCt10 LCt11 LTt9 LTt10 C1t9 C1t10 C1t11 C2t9 C2t10 C2t11 Mt9 Mt10 Mt11

Fig. 71 Dairy B T9 February, T10 March, T11 April 2015

Legend:	IDC	idrolized with chimosin
	LC	raw milk
	LT	termized milk
	C1	premature curd pH 6.8
	C2	mature curd 4,8
	M	mozzarella

UREA PAGE

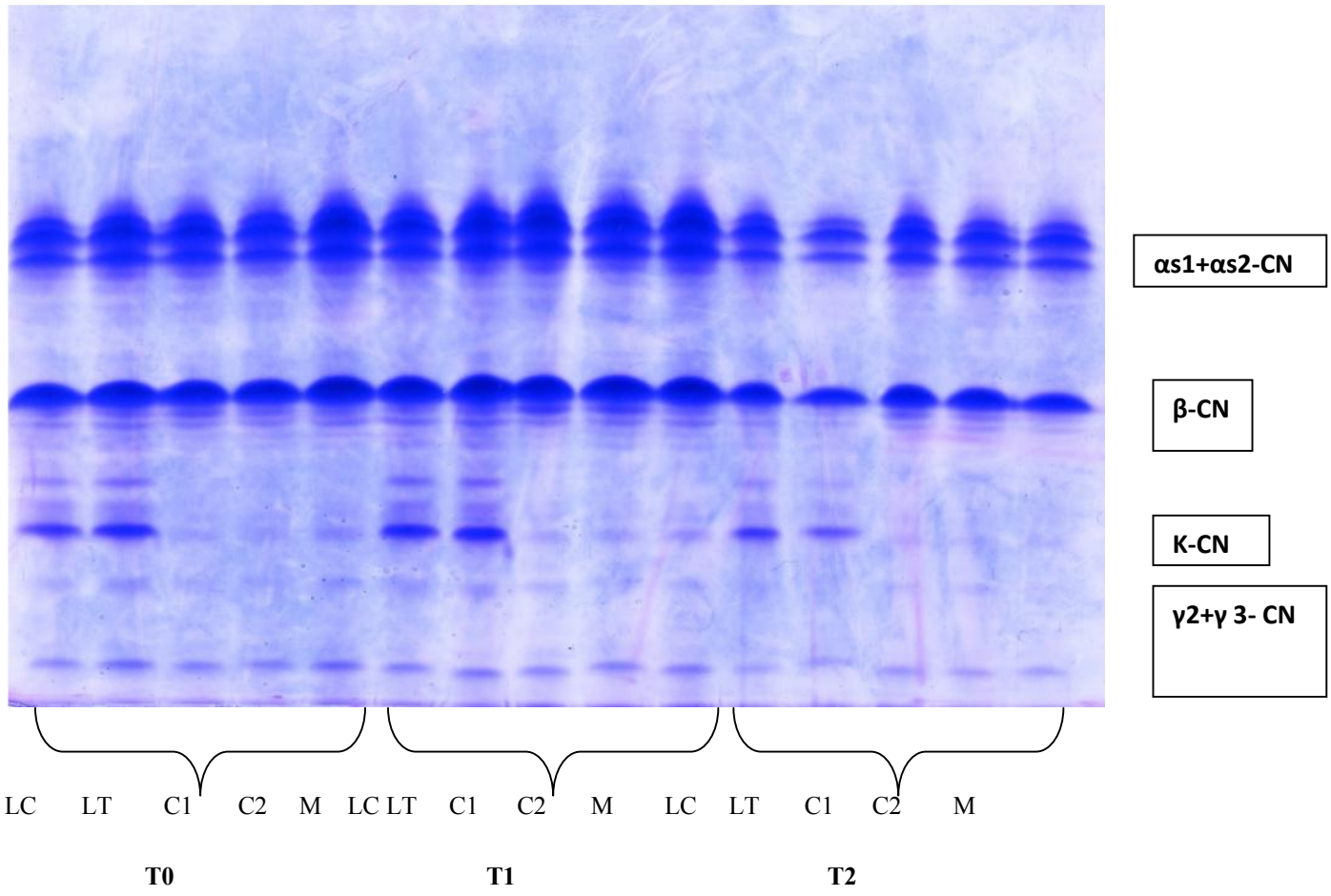


Fig. 72 Dairy C T0 July, T1 August, T2 September 2015

Legend:	LC	raw milk
	LT	termized milk
	C1	premature curd pH 6.8
	C2	mature curd 4,8
	M	mozzarella

UREA PAGE

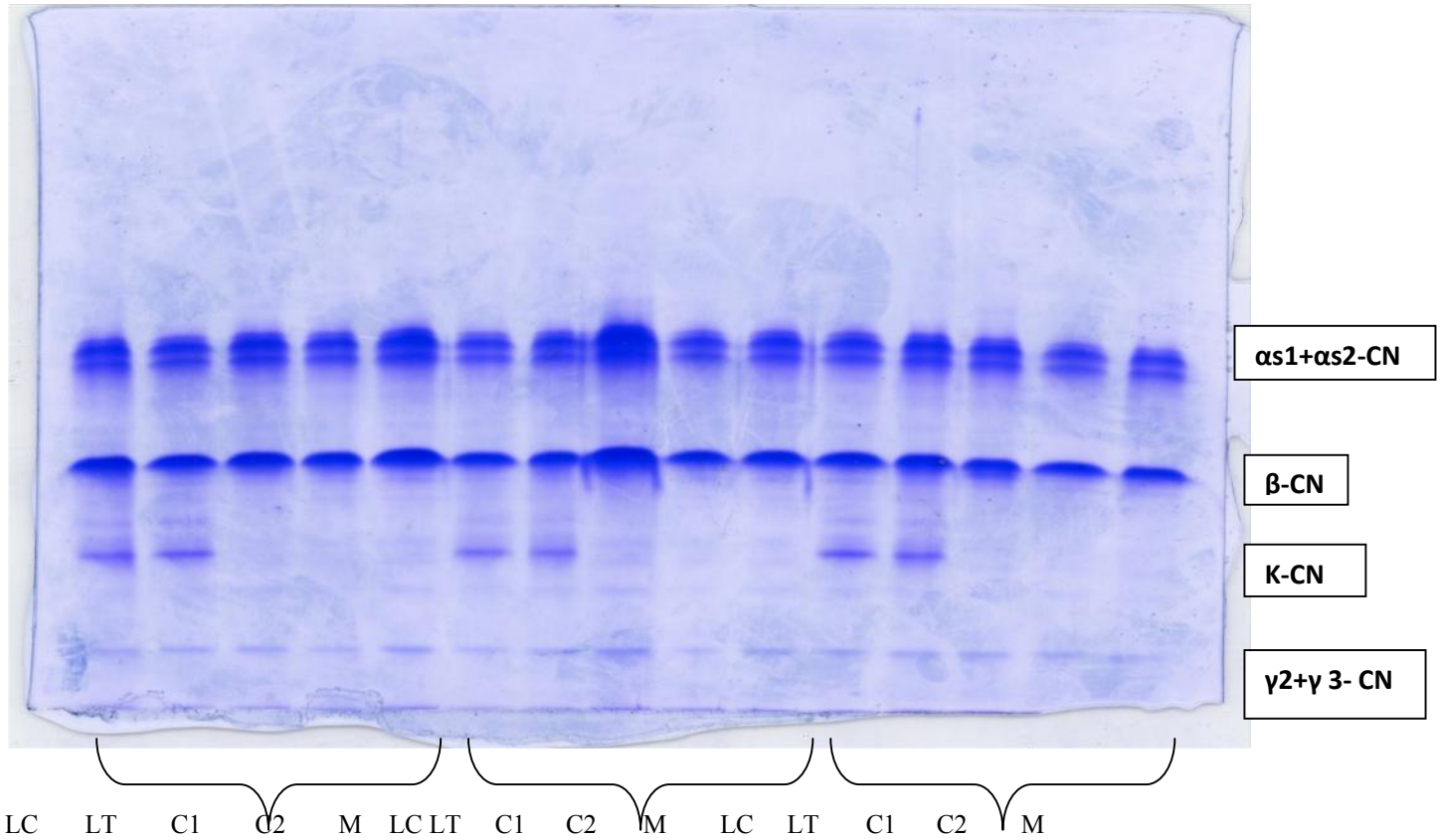


Fig. 73 Dairy C T3 October, T4 November, T5 December 2014

Legend:	LC	raw milk
	LT	termized milk
	C1	premature curd pH 6.8
	C2	mature curd 4,8
	M	mozzarella

UREA PAGE

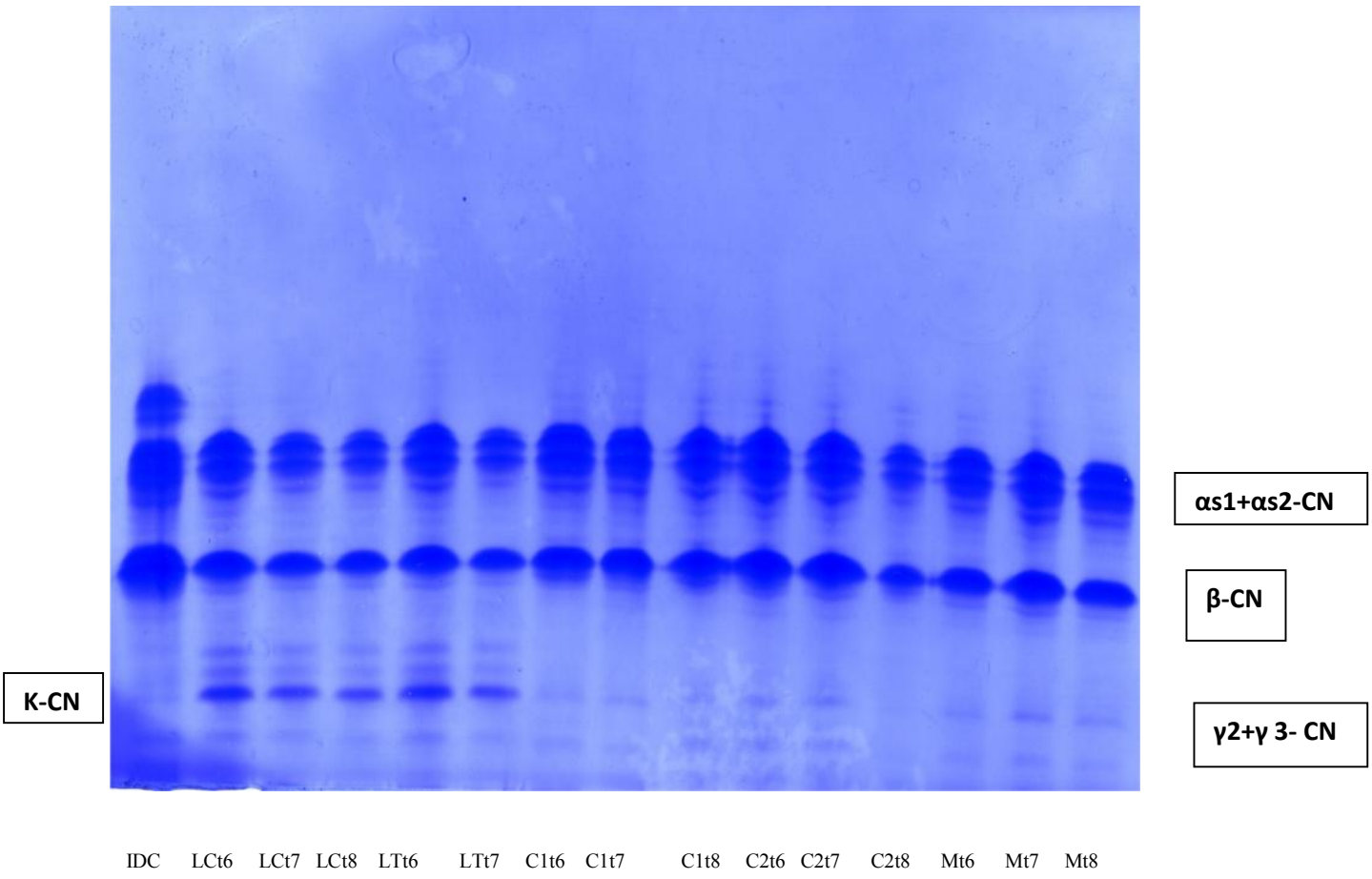


Fig. 74 Dairy C T6 January, T7 February, T8 March 2015

Legend:	IDC	idrolized with chimosin
	LC	raw milk
	LT	termized milk
	C1	premature curd pH 6.8
	C2	mature curd 4,8
	M	mozzarella

UREA PAGE

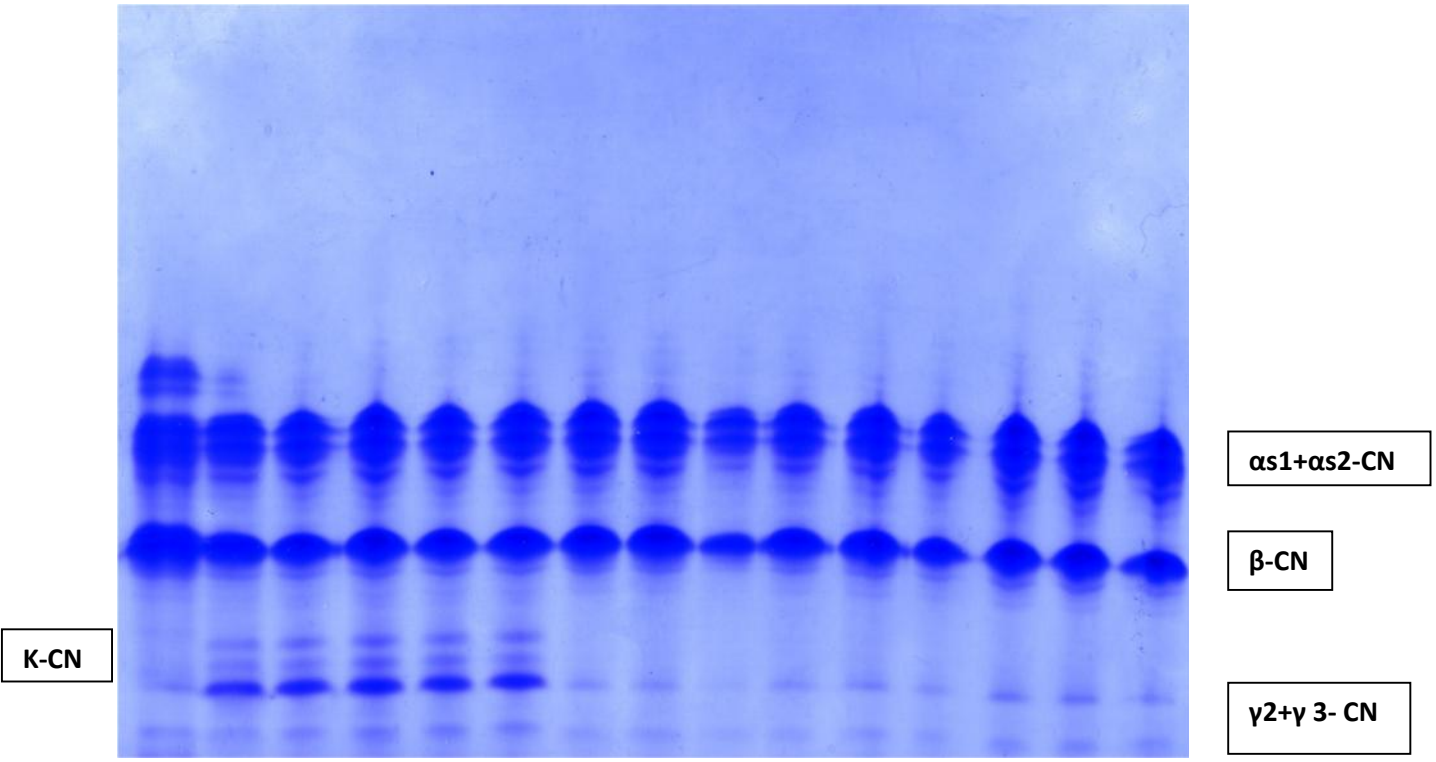


Fig.75 Dairy C T9 April, T10 May, T11 June 2015

Legend:	IDC	idrolized with chimosin
	LC	raw milk
	LT	termized milk
	C1	premature curd pH 6.8
	C2	mature curd 4,8
	M	mozzarella

17. CONCLUSION

This work studied the fragment β-CN f (69-209), identified in the bibliography as a marker to assess the traceability of milk and frozen curd into Buffalo Mozzarella Campana PDO. Depending on the conditions of the environment and of the process such as temperature, enzyme concentration, the starting milk, the proteolytic action of the loaded plasmin of the β casein, appears to be more or less intense. This fragment is the first that is formed, faster than the others due to exposure of conformational Lys_{68}-Arg_{69} site. Since the practice a PDO is based on the freezing temperature of the matrices of -20°C, the polypeptide fragment shows a steady trend with the decreasing freshness of the buffalo milk and curd. The results obtained from the electrophoretic analysis of milk and curd (fresh and frozen)used in the production Buffalo Mozzarella Campana PDO showed an absolute identity between the product obtained from fresh matrices and that obtained from frozen.

The results obtained by the electrophoretic analysis (Urea Page) of milk, semi-finished products and mozzarella, fresh and frozen, used for the production of Buffalo Mozzarella Campana PDO have shown an absolute identity between both the products obtained from fresh and frozen matrices. It was not possible to observe the αs1-I casein as was evidenced from the hydrolysate with chymosin, whereas according to the method indicated, αs1-I is separated and clearly visible on the gel.

To our knowledge, in the current state, it is not possible to identify a marker of freshnes.

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Legislative references:

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RINGRAZIAMENTI

Anche se una tesi solitamente potrebbe sembrare un lavoro individuale, è tutt'altro. Alla sua stesura collaborano molte persone a cui dobbiamo riconoscere un contributo piccolo o grande che sia .

Ringrazio il dottore Antonio Limone, commissario dell'IZSM di Portici per il sostegno dato a questo complesso lavoro di ricerca.

Un grazie al Prof. Raffaele Romano per aver creduto in questa ricerca e nell'appoggio fin dal primo momento ed in questi lunghi e complessi anni di lavoro nel corso dei quali mi ha aiutato nello sviluppo e della stesura di questa articolata ricerca.

Un grazie speciale alla dottoressa Nadia Manzo: per la sua disponibilità è stata un prezioso aiuto, sempre presente quando nel corso del lavoro mi sono imbattuta in molti ostacoli. A Nadia il merito di essermi stata sempre vicina ascoltandomi e consigliandomi con il suo fare sempre fattivo e collaborativo accompagnato sempre da un bel sorriso.

Un ringraziamento va a Michele De Pasquale e a Rachele Andolfo che pazientemente hanno camminato al mio fianco per circa un anno di ricerca.

Un grazie al personale dell'Istituto Zooprofilattico Sperimentale, con cui ho avuto, direttamente o indirettamente, occasione di collaborare. In particolare ringrazio Mario, Rosa, Salvatore, Fortuna e Antonio, che mi hanno, in differenti modi aiutata.

Ringrazio la dott.ssa Donatella Nava e il dott. Federico Capuano che hanno condiviso con me questo lungo e faticoso viaggio, che mi ha permesso di apprezzare le loro qualità non solo professionali ma soprattutto umane .

Ringrazio la dott.ssa Adalgisa Nicolai per avermi trasmesso una metodica per niente facile, ma che ha reso semplice, grazie alla sua grande passione e professionalità.

Un grazie di cuore va a mio marito Paolo, compagno di vita, con il quale ho condiviso sempre tutto, onori ed oneri, che mi ha sempre supportata e sopportata pazientemente e per avermi incoraggiata e spronata a dare il meglio, e se sono riuscita nel mio intento nel realizzare "questo piccolo grande sogno" è anche e soprattutto grazie a lui e ai miei due adorati figli Valentina e Giuseppe.

Grazie a loro due, per tutte le domeniche sacrificate a stare in casa con me, a rinunciare ad uno svago dopo una settimana laboriosa, per permettermi di studiare, incoraggiandomi a studiare e scrivere la tesi e a fare il conto alla rovescia per il raggiungimento del giorno fatidico.

La mia gratitudine più grande va a mia madre, per essere stata sempre al mio fianco, senza riserve, nel bene e nel male, nei momenti di sconforto e nei momenti di felicità, aiutandomi in ogni modo, contribuendo spesso in modo determinante.

A mia madre, a mio marito, a Valentina e Giuseppe dedico questa tesi. Grazie a tutti di cuore

Infine, un ultimo ringraziamento va a me stessa, perché ho voluto, tre anni fa, lanciarmi una sfida. E' solo grazie alla mia testardaggine e alla mia lungimiranza, ho potuto affrontare, dimostrando, ancora una volta, di possedere le qualità che mi hanno permesso di arrivare a questo desiderato traguardo. Spero di essere stata all'altezza e mi auguro che questa ricerca possa portare dei validi frutti nel prossimo futuro.

Grazie a tutti.