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# Identification and partial characterization of lactic acid bacteria isolated from Provolone del Monaco P.D.O. cheese during manufacturing and ripening

# Investigation of Biogenic Amines and Volatile Flavor compounds in aged Provolone del Monaco P.D.O. cheese

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## Abstract

Identification and characterization of Lactic Acid Bacteria (LAB) from Provolone del Monaco P.D.O. cheese has been carried out by the conventional microbiology followed by Pulse Field Gel Electrophoresis (PFGE) and 16S rDNA sequencing. The aim of this research was to acquire information about the different microbial groups involved in cheese making produced from raw milk. Lactobacilli dominated largely, especially casei/paracasei group, up to the end of cheeses ripening, together by *Lb. delbrueckii*. The dominant cocci-shaped LAB until one year of ripening were the *S. macedonicus* and *S. lutetiensis/infantarius.* Enterococci strains were also present and play a major role during manufacturing and aged cheese. Tyramine producing strains, belonging mostly to *Enterococcus* genus, were found by Decarboxylase Selective Medium (DSM). One strain of *E. faecium* tyramine positive was also enterocin A producing. Nevertheless, one strain of *L. lactis* was found to be Nisin A producing. Acidification capability test on selected isolates have shown *Lb. delbrueckii*, *S. macedonicus*, *S. thermophilus*, *L. lactis* and *S. lutetiensis* as fast acid producers. This study provided evidence that natural environments are rich in biodiversity and can be considered as proper sources of new strains and species within the LAB group.

Provolone del Monaco cheeses were also analysed for their amount of biogenic amines and volatile flavor compounds using High Performance Liquid Chromatography (HPLC) and Thermal Desorption Gas Chromatography Mass Spectrometry (TD-GC/MS), respectively. All these cheeses belonged to the same batch production of the others subjected to microbiological analysis. Biogenic amines concentration was highest in sample which showed the defect of medium-sized eyes. While the other cheeses, with same typical sensorial characteristics of Provolone del Monaco P.D.O. cheese, had the lowest and similar biogenic amines levels. Ketones, esters, acids were volatile compounds associated to the analysed cheese samples. New information regarding the flavor profile of Provolone del Monaco P.D.O. cheese are provided. Moreover, sample with medium-sized eye was associated to a very high number of esters and was found to have a different profile of volatile flavor compounds than the other cheeses, which were associated mainly to ketones and acids.

## Specific Objectives

This study has been planned in various steps as follows:

**1) Cheese microbiology**

- Description of the main dairy microflora dynamics (lactococci, lactobacilli, streptococci and enterococci) during processing and ripening of Provolone del Monaco P.D.O. cheese.
- Identification of the potential starter strains to be used for the manufacture of Provolone del Monaco P.D.O. cheese with improved and more consistent quality.
- Identification of the strains driving the ripening process of Provolone del Monaco P.D.O. cheese.

**2) Cheese safety**

- Identification of the bacteriocins producing strains and bacteriocins controlling the proliferation of pathogens or undesirable microflora.
- Identification of the strains producing biogenic amines (BA) and detection of BA in Provolone del Monaco P.D.O. cheese.

**3) Cheese technology**

- Evaluation of the strains as fast acid producers.

**4) Cheese quality**

- Detection of volatile flavor compounds in Provolone del Monaco P.D.O. cheese.

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# 1. Introduction

## 1.1. Cheese and Lactic Acid Bacteria (LAB)

The cheeses appear since ancient times in the history of food, accompanying the prehistoric man from the moment when the hunter becomes sedentary, enabling the supply of nutritious food without the slaughtering of livestock (Bogucki, 1984). Although the origins of cheese are presently unknown, written and iconographic evidence dated at the mid-third-millennium BC have partly documented the history of cheese making (Sherratt, 1981; Hallo, 1972). The bas-relief in ancient Mesopotamia Sumer, which regarded as one of the oldest document, has shown the application of dairy techniques in the third millennium BC (Battistotti et *al.*, 1983).

**Fig. 1.** Frieze of Dairy

While the true practice of milk processing could not be clearly proven, the discovery of abundant milk residues in pottery vessels from the seventh millennium sites which originated from the north-western Anatolia have provided the earliest evidence of milk processing (Evershed et *al.*, 2008). Greek mythology have featured the origin of cheese to the Nymphs, which would have taught Aristaeus, son of Apollo, the art of turning milk (Battistotti et *al.*, 1983). Nonetheless, the most famous practice on the consumption of cheese by the Greeks in Homer's Odyssey (late 8$^{th}$ century BCE) is where the Cyclops Polyphemus in his cave, prior to the act of milking sheep bleating, following to cheese making, and finally when places it in wicker baskets. "*We soon reached his cave, but he was out shepherding, so we went inside and took stock of all that we could see... His cheese-racks were loaded with cheeses, and he had more lambs and kids than his pens could hold... When he had so done he sat down and milked his ewes and goats, all in due course, and then let each*

*of them have his own young. He curdled half the milk and set it aside in wicker strainers"* (Homer, 1900).

It is from these baskets (the "*formos*") in which the Italian word cheese *"formaggio"* is derived from. The term *caseus*, used by the Romans to designate the cheese, comes from almost all European languages. For example, from English cheese, from Dutch *kaas*, from German *kase,* from Spanish *queso*, and from Portuguese *queijo*. The term *cacio* is not very commonly used in Italy except in the centre of south Italy. The most common Italian name for cheese in Italy is "*formaggio*", a word probably originated from Latin *formaticum* (Alinei, 2010).

The ancient practice to produce cheese depends on Lactic Acid Bacteria (LAB) to ferment milk; because LABs are the main microorganisms in milk and dairy products. LABs have likely been part of the diet since human first collected milk and then accidentally store the milk in a container made from the abomasum of a ruminant. In later centuries, these accidental fermentation processes are used in a controlled manner giving rise to more than 1,000 unique cheese and dairy products that are used until today (Broadbent, 2005).

The use of lactic acid fermentation for the cheese making had been developed long before Pasteur (1857) described lactic fermentation in a paper or J. Lister and O. Brefeld tried to make pure colonies of microorganisms (Benninga, 1990). Later that century, the development of pure LAB starter cultures have initiated the cheese industrialization and milk fermentation especially cheese production has undergone sustained and significant growth (Broadbent, 2005).

In 1919, Orla-Jensen had published a monograph that represents the basis of the present systematic of LAB (bacteria cellular morphology, mode of glucose fermentation, temperature ranges of growth, and sugar utilization patterns) (Orla-Jensen, 1919). At the beginning of the $20^{th}$ century the term Lactic Acid Bacteria was slowly accepted and eventually used to refer to "milk souring organism", which is different from other bacteria producing lactic acid (Carol et *al.*, 2011). The first core group of LAB was found by Orla-Jensen, who divides LAB into four main genera: *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*. These genera have appeared in the latest edition of Bergey's Manual (1986), however, some variations

on the classification have changed into the following genera: *Aerococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* (Barry, 2009). Streptococcus was distinguished into three: *Enterococcus*, *Lactococcus*, and *Streptococcus* sensu stricto (Alexander et *al.*, 2001) (Zongzhi et *al.*, 2008). Nevertheless, a new genus similar to *Lactococcus* was created namely, *Vagococcus* (Aly, 2004). The classification of genera *Lactobacillus*, *Leuconostoc* and *Pediococcus* remained the same, except for some of rod-shaped LAB belonging to the genus *Lactobacillus*, are grouped in the new genus *Carnobacterium* (Elliot et *al.*, 1991) and *Pediococcus halophilus* (Collins, 1990).

Several new classifications on Lactic Acid Bacteria belong to the phylum Firmicutes, class Bacilli, and order Lactobacillales has been proposed using the application of recent taxonomic tools. These classifications are comprises of the following: *Aerococcus, Alloiococcus, Carnobacterium, Dolosigranulum, Enterococcus, Globicatella, Lactococcus, Lactobacillus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus and Weissella* (Khalid, 2011). LAB constitutes a group of microorganism gram-positive, non-motile, chemotrophic, non-sporulating, aero tolerant or anaerobes, cocci or rods, optimum growth at pH 5.5-5.8, acid tolerant, catalase-negative, devoid of cytochromes and produces lactic acid as one of the main end product of various fermentable carbohydrates (Axelsson, 1998). The different characteristics of LAB are shown in Table 1.

**Table 1.** Common Genera of LAB and their Differential Characteristics

| **Family** | **Genera** | **Characteristics** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Shape | $CO_2$ from Glucose | Growth at 10°C | Growth at 45°C | Growth in 6.5% NaCl | Growth in 18% NaCl | Growth at pH 4.4 | Growth at pH 9.6 | Type of Lactic Acid |
| *Aerococcaceae* | *Aerococcus* | Cocci (tetrads) | - | + | - | + | - | - | + | L |
| *Carnobacteriaceae* | *Carnobacterium* | Rods | - | + | - | ND | - | ND | - | L |
| *Enterococcaceae* | *Enterococcus* | Cocci | - | + | + | + | - | + | + | L |
| | *Tetrageonococcus* | Cocci (tetrads) | | + | - | + | + | Variable | + | |
| | *Vagococcus* | Cocci | | + | - | - | - | | - | |
| *Lactobacillaceae* | *Lactobacillus* | Rods | Variable | Variable | Variable | Variable | - | Variable | - | D, L, DL |
| | *Pediococcus* | Cocci (tetrads) | - | Variable | Variable | Variable | - | + | - | L, DL |
| *Leuconostocaceae* | *Leuconostoc* | Cocci | + | + | - | Variable | - | Variable | - | D |
| | *Oenococcus* | | + | + | - | Variable | - | Variable | - | D |
| | *Weissella* | | + | + | - | Variable | - | Variable | - | D, DL |
| *Streptococcaceae* | *Lactococcus* | Cocci | - | + | - | - | - | Variable | - | L |
| | *Streptococcus* | | - | - | Variable | - | - | - | - | L |

LAB can be distinguished, according to their metabolism, into two major physiological groups: homofermentative LAB (such as *Lactococcus* and *Streptococcus*) and heterofermentative LAB (such as *Leuconostoc* and *Weissella*) (Stackebrandt et *al.*, 1988; Thompson, 1988). The homofermentative lactic acid bacteria produce lactic acid as a final fermentative substance, while the heterofermentative produce lactic acid, ethanol and $CO_2$ at an approximate equimolar amount (Caplice et *al.*, 1999; Jay, 2000; Kuipers et *al.*, 2000).

The LAB that associates with cheese ripening has different properties, hence it is probably best to divide them into different groups: Starter Lactic Acid Bacteria (SLAB) and Non Starter Lactic Acid Bacteria (NSLAB). SLAB has the ability to produce enough acid during cheese manufacture resulting in a significant reduce of the milk pH. Furthermore, SLAB has the ability to produce enzymes which are responsible for lipolysis and proteolysis reactions as well as forming flavor compounds (mainly microflora) (Beresford et *al.*, 2001). SLAB is composed of a great deal of microorganisms encompassing different types (bacteria, yeast, moulds) and species.

SLAB is mostly belonging to genera *Lactococcus*, *Lactobacillus* and *Streptococcus*. In general, SLAB is classified according to their optimum temperature for growth. First is mesophilic that has an optimal growth of ~30 °C (for example, *Lactococcus lactis subsp. cremoris* and *Lactococcus lactis subsp. lactis)*. Second is thermophilic that has an optimal growth of~42° C, but often used for higher temperature such as 50-55°C (for example, *Streptococcus thermophilus, Lactobacillus delbrueckii subsp. delbruekii, Lactobacillus delbrueckii subsp. bulgaricus, Lactobacillus delbrueckii subsp. lactis* and *Lactobacillus helveticus*). Nevertheless, both classifications often found (or used) independently of the temperatures which reaches during the cheese manufacture (Beresford et *al.*, 2001; Limsowi et *al.*, 1996; Parente et *al*, 1997). Starters cultures can be found in raw milk for making traditional artisanal cheeses such as Conciato Romano (Mormile et *al.*, 2013). They can be added directly to milk or whey, and their regular usage in cheese production leads to the standardization of final products related to organoleptic properties (Holzapfel et *al.*, 2002a; Hansen, 2002). Starter cultures are classified into natural or artisanal starters, commercial mixed-strain starters (MSS), and defined-strain starters (DSS). Natural starters are from the production of the previous day like

Mozzarella that made from whole raw water buffalo's milk by adding natural whey culture (NWC) for the curd acidification (De Filippis et *al.*, 2014). MSS are derived from the "best" natural starters produced by Dairy Centres or commercial starter companies with undefined composition and a strong control reproduction for reducing variability to prevent phage infection (Stadhouders et *al.*, 1984; Bissonnette et *al.*, 2000). Moreover, natural or artisanal and commercial mixed-strain starters are called traditional starters (MSS) (Limsowtin et *al.*, 1996). DSS are prepared by a limited number of strains (commonly 2-6) and are rotated daily (3-day rotations) to keep their phage-resistance (Limsowtin et *al.*, 1977). Virulent lactic acid bacteriophages are the cause of serious problems during manufactures of Italian soft cheese and yogurt, and industries are constantly looking for a solution against these viruses to keep them under control, in particular against phage infections of *Streptococcus thermophilus* (Chirico et *al.*, 2014).

NSLAB has poor ability to grow in milk and any active function during cheese manufacture period, but it grows well mostly during cheese ripening (Cogan et *al.*, 1997). Typical NSLAB includes NS-lactobacilli, *Pediococcus*, *Leuconostoc* and *Enterococcus*, with the former having the greatest proportion within NSALB population in many cheeses during ripening (Beresford et *al.*, 2001). NSLAB is always present in cheese made with raw milk but not in pasteurised milk. However, contamination does appear post-pasteurisation resulting in the activation of NSLAB (Turner et *al.*, 1983).

*Lactobacillus paracasei subsp. paracasei* is one of the major NSLAB found in most of cheese varieties. They are able to survive pasteurisation or be presence in the cheese, milk or curd as post-contaminants. Additionally, they are resistant to adverse conditions in the interior of the cheese which has an acid pH, a high salt concentration, low fermentable carbohydrate, and anaerobic environment (Fox et *al.*, 1998). Another species of mesophilic NS-lactobacilli is isolated frequently from cheeses such as *Lb. plantarum, Lb. rhamnosus* and *Lb. curvatus*. Another species like pediococci may also encountered in cheese such as, *Pediococcus acidilactici* and *Pe. pentosaceus* (Jordan et *al.*, 1993; Coppola et *al.*, 1997; Fitzsimons et *al.*, 1999; Beresford et *al.*, 2001). *Leuconostoc spp.* is adventitious bacteria which grow during cheese ripening and is different from NSLAB because they are used as part of secondary added cultures (Fox et *al.*, 1998). According to El Soda et *al.* (2000),

NSLAB can be considered as a “good” contaminant on either milk or cheese production due to its contribution to flavor development in some varieties of cheese (El Soda et *al.*, 2000). Propionic acid bacteria (PAB), moulds and yeast are bacteria that contribute in cheese ripening process without being involved during cheese manufacturing like NSLAB. These bacteria along with NSLAB are called secondary microflora (Beresford et *al.*, 2001).

## 1.2. Taxonomy

### 1.2.1. Genus *Lactococcus*

Lactococci are gram-positive cocci with size of 0.5–1.5 μm linked in short chain. They are mesophilic and ferment hexoses homofermentatively producing L (+) lactic acid. There are different species of *Lactococcus (L.)* (previously belong to "Group N Streptococci") namely; *Lactococcus lactis* (distinguished in the *subspecies cremoris, lactis*, and *hordniae*), *L. garvieae*, *L. piscium*, *L. raffinolactis* (Batt, 2000; Schleifer et *al.*, 1985; Doménech et *al.,* 1993; Williams et *al.,* 1990), *L. chungangensis* (Cho et *al.,* 2008), *L. fujiensis* (Cai, 2011), *L. taiwanensis* (Chen et *al.*, 2013), and *L. formosensis* (Chen et *al.*, 2014).

*L. lactis* is mostly isolated from raw or dairy milk products and is originated from the forage. *L. lactis subsp. lactis* and *cremoris,* which are the lactococci traditionally used like starters, are mostly used with *Leuconostoc mesenteroides subsp. cremoris* for fermented milk and cheese production (Stanley, 1998; Courtney, 2000). The subspecies *lactis* is different from the *cremoris* by their salt tolerance (growth at 4% NaCl) and ability to hydrolyze arginine. In addition to these subspecies, there are also other subspecies that has the ability to produce diatecyl variants of *L. lactis subsp. lactis* referred as *biovar. diacetylactis* (Batt, 2000). *L. lactis* strain can be used as single starter which referred to 0-starter, while a single of *L. lactis subsp. lactis biovar. diacetylactis* or *Lc. mesenteroides*, or both, can be added as starters and also capable of producing aroma (Stanley, 1998; Courtney, 2000). Besides its role in the development of subtle aroma and flavor, *L. lactis* also produce a bacteriocin called nisin which contribute to the safety (Deedana et *al.*, 2006). Bacteriocins are substances of protein structure (either proteins or polypeptides) with antimicrobial activities (Zacharof et *al.*, 2012).

*L. garvieae* was originally isolated from *mastitis* (Collins et *al.,* 1983), but it has been found recently in dairy food (Fortina et *al.*, 2003) and in fish species (Venderell, 2006). Fortina et *al*. (2009) have suggested that *L. garvieae* from the dairy food ferments lactose, while no lactose has been fermented from fish species (Fortina et *al.*, 2009). *L. piscium* is another fish pathogen like *L. garviae*, they grow below 15°C and at warm temperatures, respectively (Williams et *al.*, 1990). Another

*Lactococcus* species is *L. raffinolactis,* it was found in raw milk but is not used by the dairy industry due to its lack of caseinolytic activity. Although proteinase-associated plasmid from *L. lactis subsp. cremoris* has been introduced to this species, the caseinolytic activity was not found (Holler et *al.*, 1995; Holt et *al.*, 1994; Schleifer et *al.*, 1985).

*L. chungangensis, L. fujiensis, L. taiwanensis* and *L. formosensis* were isolated from activated sludge foam, vegetable matter, fresh cummingcordia (which is the main ingredient of pobuzihi, a traditional fermented food in Taiwan) and yan-tsai-shin (fermented broccoli stems), respectively (Cho et *al.*, 2008; Cai et *al.*, 2011, Chen et *al.*, 2013; Chen et *al.*, 2014).

*Lactococcus lactis subsp. lactis* and *cremoris* are the only species used in the manufacture of fermented dairy products. Holler and Steele (1995) have investigated the potential use of *L. lactis subsp. hordniae, L. raffinolactis, L. garvieae* and *L. piscium,* as starter cultures for dairy applications. They were assessed for phage resistance, lactose fermentation, production of inhibitory substances, and their growth in milk supplemented with glucose and casein hydrolysate. Although there was no caseinolytic proteinase activity detected, *L. raffinolactis* was found to be the most potential species for use as a starter. According to Fortina et *al.,* (2007), *L. garvieae* can be used for starter strain preparations due to its presence in artisanal Italian chesses and influence on the sensory characteristic of cheeses.

Lactococci are part of the dominant bacterial population in vegetables and forage crops, therefore they have also potential application in vegetable pickling and silage fermentation adding a new possible field of application (Cai et *al.*, 2011).

### 1.2.2. Genus *Lactobacillus*

Lactobacilli are gram-positive rods with maximum dimensions length of 1-10 µm. Some Lactobacilli appear as thin rods, long, sometimes curved; while others are short and stubby (coccobacilli). The short and stubby shape may attribute to the genus *Leuconostoc (Lc.)*, which is a coccus. However, within each species, the length can change with the age of the crop, with the composition of the medium, and oxygen concentration. *Lactobacillus brevis* and *Lactobacillus fermentum* are long and short rods (Kandler et *al.*, 1986). They are microaerophilic and are distinguished in: Homofermentative, Facultative Heterofermentative and Obligatory Heterofermentative (Table 2).

**Table 2.** Mode of sugar fermentation in *Lactobacillus* species

| **Homofermentative** | **Facultative Heterofermentative** | **Obligatory Heterofermentative** |
|---|---|---|
| *Lb. acidophilus* | *Lb. casei* | *Lb. brevis* |
| *Lb. delbrueckii* | *Lb. curvatus* | *Lb. bunchneri* |
| *Lb. helveticus* | *Lb. plantarum* | *Lb. fermentum* |
| *Lb. salivarius* | *Lb. sakei* | *Lb. reuteri* |
| | *Lb. rhamnosus* | *Lb. pontis* |

The genus *Lactobacillus (Lb.)* belongs to the phylum Firmicutes, the class Bacilli, and the order Lactobacillales. They are widely distributed in nature and commonly found in nutrient-rich dairy environments, fresh meat, fish (Chaillou et *al.,* 2005), plants / vegetables (Yang et *al.*, 2010a), soil (Chen et *al.*, 2005), and human mucosal surfaces (Yang et *al.*, 2010b; Ravel et *al.*, 2011). The numerousity of the ecological niches of lactobacilli is reflected the heterogenic phylogeny of the genus. The genus comprises over a hundred different species and many of them are well-characterized such as *Lb. acidophilus, Lb. casei, Lb. delbrueckii subsp. bulgaricus, Lb. plantarum, Lb. rhamnosus, and Lb. salivarius* (Kleerebezem et *al.*, 2009).

*Lb. casei, Lb. plantarum* and *Lb. helveticus* have been recently (2014) isolated from artisanal Travnik young cheeses, sweet creams and sweet kajmaks at four different seasons (Terzic-Vidojevic et. *al*, 2014). *Lb. paracasei*, *Lb. plantarum*,

*Lb. brevis*, *Lb. fermentum* and *Lb. pentosus* were identified in a traditional Turkish Kargi tulum cheese (Kunduhoglu et *al.*, 2014).

Some *Lactobacillus* species were identified from Piedmont hard cheese made of raw milk without thermal treatment and addition of industrial starter. These species, according to the author, are identified as *Lb. rhamnosus, Lb. helveticus, Lb. fermentum, Lb. delbrueckii, Lb. pontis, Lb. gasseri* and *Lb. reuteri* (Bautista-Gallego et *al.*, 2014).

*Lb. salivarius* and *Lb. acidophilus* were often found in gastrointestinal tract especially intestine and feces, respectively (Claesson et *al.*, 2006; Altermann et *al.*, 2005).

*Lb. pobuzihii sp. nov*. (2010) isolated from pobuzihi (fermented cummingcordia) and *Lb. nasuensis sp. nov* (2012) from silage have added two novel species in the genus *Lactobacillus* (Chen et *al.*, 2010; Cai et *al.*, 2012).

Lactobacilli are found in most of fermented food, particularly in dairy product such as yoghurt, cheese, and fermented milk. Many traditional regional fermented product, such as the Korean kimchi and the Caucasian kefir, are fermented with lactic acid bacteria (LAB) using *Lactobacillus* strain (Park, et *al.*, 2001; Heller et *al.*, 2001).

Lactobacilli are also used as starter cultures in vegetable fermentations and in the making of sourdough bread. *Lb. sakei* is currently used in fermented meat product and was found in fresh meat and fish (Chaillou et *al.,* 2005).

Species such as *Lb. plantarum* and *Lb. buchneri* are used in the production of silage, which is a fermented animal feed (Hu et *al.*, 2009). *Lb. plantarum* is able to produce bacteriocins. Zhou et *al.* (2014) have found that *Lb. plantarum* can produce a bacteriocin C8 with molecular weight 16.5 kDa, which is not the typical molecular weight range for most bacteriocins .

Members of the genus *Lactobacillus* keep natural niches and widely used in food and feed manufacturing. In addition, they are also used commercially as health-promoting microbes (i.e., probiotics). Probiotics are used as human dietary supplements or feed for farm animals and pets. Although Bifidobacteria is one of the human probiotics that widely used, animal probiotics have a greater variability. Nevertheless, the use of LAB as probiotics has also been found in different animal species such as cattle, poultry, and pigs (Gaggia et *al.*, 2010).

### 1.2.3. Genus *Streptococcus*

Streptococci are gram-positive spherical or ovoid bacteria (coccoid shape) with >2 µm in diameter. The word "streptococcus" derives from Greek words *strepos* (meaning "easily twisted like a chain") and *kokkos* (meaning "grain or seed") (Billroth, 1874). Their Cell division is along a single axis which makes them grow as long chain (50 or more cocci) especially in liquid medium (Ekstedt et *al.*, 1960). Some coccoid cells can form cross-walls that look like many cells firmly united with each other within the chain. On solid media they often appear as short rod forms. Taxonomically the genus *Streptococcus (S.)* belongs to the phylum Firmicutes and their members are within the lactic acid bacteria group (Ekstedt et *al.*, 1960). The optimum temperature for most streptococci is around 37°C, though some species such as *Streptococcus macedonicus* and *Streptococcus thermophilus* have ability to grow better at higher temperatures such as 45°C (Tsakalidou et *al.*, 1998). Streptococci are facultative anaerobes bacteria and some of them can grow better under anaerobic conditions. Similar to LAB, they need energy from the fermentation of carbohydrates, but are not able to do respiratory metabolism.

To date, there are many species that has been described in literature. However, only few of them were isolated. According to the literature, streptococci may be divided into the following seven groups:

1. Pyogenic group (β-hemolytic species, pathogens);

2. Anginosus group (commensals and occasional opportunistic pathogens);

3. Mitis group (commensals, pathogens);

4. **Salivarius group (commensals and dairy bacteria);**

5. **Bovis group** (gastrenteric bacteria);

6. Mutans group (species phenotypically similar and genetically heterogeneous);

7. Currently ill-defined group (species that are not yet grouped or of uncertain phylogenetic relationships).

The salivarius group includes three species, namely *S. salivarius*, *S. vestibularis* and *S. thermophilus* (Facklam, 2002). The latter speciesis a thermophilic LAB and is frequently added as starter in the manufacture of dairy products. According to Hols et *al.*, (2005), *S. thermophilus* may be regarded as the second most important strain used in cheese production after *Lactococcus lactis.* Fermentation activity of *Streptococcus thermophilus* is indicated by the rapid metabolization of lactose into lactic acid, thus lowering the pH and the production of metabolic substances useful for their technological peculiarities.

Strains belonging to *S. thermophilus* can synthesize exopolysaccharides (EPS) which are responsible for rheological properties, particularly viscous texture of fermented milk products like yogurt (Broadbent et *al.*, 2003). EPS' mechanism of action is not very known and Mende et *al.,* (2014) have recently characterized the different purified EPS fractions (free EPS, capsular derived EPS and the entire total EPS) from *S. thermophilus* ST-143 to obtain information on their physicochemical properties in solution and to understand their role in fermented dairy products (Mende et *al.*, 2014).

*S. thermophilus* strains are also bacteriocin-producing bacteria and their bacteriocins are active against *Pediococcus acidilactici, Clostridium tyrobutyricum*, *Clostridium sporogenes*, *Clostridium botulinum, Bacillus cereus* and *Listeria monocytogenes*. Some of them could be added like starters only if they are not harmful to other thermophilic LAB (Aktypis et *al.*, 1998; Gilbreth et *al.*, 2005; Marciset et *al.*, 1997; Mathot et *al.*, 2003).

*S. thermophilus* strains are also probiotics and have a positive effect on diarrhea in children, enterocolitis in premature neonates and inflammatory gut disease (Bibiloni et *al.*, 2005; Bin-Nun et *al.*, 2005; Shamir et *al.*, 2005).

*S. thermophilus* was classified as *Streptococcus salivarius subsp. thermophilus* for many years (Farrow et *al.*, 1984; Sherman, 1937). However, a research on its right taxonomic status has been carried out by Schleifer et *al.* (1991) based on DNA–DNA reassociation. Pulsed Field Gel Electrophoresis (PFGE) and RAPD-PCR typing methods were used to prove a high degree of variability within the *S. thermophilus* species (Colmin et *al.*, 1991; Giraffa et *al.*, 2001; Mora et *al.*,

2002; Moschetti et *al.*, 1998), while slight variability within this species was observed in the following studies by comparing the lacSZ operon sequences of 29 *S. thermophilus* strains counteracting the first results (Ercolini et *al.*, 2005).

*S. thermophilus* is among species of the same group that originate from dairy product. It is a "Generally Recognized As Safe" (GRAS) species; instead *S. salivarius* and *S. vestibularis* are associated with infections in humans like meningitis, endocarditis and bacteremia (Doyuk et *al.*, 2002; Idigoras et *al.*, 2001; Ruoff et *al.*, 1989). The salivarius group is very similar to the group bovis, two species (*Streptococcus infantarius* and *S. alactolyticus*) from the salivarius group, were reclassified taxonomically and included in the group bovis (Facklam, 2002). *S. salivarius* is finding application in the field of probiotics, its ability to compete with the oral flora seems attributed to the production of of bacteriocin-like inhibitory substances (BLIS) (Wescombe et *al.*, 2006).

Apart from *S. thermophilus*, other streptococci were found in milk and dairy products mainly belong to the bovis group which designated as S*treptococcus bovis/Streptococcus equinus* complex (SBSEC) (Jans et *al.*, 2012). There is no clear reason behind their presence in milk; they are naturally in gastrointestinal tract, teat skin in animals lactiferous and maybe they could contaminate the milk for passive transmission. Furthermore, some species of the bovis group caused cases of endocarditis, meningitis, bacteremia and colon cancer in humans (Galdy et *al.*, 2012; Abdulamir et *al.*, 2011). The isolates from human infections had previously been divided into S. bovis biotypes I, II/1, and II/2. The biotype I isolates were reclassified as *Streptococcus gallolyticus subsp. gallolyticus* (Schlegel et *al.*, 2003), the biotype II/1 as *S. infantarius* (Schlegel et *al.*, 2000) and *Streptococcus lutetiensis* (Poyart et *al.*, 2002), and the biotype II/2 as *Streptococcus gallolyticus subsp. pasteurianus* (Schlegel et *al.*, 2003).

*S. macedonicus*, isolated the first time from naturally fermented Greek Kasseri cheese (Tsakalidou et *al.*, 1998), appeared phylogenetically related to *Streptococcus gallolyticus subsp. gallolyticus and subsp. pasteurianus*, so they named *S. gallolyticus subsp. macedonicus. Streptococcus gallolyticus subsp. gallolyticus* and *subsp. pasteurianus* are considered pathogenic (Danne et *al.*, 2011).

In addition, *Streptococcus infantarius subsp. infantarius* has been isolated recently (2013) and has been the predominant flora in East African camel milk spontaneously fermented (suusac) (Jans et *al.*, 2013); *Streptococcus lutetiensis* was dominant bacteria found in a Turkish cheese “armola” (Hande et *al.*, 2012).

Some strains of the SBSEC complex are certainly pathogens and for this reason Papadimitriou et *al.*, (2014) did the first complete genome sequence of *S. macedonicus* and they investigated its potential pathogenicity comparing the genome sequence of the dairy isolate (*S. macedonicus* ACA-DC 198) to the other SBSEC genomes. This step is important to get more information about the safety of this specie and whether it can be used as a starter or some technological measures are necessary to prevent its presence in cheese.

### 1.2.4. Genus *Enterococcus*

Enterococci are gram-positive cocci, mesophilic and ferment hexoses homofermentatively producing L (+) lactic acid. The genus *Enterococcus (E)* belongs to the phylum Firmicutes, but commonly regarded as members of LAB. They are ubiquitous bacteria, but are associated mostly with habitat in the gastrointestinal tract of humans and animals (Devriese et *al.*, 1995). Enterococci are also found in different fermented food like dairy products as contamination from the environment but are not of great value as hygiene indicators colonies in food industry (Birollo et *al.*, 2001). Their ability to be in different ecological niches depends on their resistancy towards acidity, salt, drying, heat, and chemical sanitizing agents (Holzapfel et *al.*, 2002b). The genus comprises of different species and among those most frequently found in cheese are: *Enterococcus durans*, *Enterococcus faecium* and *Enterococcus faecalis* (Terzic-Vidojevic et *al.*, 2014).

Enterococci are particularly active in Biogenic Amines production. Biogenic amines (BA) are natural anti-nutrition factors occurring in foods such as histamine, putrescine, cadaverine, tyramine, tryptamine, p-phenylethylamine, spermine, and spermidine. They can be an agent in a number of food poisoning phenomenon, particularly histamine toxicity 'scombroid poisoning' and tyramine toxicity 'cheese reaction' (Shalaby, 1995; 1996). Nevertheless, their presence can induce different reactions for susceptible consumers, such as nausea, respiratory distress, hot flushes, sweating, heart palpitation, headache, bright red rash, oral burning, hyper- or hypotension (Ladero et *al.*, 2010a). Kučerová et *al.* (2009) proved that all *Enterococcus* strains isolated from samples of raw milk, fresh cheeses and semi-hard cheeses were mostly tyramine producers (tyrosin decarboxylase activity) and to some extent histamine producers (histidine decarboxylase activity). *Enterococcus* with tyramine production were isolated in dutch semihard cheese especially those belong to genus *E. durans, E. faecalis* and *E. casseliflavus* (Komprda et *al.*, 2008).

*Enterococcus* could have probiotic action and in this case, their safety must be taken into consideration. Virulence determinants need to know for each strain (hemolysin, gelatinase, Esp protein, etc.) and low incidence of virulence factors were observed in isolated colonies from food (Franz et *al.,* 2001). Furthermore, the German Chemical Industry bacteria have published a classification of

microorganisms according to the risk of animal and human health (Berufsgenossenschaft der Chemischen Industrie, 1995). The first group in the classification included many LAB and all those microorganisms that do not cause risk to human and animal health. The second group included bacteria that are potentially pathogenic, which pathogenicity depends mainly on the health of the host. Most of the bacteria of the genus *Enterococcus* belong to the second group except *Enterococcus faecium* and *E. durans* which are not risky organisms (Group 1) (Berufsgenossenschaft der Chemischen Industrie, 1995).

Enterococci are also producers of bacteriocins, particularly enterocins, which can potentially provide useful benefits (Gálvez et *al.*, 1989; Lauková et *al.*, 1993; Aymerich et *al.*, 1996; Casaus et *al.*, 1997; Cintas et *al.*, 1997, 1998, 2000; Floriano et *al.*, 1998; Herranz et *al.*, 2001; Foulquié-Moreno et *al.*, 2003; Mareková et *al.*, 2007; Maldonado-Barragán et *al.*, 2009; Khan et *al.*, 2010). Probiotic and Bacteriocin-Producing Enterococci have some potential benefits in the dairy products. They can be used as nonstarter microbiota in milk, whey/cheese starter cultures or cheeses which influences the organoleptic characteristics of the final product (Giraffa et *al.*, 2002).

### 1.3. Pulse Field Gel Electrophoresis (PFGE), DNA sequencing and Polymerase Chain Reaction (PCR)

Microbial diversity can be studied using traditional microbiological method and also by the application of molecular biological techniques and increasing knowledge of gene sequences.

Fingerprinting is one of molecular biological techniques. It analyses protein or nucleic acid content providing a pattern of microbial communities or individual strains. Genetic fingerprinting techniques based on analysis of DNA or RNA can be divided into direct methods, where nucleic acids are extracted and analysed or indirect methods which require amplification of DNA / RNA.

PFGE is a direct fingerprinting method. It was described as the "gold standard" of fingerprinting techniques for its high discriminatory power and reproducibility (Turabelidze et *al.*, 2000). PFGE is based on using of restriction enzymes which cut DNA in large fragments up to 12 Mb. DNA fragments of discrete size are generated due to the prevention of random breaks caused by shearing during DNA extraction procedures. PFGE starts with bacterial cell trapped in small agarose plugs and then cell lysis is performed and contaminating proteins are digested. This steps are followed by the addition of a restriction endonuclease, which penetrates the agarose network and digestion occurs in *situ.* The bacterial genome (approximately 2,000 to 5,000 kb pairs) has specific recognition sites where is digested with a restriction enzyme resulting restriction fragments from 10 to 800 kb in size. Restriction enzymes used in PFGE are able to recognise 8-base sequences which are estimated to occur randomly within DNA intervals of 65 kb or restriction enzymes of extreme GC content. For example, Sma I can be used with chromosomal DNA of low GC content and be expected to cut the DNA into relatively large fragments and it recognises the sequence GGGCCC (Tanskanen et *al.*, 1990). Cleaved DNA fragments are run and electrophoresis patterns are visualised after staining whit a fluorescent dye. Classical gel electrophoresis uses a continuous unidirectional current and separates molecules by size in a sieving manner in an agarose gel matrix. This method determinates the separation of molecules below 50 kb and all molecules below this size migrate with same mobility, demonstrated by a large band on gel. PFGE electrophoresis separates DNA fragments in the 50kb - 12Mb range. PFGE

uses alternating electric currents which force DNA molecules due to continuously change their direction of migration. The smaller molecules are able to separate and orientate faster than lager molecules when there is change of electric current direction. Therefore, smaller molecules spend a longer proportion of the pulse interval (the time the electric current is applied in one direction) migrating in the gel. The pulse intervals increased gradually over the run time of the gel, which ensures that each DNA fragment size is subjected to optimal separation conditions.

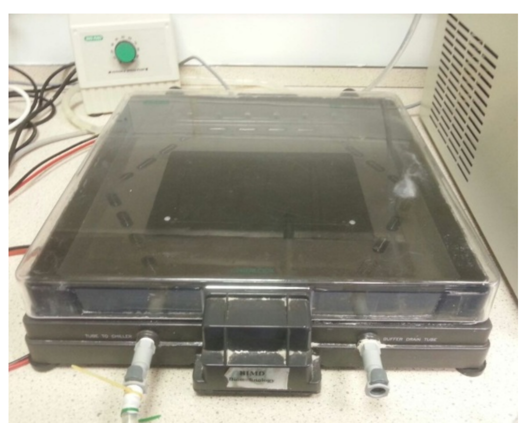


**Fig. 2.** Contoured-clamp Homogeneous Electric Field (CHEF) system

Separation and resolution of DNA molecules was influenced by many factors: occurs size of DNA molecules, agarose type and concentration, electric field strength, field angle and shape, ionic strength, , pulse time and temperature (Farber, 1996). A number of variations to the initial description of PFGE by Schwartz and Cantor (1984) are available. These variations are all based on changing the direction of migration of the DNA fragments. The primary difference between the method is the position of the electrodes. The contoured-clamp homogeneous electric field (CHEF) show in Figure 2 is the most popular pulse field system. The 24 electrodes are arranged in a hexagonal array, which allows the electric field to switch through a fixed orientation of 120° between pulses. The electrodes are clamped to a particular voltage, which provides a uniform electrical field. The homogenous electric field result in straight lines and good resolution of large DNA fragments (>5Mb), which allows comparison of different samples. Analysis of gel images can be carried out using one of commercially available software packages for example Applied Maths, Bio-Rad, or BioSystems.

Using these system extensive database of strains can be generated. A system for standardising the interpretation of PFGE patterns for studies with limited genetic variation was proposed by Tenover et *al.,* (1995). This system considered bacterial isolated to be same strains if PFGE patterns were identical. A difference of one to three bands indicated closely related bacterial isolates differing by one genetic event. More distantly related isolates different by four to six or more bands were considered unrelated. Band differences between strains may be due to DNA insertions or deletions into the chromosome or random mutation, which cause the appearance or disappearance of restriction enzyme sites. Plasmid or bacteriophage DNA may also cause the presence or absence of additional bands (Towner et *al.*, 1993).

PFGE can be used for many different applications such as for species and strain identification, the number and size of chromosomes, construction of physical and genetic maps, investigation *in vivo* of chromosome breakage and degradation, identification of restriction fragment lenght polymorphisms, and cloning and characterisation of genes (Bouton et *al.,* 2002; Brennan et *al.,* 2002; Belli et *al.*, 2001; Suzuki et *al.*, 1999; Johnston et *al.*, 2000). PFGE is a useful tool for the study of species and strains as it has been reported in many study of cheese characterization. This fingerprinting method has been used to identify enterococci from Cheddar cheese (Gelsomino et *al.*, 2001) and selected lactobacilli starter during ripening of Comté cheese (Bouton et *al.*, 2002). It was applied to characterize nisin-producing bacteria during traditional Fior di latte cheese-making (Moschetti et *al.*, 2001) and to investigate the bacterial flora surface of smear ripened cheeses (Brennan et *al.*, 2002). PFGE showed also to be used like a predicting method for biochemical proprieties according to O'Sullivan et *al.*, (1998) who observed that PFGE using 16 *S. thermophilus* strains, isolated from different dairy products, generated restriction patterns enabled the clustering of strains into two groups corresponding to biochemical aspects previously examined (O'Sullivan et *al.*, 1998).

PFGE is reproducible, easy to interpret, and highly discriminative; on the other hand, is difficult to apply in industry because they are cumbersome, difficult to use, and expensive (Olive et *al.*, 1999). The time required to complete the procedure can limit the number of isolates capable of being processed by PFGE. Some technical inconvenient problems such as inadequate cell lysis or incomplete protenase digestion can happen (Farber, 1996). Some bacteria such as *Clostridium*

*difficile* are nontypable by PFGE, due to the degradation of chromosomal DNA presumably by endogeneous nucleases prior to the action of restriction enzymes (Kato et *al*., 1994). PFGE of small and very similar fragments may generate poor resolution profiles.

DNA sequencing is considered the gold standard for microbial identification and the introduction of automated DNA sequencing machines and the development of bioinformatics have allowed laboratories to increase their output of DNA sequences from a few thousand base pairs per week to millions of base pairs per week. Phylogenetic relationships between organism can be established through the analysis of molecular chronometers, molecules showing good clocklike behaviour. Sequences within these molecule change randomly over time, reflecting evolutionary changes. Molecules representing highly constrained functions are the most useful for phylogenetic studies as they span the full evolutionary spectrum (Woese, 1987). Ribosomal RNA (rRNA) is an essential component of all organism as it is involved in protein synthesis. They are ideal chronometers due to inherent characteristics such as size, abundance within cells, universality, and their varying level of conservation and relative ease of sequencing. Different positions in their sequence evolve at different rates, therefore sequence variations occur between organism. Highly conserved sequence regions exit among the most distant organism. Closely related organism have a high level of homology, whereas distantly related organism have a low level of homology (Woese, 1987). 5S, 16S, 23S rRNA and their spacer regions are the sequences mostly detected in phylogenetic investigation. Among them, 16S r RNA is often chosen for sequencing in characterization studies of microbial population. It is actually known that 16S rRNA sequencing needs to be supported from other tests because some discordant results were found from Fox et *al*., (1992). They observed that strains showed 99.5% of similarity in sequence where not identical by DNA-DNA hybridisation results. Nevertheless, 16 rRNA sequence allow to know which species a strain probably belongs to once relevant species compared with 16S rRNA sequence database.

The advent of the polymerase chain reaction (PCR) developed by Mullis and Faloona, (1992) marked a major milestone towards the rapid identification of bacterial isolates. Today PCR is a popular molecular tool used to amplify DNA segments. Which can be used to identify individual species. Two oligonucleotide

primers are synthesised, one of which is complementary to the 3' end of the forward strand of DNA, and the other complementary to the 3' end of the reverse strand of DNA. A PCR requires a mix of high-temperature resistant DNA polymerase, primers a DNA template, free nucleotides and buffer. Through cycling the temperature of the PCR mix, DNA is polymerized and a complementary copy of the template is made. Typically PCR consists of repetitive cycle, whit each cycle consisting of three steps:

- Step 1 : double stranded DNA is denatured into single strands.
- Step 2: primers anneal to single strand DNA sequences.
- Step 3: DNA synthesis along each strand of template DNA in an extension reaction.

At each of the above steps temperature is critical. Through repeating PCR cycles, a nearly exponential increase in the quantity of desired DNA is obtained, whit the quantity of DNA doubling whit each cycle. Various types of PCR have been developed for various applications. In specific PCR (Predictable Amplicon Size Specific PCR), the intermediate area of know size between the binding sites of a pair of forward and reverse primer is amplified during PCR. Through the choice of specific primers, regions with different levels of phylogenetic relatedness can be amplified. Members of different genera can be clearly differentiated through amplified gene sequences. Conserved regions unique to specific genera area first identified after which genus specific primers are designed to. Produce PCR fragments Deasy et *al.*, (2000) developed such a PCR based method to distinguish between *Lactococcus* and *Enterococcus*, two genera commonly found in cheese products. Species-specific primers have also been developed to distinguish between the *Lactobacillus* species*; Lb. zeae, Lb. paracasei,* and *Lb. rhamnosus* common to Comté cheese (Berthier et *al*., 2001).

### 1.4. Bacteriocins

Bacteriocins are represented from a heterogeneous group peptides or proteins synthesized at ribosome's level and which have antimicrobial activity against other microorganism (Klaenhammer, 1993). Although bacteriocins possess an antimicrobial activity, they are not antibiotics. Bacteriocins act to restrict their activity to strains of species related to the producing species and particularly to strains of the same species. It has been known that antibiotics have a wider activity spectrum and do not show any preferential effect on closely related strains (Zacharof et *al.*, 2012).

Research related to LAB-bacteriocins, especially for their action as biopreservatives in food to inihibit the growth of spoilage organisms and food-borne pathogens like *Listeria monocytogenes,* has existed for the past few decades (Tagg et *al.*, 1976). Although LAB are not the only species that can produce bacteriocins, the use of it on industries has been growing (Nettles et *al.*, 1993) due its safety recognition by USFDA (United States Food and Drug Administration) (Dimov et *al.*, 2005).

**Table 3.** Classification of bacteriocins from lactic acid bacteria

| **Main** | **Category and Subcategory** |
|---|---|
| Class I - lantibiotics | Type A: elongated molecole<br>Subtype A1: leader peptides are cleaved by a dedicated serin proteinase<br>Subtype A2: leader peptides are cleaved by a dedicated ABC-transporter<br>Type B: globular molecole |
| Class II - nonmodified, heat-stable bacteriocins | Class IIa: pediocin-like bacteriocins<br>Class IIb: two-peptide bacteriocins<br>Class IIc: sec-dependent bacteriocins<br>Class IId: other bacteriocins |
| Class III - large, heat-labile bacteriocins | |

(Sources: de Vos et *al.*, 1995; Nes et *al.*, 1996, Moll et *al.*, 1999b)

LAB bacteriocins are classified into three different classes based on its characteristics (Nes et *al.*, 1996; Moll et *al.*, 1999a). First is lantibiotics (type A and B) (Class I) with characteristic small in size, heat-stable polycyclic peptides containing unusual and post-translationally modified amino acids; second is non-lantibiotics (Class II) with characteristic small in size (<10 kDa), heat-stable, and are

further subdivided into four subgroups: IIa (bacteriocins with a strong anti-*Listeria* activity called pediocin-like), IIb (two-peptide bacteriocins), IIc (sec-dependent bacteriocins) and IId (Class II bacteriocins not included in the previous groups). Third is LAB bacteriocins included as (Class III) with characteristic large in size and (>30 kDa) heat-labile (Table 3 and 4).

**Table 4.** Overview of biochemically and genetically characterized bacteriocins

| Bacteriocin | Producer Organism | Reference(s) |
|---|---|---|
| **Class I (lantibiotics)** | | |
| Nisin A | *L. lactis* | de Vuyst et *al.*, 1994 |
| Nisin Z | *L. lactis* | de Vuyst et *al.*, 1994b |
| Lactococcin DR (*) | *L. lactis* ADRIA 85L030 | Dufour et *al.*, 1991 |
| Lacticin 481 (*) | *L. lactis* CNRZ481 | Piard et *al.*, 1992 |
| Lacticin 3147 (LtnA1 and LtnA2) | *L. lactis* DPC3147 | Dougherty et *al.*, 1998 |
| Cytolysin (CylL1 and CylL2) | *E. faecalis* | Gilmore et *al.*, 1994 |
| Lactocin S | *Lb. sakei* L45 | Mortvedt et *al.*, 1991 |
| **Class II** | | |
| **Class IIa (pediocin-like bacteriocins)** | | |
| Acidocin A | *Lb. acidophilus* TK9201 | Kanatani et *al.*, 1995 |
| Curvacin A (‡) | *Lb. curvatus* LTH1174 | Tichaczek et *al.*, 1992 |
| Sakacin A (‡) | *Lb. sakei* LB706 | Holck et *al.*, 1992 |
| Sakacin P (§) | *Lb. sakei* LTH673 | Tichaczek et *al.*, 1992 |
| Sakacin 674 (§) | *Lb. sakei* LB674 | Holck et *al.*, 1994 |
| Enterocin A | *E. faecium* CTC492 | Aymerich et *al.*, 1996 |
| **Class IIb (two-peptide bacteriocins)** | | |
| Lactococcin M (LcnM and LcnN) | *L. cremoris* 9B4 | Van Belkum et *al.*, 1991a |
| Lactococcin G (LcnGa and LcnGb) | *L. lactis* LMG2081 | Nissen-Meyer et *al.*, 1992 |
| Acidocin J1132 (a,b) | *Lb. acidophilus* JCM1132 | Tahara et *al.*, 1996 |
| Plantaricin S (Plsa and Plsb) | *Lb. plantarum* LCPO10 | Jimenez-Diaz et *al.*, 1995 |
| Plantaricins EF (PlnE and PlnF) | *Lb. plantarum* C11 | Diep et *al.*, 1996 |
| Plantaricins JK (PlnJ and PlnK) | *Lb. plantarum* C11 | Diep et *al.*, 1996 |
| Leucocin H (a and b) | *Leuconostoc sp.* MF215B | Blom et *al.*, 1999 |
| Termophilin 13 (ThmA/ThmB) | *S. thermophilus* SPi13 | Marciset et *al.*, 1997 |
| **Class IIc (sec-dependent bacteriocins)** | | |
| Acidocin B | *Lb. acidophilus* M46 | Leer et *al.*, 1995 |
| Bacteriocin 31a | *E. faecalis* YI17 | Tomita et *al.*, 1996 |
| Enterocin Pa | *E. faecium* P13 | Cintas et *al.*, 1997 |
| Lactococcin 972 | *L. lactis* IPLA972 | Martinez et *al.*, 1999 |
| **Class IId** | | |
| Lactococcins A and B | *L. cremoris* 9B4 | Van Belkum et *al.,* 1991a,b |
| | *L. lactis* WM4 | Stoddard et *al.*, 1992 |
| | *L. cremoris* LMG2130 | Holo et *al.*, 1991 |
| Diacetin B | *L. lactis subsp. diacetylactis* UL720 | Ali et *al.*, 1995 |
| Acidocin 8912 | *Lb. acidophilus* TK8192 | Kanatani et *al.*, 1995 |
| Peptide A | *Lb. acidophilus* LF221 | Bogovic-Matijasic et *al.*, 1998 |
| Peptide B | *Lb. acidophilus* LF221 | Bogovic-Matijasic et *al.*, 1998 |
| Lactocin 705 | *Lb. casei* CRL 705 | Palacios et *al.*, 1999 |
| Gassericin B3 | *Lb. gasseri* HCM2124 | Tahara et *al.,* 1997 |
| Plantaricin 1.25a | *Lb. plantarum* TMW1.25 | Ehrmann et *al.*, 2000, Remiger et *al.*, 1999 |
| Plantaricin 1.25b | *Lb. plantarum* TMW1.25 | Ehrmann et *al.*, 2000, Remiger et *al.*, 1999 |
| Enterocin B | *E. faecium* T136 | Casaus et *al.,* 1997 |
| Enterocins L50 (EntL50A and EntL50B)b | *E. faecium* L50 | Cintas et *al.*, 1998b |
| Enterocin Q | *E. faecium* L50 | Cintas et *al.*, 2000 |
| **Class III** | | |
| Helveticin J | *Lb. helveticus* 481 | Joerger et *al.*, 1986 |
| Caseicin 80 | *Lb. casei* B80 | Rammelsberg et *al.*, 1990 |
| Enterolysin A | *E. faecalis* LMG2333 | Nilsen et *al.*, 2003 |

The interaction between bacteriocin and cell is done by recognizing general or specific receptors on the plasma membrane of sensitive cells that allows a link and pore formation (Venema et *al.*, 1995; Fimland et *al.*, 2005; Hechard et *al.*, 2002).

Some bacteriocins may have a bactericidal effect, their action is influenced by several factors such as, bacteriocin quantity and degree of purification, physiological status of the indicator cells (e.g. growth phase), and experimental parameters (e.g. temperature, pH, antimicrobial agents or presence of compounds modifying cell wall integrity (Cintas et *al.*, 2001). Some bacteriocins have shown to act through a bacteriostatic activity against sensitive microorganisms, e.g. lactocin 27 (Upreti et *al.*, 1975), leucocin A-UAL187 (Hastings et *al.*, 1991) and leucocin S (Lewus et *al.*, 1992). Bacteriocins exerted a bacteriocidal action that may cause or may not cause the lysis of sensitive cells. Bacteriocins that cause lysis is called bacteriolytic bacteriocins, such as nisin A (Bierbaum et *al.*, 1991), plantaricin C (González et *al.*, 1994) and enterocin EFS2 (Maisnier-Patin, 1996). On the other hand, bacteriocins that does not cause lysis is called lactococcin A (Van Belkum et *al.*, 1991b), lactacin B (Barefoot et *al.*, 1983), lactostrepcins (Kozak et *al.*, 1978), lacticin 481 (Piard et *al.*, 1990), helveticin J (Joerger et *al.*, 1986), sakacin P (Schillinger et *al.*, 1989), plantaricin S (Jiménez-Díaz et *al.*, 1993), acidocin B (Ten Brink et *al.*, 1994), enterocin 226NWC (Villani et *al.*, 1993), enterocin A (Aymerich, 1996) and enterocin 900 (which is identical to enterocin B) (Franz et *al.*, 1996, 1999).

The antimicrobial spectrum of bacteriocins is very wide and heterogenic, and for this reason, it is distinguished into three different main bacteriocins groups: with narrow, intermediate and broad antimicrobial spectrum bacteriocins. Bacteriocins of the first group (narrow action) act in a limited way and namely against microorganisms that belong to the same species that produces such as caseicin 80 (Rammelsberg et *al.*, 1990), lactococcin A (Holo et *al.*, 1991) and / or to species within the same genera, such as lactacin B (Barefoot et *al.*, 1983) and lactocin 27 (Upreti, 1994). Bacteriocins of the second group (intermediate action) are able to inhibit other LAB and a large number of Gram positive among which are some food-borne pathogens such as, *Listeria monocytogenes*, *Staphilococcus aureus*, *Clostridium perfringens* and *Clostridium botulinum*. Bacteriocins belong to this group are lactacin F (Muriana et *al.*, 1987), lacticin 481 (Piard et *al.*, 1990), plantaricins S and T (Jiménez-Díaz et *al.*, 1993) and plantaricin C (González et *al.*, 1994). Bacteriocins of the third group (broad action) have a spectrum of action wider than the first or second groups well as other bacteria mentioned above plus *Propionibacterium spp.* and *Bacillus spp.* Examples of bacteriocins belonging to the

third one are nisin A and Z, which also inhibit spore outgrowth of *Clostridium spp*. and *Bacillus spp.* (Hurst, 1981; Harris et *al.*, 1992; Vandenbergh, 1993; Cintas, 1995; Cintas et *al.*, 1998a), pediocin AcH/PA-1 (Bhunia et *al.,*1987, 1988; Henderson et *al.*, 1992), leucocin S (Lewus et *al.*, 1991) and enterocins L50 (Cintas et *al.*, 1995; 1998a).

Several observations were made on the different mode of antimicrobial action of low-molecular-weight bacteriocins (Jack et *al.*, 1995). It was found that some of the strains belonging to the same species may be sensitive and others may be resistant to a bacteriocin. Additionally, some of the strains belonging to the same population may be sensitive and others may be resistant to the same bacteriocin. It was also found that a strain can be sensitive to a particular bacteriocin and resistant to another very similar to the previous one or a bacteriocin-producing strain may be sensitive to another bacteriocin or cells of a sporogenous strain can be always sensitive to a bacteriocin, while its spores are resistant that become sensitive with germination. Moreover, LAB-bacteriocins are not active against Gram-negative in a normal condition, but they can have an antimicrobial action after purification with a presence of chemical compounds (e.g. chelating agents, organic acids) or in the certain stress conditions (pH, temperature, high hydrostatic pressure) (Kalchayanand et *al.*, 1992, 1998; Stevens et *al.*, 1991).

Bacteriocin LAB producing are equipped with a self-defense mechanism (immunity) towards their own bacteriocins (Tagg et *al.*, 1976; Klaenhammer, 1993; Jack et *al.*, 1995). The immunity exists due to a protein which is expressed simultaneously with the bacteriocin, hence it does not need a system to be processed and transported outside the cell (Nes et *al.*, 1996). The presence of the immunity is also facilitated by intracellular proteases which can block bacteriocin action inside the cell producer (Abee, 1995; Jack et *al.*, 1995; Allison et *al.*, 1996).

The great part of LAB-bacteriocins are synthesized like inactive precursor peptides, namely prebacteriocins or prepropeptides, from ribosomes. Prebacteriocins are made of a C-terminal propeptidic domain, called probacteriocin, and an N-terminal extension (Jack et *al.*, 1995; Nes et *al.*, 1996). The N-terminal extension is outsourced and binds to specific sites of transformation where changes into its active peptide called mature bacteriocin (Schnell et *al.*, 1988; Holo et *al.*, 1991; Freund et

*al.*, 1992; Van Belkum et *al.*, 1997). The N-terminal extensions have different functions, such as recognition and processing of the preprobacteriocins, transport of the probacteriocin and its defends interior of the cell through inactivating bacteriocin (Jack et *al.*, 1995; Nes et *al.*, 1996).

LAB bacteriocin (Class I) that has been studied comprehensively is nisin. It is produced by certain strains of *Lactococcus lactis* that were isolated from different food products like milk, vegetable-based products (Hurst, 1981; Harris et *al.*, 1992) and Spanish-dry fermented sausages (Cintas, 1995; Rodríguez et *al.*, 1995; Cintas et *al.*, 1998a). Nisin has a bactericidal activity with a broad spectrum of action against a wide range of Gram-positive bacteria, most prominent are *Staphylococcus aureus* and *Listeria monocytogenes* (Hurst, 1981; Cintas et *al.*, 1998a). It also prevents spore outgrowth and inhibits vegetative cells of *Bacillus spp*. and *Clostridium spp.* (Hurst, 1981; Abee et *al.*, 1995). The mechanism of action of nisin has been known in detail. The action of nisin cause cell membrane pores formation which destroys the proton motive force, the pH balance and eventually after causing leakage of ions destroys hydrolysis of ATP leading to cell death (Benz et *al.*, 1991; Sahl et *al.*, 1987). Its long history of safe compound, food-grade status, and proven efficacy make it one of only a few commercially applied bacteriocins. Nisin is widely used as a food-preservative (The EFSA Journal, 2005) in certain processed cheeses, dairy products and canned foods (Delves-Broughton et *al.*, 1996). Even pediocin-like bacteriocins are allowed to be used in food products when they are produced by GRAS microorganisms that normally added for the manufacture of cultures in milk or whey that have the status of food ingredient (Deegan et *al.*, 2006).

The use of bacteria LAB recognized as GRAS and their bacteriocins in preserving foods is becoming more and more attractive (Ghrairi *et al.* 2012). However, but many other LAB bacteriocins producing are lacking GRAS status, thus are broadly explored for hypothetical application in food preservation. The genus *Enterococcus* belongs to LAB (no GRAS recognized) and many of its species represent non-pathogenic flora found in many dairy products (Morandi *et al.* 2006). The first observation on the bacteriocinogenic property of enterococchi was done by Kjems in 1955. Afterwards, a great number of scientists have discovered, described as well as characterized a large number of bacteriocins produced by enterococci (often named enterocins) (Franz et *al.,* 2007). The enterocins have been

distinguished, like other bacteriocins, into three main classes. Among the three main classes Enterocins belonging to classes II and III, especially enterocin AS-48, are the subject of an increasing interest and potential use in food products because they are able not only to inhibit the growth of related species but also Gram-positive food spoilage and food borne bacteria (Cotter et *al.,* 2005). Recently, various strains that have the ability to inhibit the growh of Gram-negative bacteria has been found (Line et *al.,* 2008; Svetoch et *al.,* 2011; Messaoudi et *al.,* 2013). Several species of enterococci are quite unique in producing a large number of antimicrobial substances and often a same strain can produce various peptide fractions. Some of them are still unknown and atypical like enterocin Q, enterocins L50A and L50B (Cintas et *al.,* 1997; 1998), which not fall under the classification known, have led to a revision of the class II bacteriocins (Franz et *al.,* 2007).

Bacteriocin genes are generally linked and may be located on plasmids (which may be conjugative or not) or in the bacterial chromosome (often integrating a composed transposon) (Jack et *al.*, 1995). The lantibiotics nisin A and Z (Horn et *al.*, 1991; De Vos et *al.*, 1993) and enterocin A (Aymerich et *al.*, 1996) are chromosomally-encoded.

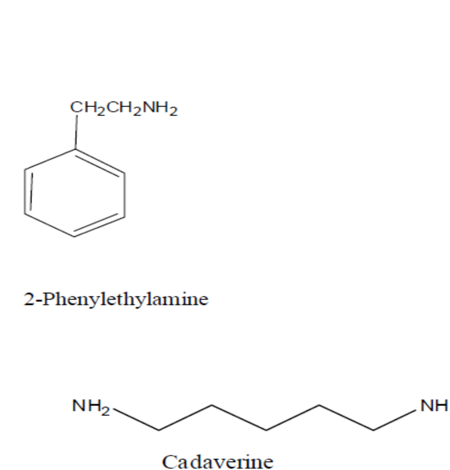
## 1.5. Biogenic Amines

Biogenic amines (BA) are organic, nitrogenous, low molecular weight compounds endowed with biological activity, mainly formed by the decarboxylation of amino acids (Valsamaki et *al.*, 2000; Shalaby, 1996).

They are involved in several physiological processes such as cellular growth, synaptic transmission, blood pressure and allergic response. However, if their amounts reached critical threshold, they could be very dangerous for human health (Russo et *al.*, 2010).

Exogenous BA are present in a wide range of non-fermented and fermented foods. Excessive consumption and the absorption of food containing high levels of exogenous BA can cause some adverse reactions such as nausea, headaches, changes in blood pressure and rashes (Shalaby, 1996; Premont et *al.*, 2001). The efficiency of detoxification varies considerably from individual to individual and can be conditioned or inhibited by several factors such as genetic constitution and / or their hiring medicines (Spano et *al.*, 2010).

**Fig. 3.** Structural chemical formula of Biogenic Amines



The most important BA, both qualitatively and quantitatively, encountered in foods and beverages are histamine, tyramine, putrescine, cadaverine, and β-phenyl ethylamine, deriving from the decarboxylation of histidine, tyrosine, ornithine, lysine, and β-phenylalanine, respectively. Amines can be distinguished according to the chemical composition in aliphatic (putrescine, cadaverine, spermine, and spermidine), aromatic (tyramine, phenylethylamine) or heterocyclic (histamine, tryptamine) compounds; and according to the number of amino groups in monoamines (tyramine, phenylethylamine), diamines (putrescine, cadaverine), or polyamines (spermine and spermidine) (Linares et *al.*, 2011; Bardócz et *al.*, 1999).

Lactic acid bacteria are able to increase their acid resistance or produce metabolic energy by using catabolic pathways that convert amino acids into compounds containing amino groups, designated biogenic amines (Molenaar et *al.*, 1993; Fernández et *al.*, 2006a).

**Table 5.** BA-producing microorganisms from dairy products

| **Biogenic Amines** | **BA - producing LAB** | **References** |
|---|---|---|
| Histamine | *Lb. buchneri, Lb. curvatus*<br>*Lb. helveticus, Lb. parabuchneri,*<br>*S. thermophilus* | Martín et *al.,* 2005<br>Burdychova et *al.*, 2007<br>Calles-Enríquez et *al.,* 2010 |
| Tyramine | *E. casseliflavus, E. durans,*<br>*E. faecalis, E. faecium,*<br>*E. hirae, Lb. brevis,*<br>*Lb. curvatus, S. thermophilus* | Fernández et *al.,* 2007b<br>Lucas et *al.,* 2007<br>Bonetta et *al.,* 2008<br>Bunková et *al.,* 2009<br>La Gioia et *al.*, 2011<br>Ladero et *al.*, 2012° |
| Putrescine | *E. faecalis, E. hirae,*<br>*Lb. brevis, Lb. curvatus,*<br>*L. lactis* | Llacer et *al.,* 2007<br>Lucas et *al.,* 2007<br>Ladero et *al.*, 2011a, b |
| Cadaverine | – | |
| Tryptamine | – | |
| Phenylethylamine | Enterococci | Marcobal et *al.*, 2006b |

Many LAB are main histamine and tyramine producers; *Enterococcus*, *Lactobacillus*, *Leuconostoc*, and *Streptococcus* are involved in BA production. They derived from different origins; such as contamination before, during, or after dairy manufacturing; or from expression of a decarboxylase activity originate from starter cultures (Bottazzi, 1993; Burdychova et *al.*, 2007; Komprda et *al.*, 2008).

However, it is difficult to find direct correlation between number of microorganisms and BA content since the ability to produce amines by different strains varies greatly (Innocente et *al.*, 2002; Schirone et *al.*, 2011). Isolated strains from dairy products such as *Streptococcus thermophilus* (Calles-Enríquez et *al.*, 2010; La Gioia et *al.*, 2011), *Lb. brevis* and *Lb. curvatus* (Ladero et *al.*, 2011b), and *Lactococcus lactis* (Ladero et *al.*, 2011a) has been found to produce histamine, tyramine or putrescine, respectively.

However, it was reported that *E. faecalis* produced 2-phenylethylamine and also substantial amounts of tyramine in skim milk, which is the main raw material of fermented dairy products (O'Brien et *al.*, 2004). *Enterococcus spp*. isolates with tyramine production were detected also in dutch type semihard cheese, especially by *E. durans, E. faecalis* and *E. casseliflavus* (Komprda et *al.,* 2008). *E. faecium* and *E. faecalis* isolated from a semi-hard cheeses were identified and a strong decarboxylase activity was found (Kucerova et *al.*, 2009).

*Lb. helveticus* was selected from starter cultures and its histamine-producing capability was proven (Burdychova et *al.*, 2007). This aspect should not be underestimated because some of the strains before cited belong to the same species of SLAB added for cheese production. It is recommended not to use strains producing-amine as starter cultures as it may increased the problem about the introducing of the qualification criteria "*absence of BA production and BA production associated*" in the "Qualified Presumption of Safety" (QPS) assessment scheme introduced By the European Food Safety Agency (EFSA). (Crow et *al.*, 2001; Linares et *al.*, 2011; EFSA Panel on Biological Hazards BIOHAZ, 2011). For this evaluation, methods for the characterization and quantification of BA producing bacteria has been known (Fernández et *al.*, 2006b; Torriani et *al.*, 2008; Ladero et *al.*, 2010b, 2012b). The presence of Biogenic Amines in cheese adding strains, which are able to catabolize BA, can be reduced (Leuschner et *al.*, 1998; Naila et *al.*, 2010). *Brevibacterium linens* was used to degrade histamine and tyramine during Muster cheese making (elaboration) (Leuschner et *al.*, 1998).

Environmental conditions that involved in BA production are pH, temperature, NaCl. There are two contradictive mechanisms about pH action; firstly is fast acidification of the medium leaading to decrease in decarboxylating bacteria

and BA production (Gardini et *al.*, 2001). Secondly, it has been found that acid pH facilitates BA synthesis (Marcobal et *al.*, 2006b; Fernández et *al.*, 2007b). A research by Fernández et *al.*, (2007) have shown that the ability of *Enterococcus durans* BA-producing strain, isolated from cheese, to produce tyramine is pH dependent. Higher amount of tyramine was found in a medium at pH 5.0 than 7.0 (Fernández et *al.*, 2007b). Linares et *al.*, (2009) have also proved an increasing BA production by *Enterococcus durans* under low pH conditions due to expression of decarboxylases and transporter genes (Linares et *al.*, 2009). The quantitative production of biogenic amines was also reported to decrease in cheeses kept at lower storage temperature (20°C instead of 32°C) and made with adding NaCl. The definition of "Storage of the cheese" is the period from the last step of cheese making to the ripening and until consumption (Santos et *al.*, 2003). Lower levels of histamine was observed in milk inoculated with a strain of *Streptococcus thermophilus* histamine-producing and incubated at 4˚C. The decreased was due to reduction in histamine activity instead to a cell number in diminution (Calles-Enríquez et *al.*, 2010). Same effects were obtained in cheese made with milk containing high level of sodium chloride (5%) and with adding *Lb. bulgaricus* (Chander et *al.*, 1989) or *Lb. buchneri* (Sumner et *al.*, 1990).

**Table 6.** Biogenic amines content in some cheeses*

| Biogenic Amines (mg/kg) | | | | | |
|---|---|---|---|---|---|
| **Type of Cheese** | **Milk** | **HI** | **TY** | **PU** | **CD** |
| Ripened cheese | Raw | 510,2 | 453,7 | 176,3 | 328,4 |
| | Pasteurized | 65,4 | 301 | 175,3 | n.d. |
| Unripened cheese | Raw | 110,8 | 233,3 | 38,7 | 96,3 |
| | Pasteurized | 60,2 | 22 | n.d.** | n.d. |
| Bluee cheese | raw | 1041 | 1051 | 875,8 | 756,7 |
| | pasteurized | 127 | 526,6 | 237,5 | 89,4 |

Histamine (HI), tyramine (TY), putrescine (PU), cadaverine (CD)
**n.d., no detected
*Fernández et *al.*, 2007

Cheese made from raw milk has been found to have a higher histamine, tyramine and putrescine contents than those derived from pasteurized milk (Table 6) (Ladero et *al.*, 2008; 2010b; 2012b; Novella-Rodríguez et *al.*, 2004; Fernández et *al.*, 2007b). Other scientists attributed the differences in BA concentration in cheese, which produced from pasteurized and non-pasteurized milk, to the heat sensitivity of certain cofactors (e.g. pyridoxal 5-phosphate) needed for amino acid decarboxylation

reaction (Joosten et *al.*, 1987). Moreover, the ripening period has an incisive influence on the accumulation of BA, in particular on histamine levels. This happens because the substrate amino acids of the decarboxylating enzymes are released from casein during cheese ripening, proteolysis represents the biochemical reaction which happens during cheese ripening and responsible of BA production. Similary, Ladero et *al.,* (2008) also observed a high level of histamine concentrations during long period of ripening cheese, particularly blue cheese where there was a strong occurrences of proteolysis.

There is specific legislation concerning the safe-amount of BA in foods. Histamine in fishery products from fish species associated with a high amount of histidine is satisfactory if the mean value is ≤ 200 mg/kg (COMMISSION REGULATION (EU) N. 1019/2013 of 23 October 2013 amending Annex I to Regulation (EC) N. 2073/2005 as regards histamine in fishery products). Other foods have only been recommended or suggested an upper limit of 100 mg/Kg of histamine; whereas a limit of between 100 and 800 mg/Kg and of 30 mg/Kg has been recommended for tyramine or β-phenylethylamine, respectively (Ten Brink et *al.*, 1990; Halász et *al.*, 1994). Some levels of biogenic amines resulting in an exposure from a single food consumption within the toxicological threshold are reported by EFSA. According to EFSA (2011), a level of 50 mg of histamine is recommended for healthy individuals. On the other hand, a level of 600 mg of tyramine is recommended for healthy individuals or 50 mg for individual under Reversible Monoamine Oxidase Inhibitor (RIMA) medications. RIMA medications are drugs that reversibly inhibit the enzymatic activity of monoamine oxidase (MAO), those enzymes responsible for the degradation and inactivation of biogenic amines.

A recent publication that evaluates the competitive effects of three bacteriocin producing strains of *Lactococcus lactis subsp. lactis* against two aminobiogenic lactic acid bacteria (e.g. tyramine producing strain *Enterococcus faecalis* EF37 and histamine producing strain *Streptococcus thermophilus* PRI60), showed the relation between bacteriocinogenic and aminobiogenic strains with respect to possibility to accumulate BA and in the same time observed the different action of the three bacteriocins on the same strain BA producing. The use of bacteriocinogenic lactococci could be a predictable strategy against aminobiogenic bacteria in cheese production (Tabanelli et *al.*, 2014).

## 1.6. Volatile Flavor Compounds in Cheese

Flavor perception is the sensation resulting from the interaction of different signals produced from sensing chemical compounds through taste and smell (Laing et *al.*, 1996). Many receptors (300-400) in the nose are naturally able to distinguish numerous volatile flavor compounds (Brand et *al.*, 1994). Taste-receptor cells in the mouth are sensitive to five basic differences like sweet, bitter, salt, acid and umami and as well as cold and hot sensations (Ninomiya, 2002).

Mature cheeses contain a wide range of flavor compounds, including volatile and non-volatile compounds (Curioni et *al.*, 2002; Urbach et *al.*, 1997). The flavor-active compounds have been identified in several cheese varieties, including Emmental, Gruyere and Camembert cheese (Kubickova et *al.*,1998; Preininger et *al.*,1994; Preininger et *al.*, 1996; Rychlik et *al.*, 2001).

Cheese is a biochemically dynamic product, meaning that it undergoes a series of changes during cheese ripening. In point of fact, the curds of a certain number of cheeses can be similar, and it is only during ageing that the cheeses acquire their ultimately distinctive sensory characteristics (Larráyoz et *al.*, 2002). It is now commonly accepted that the flavor of most cheeses results from the combination of a large number of different volatile compounds present in the correct proportions and concentrations ('component balance theory') (Urbach, 1993; Kosikowski et *al.*, 1958). Flavor compounds in cheese can be produced from enzymatic reaction arising from rennet, milk, SLAB, secondary microflora (among them, NSLAB). Cheese flavor cannot be produced without starter bacteria. In aged cheese, SLAB die out quickly and release their cellular enzymes; this step occurs with a speed which has an influence on the rate of free amino acids formation. Rennet is mainly responsible for the formation of large, medium and small peptides supported from other enzymes action. Free amino acids, together with the products of glycolysis, form substrates for secondary flora, the nature of which, determines the cheese variety (Urbach, 1995). During cheese ripening, the development of flavor compounds is very slow and involves biochemical reactions like glycolysis, lipolysis, proteolysis, and metabolism of citrate (Smit et *al.*, 2005).

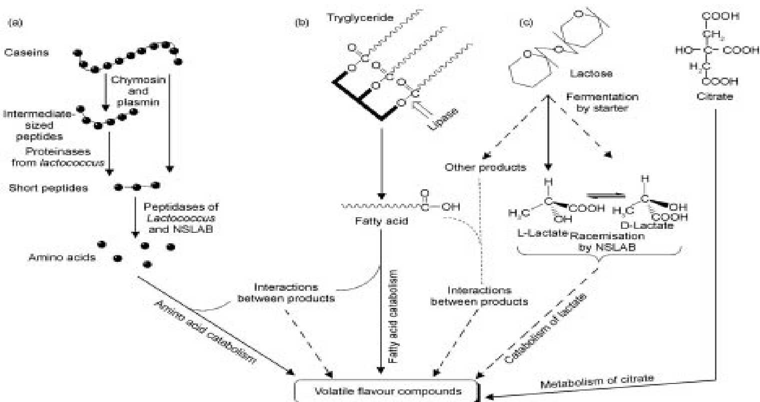



**Fig. 4.** Schematic representation of the principal biochemical pathways that occur in cheese during ripening (from McSweeney, 2004)

Regarding glycolysis, lactose is fermented in lactate by LAB and also in the intermediate pyruvate; this last substance can be converted in different compounds like diacetyl, acetoin, acetaldehyde, or acetic acid, some of which contribute to typical yoghurt flavors (Smit et *al.*, 2005). Lypolysis is an enzymatic reaction which lead the conversion of fat in free fatty acids (FFA). FFA can be precursors of flavor compounds methylketones, secondary alcohols, esteres and lactones. LAB contribute little in this kind of reaction, instead moulds, present on the surface of a ripening cheese, can have strong lypolysis activity (Molimard et *al.*, 1996). Various flavors in soft cheeses like Camembert and Roquefort can be derived from lypolysis (Smit et *al.*, 2005). Proteolysis is the most important biochemical pathway through the degradation of casein and for flavors production especially in hard-type and semi-hard-type cheeses. Casein can be degraded in peptides and free amino acids (FAA) either by rennet enzymes and either LAB cell envelope-associated proteinase and peptidases. FAA are converted further in flavor compounds such as alcohols, aldehydes, acids, esters and sulphur compounds (Smit et *al.*, 2005). Citrate is metabolized by citrate-positive strains of lactococci, such as *Streptococcus diacetylactis* or *Lactococcus lactis subsp. lactis* biovar *diacetylactis,* which can transport the citrate into the cell (McSweeney, 2004). Citrate contributes to the formation of diacetyl and acetoin in some cheese varieties, as well as it contributes to carbon dioxide formation, which is responsible for the characteristic eye in certain Dutch cheese (Hugenholtz, 1993).

LAB are the main microrganisms producing enzymes for flavor compounds formation in cheese, especially *Lactococcus lactis*, *Streptococcus thermophilus,*

*Lactobacillus* species (mesophilic lactobacilli, originating from the milk environment) and *Leuconostoc mesenteroides*. However, also additional cultures are used, such as *Propionibacterium* in the case of Swiss-type and Maasdammer-type of cheeses, and various aerobic cultures (e.g., *Brevibacterium, Arthrobacter, Staphylococcus, Penicillium, Debaromyces*) for surface-ripened cheeses (Bockelmann, 1997, 2001; Molimard et *al.*, 1996). The formation of volatile compounds by LAB derive mostly from amino acid catabolism (Laing et *al.*, 1996; Moio et *al.*, 1998). Hundreds of volatile compounds are involved in cheese flavor (Sable et *al.*, 1999) (Table 7).

**Table 7.** Volatile Flavor compounds in some cheese

| Name | Compound | Cheese | Reference in cheese |
|---|---|---|---|
| **Esters** | Diethyl ether | Cheddar Cheese | Urbach, 1995 |
| | n-Propyl acetate | Swiss cheeses | Thierry et *al.*, 2005 |
| | Ethyl Acetate | Swiss cheeses | Thierry et *al.,* 2005 |
| | Propanoic acid, ethyl ester (ethyl propionate) | Roncal cheese | Larrayoz et *al.*, 2001 |
| | Propanoic acid, propyl ester (propyl propionate) | Manchego, Ragusano | Mallia et a*l.,* 2005 |
| | Butanoic acid, ethyl ester (ethyl butanoate) | Roncal cheese | Larrayoz et al., 2001 |
| | Butanoic acid, propyl ester (propyl butanoate) | Roncal cheese | Larrayoz et al., 2001 |
| | Hexanoic acid, ethyl ester (ethyl hexanoate) | Roncal cheese | Larrayoz et *al.*, 2001 |
| | Hexanoic acid, propyl ester (propyl hexanoate) | Roncal cheese | Larrayoz et *al* 2001 |
| | Heptanoic acid, ethyl ester (ethyl heptanoate) | Parmigiano-Reggiano | Qian et *al.*, 2002 |
| | Octanoic acid, ethyl ester (ethyl octanoate) | Roncal cheese | Larrayoz et *al.*, 2001 |
| | Octanoic acid, methyl ester (methyl octanoate) | Beaten cheese (Bieno sirenje) | Sulejmani et *al.*, 2014 |
| **Ketones** | Acetone | Cheddar Cheese | Urbach, 1995 |
| | 2-Butanone | Hispánico cheese | Garde et *al.,* 2002 |
| | 2-Pentanone | Swiss cheeses | Thierry et *al.*, 2005 |
| | 2-Heptanone | Swiss cheeses | Thierry et *al.,* 2005 |
| | 8-Nonen-2-one | Swiss cheeses | Thierry et *al.,* 2005 |
| | 2-Octanone | Swiss cheeses | Thierry et *al.*, 2005 |
| | 2-Nonanone | Swiss cheeses | Thierry et *al.,* 2005 |
| **Aldehydes** | Butanal, 2-methyl- | Swiss cheeses | Thierry et *al.,* 2005 |
| | Butanal, 3-methyl- | Swiss cheeses | Thierry et *al.,* 2005 |
| | Propanal, 2-methyl- | Swiss cheeses | Thierry et *al.,* 2005 |
| **Alcohol** | 2-Butanol | Swiss cheeses | Thierry et *al.,* 2005 |
| **Acids** | Acetic acid | Cheddar Cheese | Urbach, 1995 |
| | Propanoic acid | Cheddar Cheese | Urbach, 1995 |
| | Butanoic acid | Swiss cheeses | Thierry et *al.,* 2005 |
| | Hexanoic acid | Cheddar Cheese | Urbach, 1995 |
| | Heptanoic acid | Cheddar Cheese | Urbach, 1995 |
| | Octanoic acid | Cheddar Cheese | Urbach, 1995 |
| | Decanoic acid | Cheddar Cheese | Urbach, 1995 |
| **Benzenes** | Benzene | Cheddar Cheese | Urbach, 1995 |
| | Styrene | Camembert-type cheeses | McSweeney, 2007 |

### 1.7. Pasta filata Cheese

Pasta filata cheeses includes a group of a variety of cheeses that originated especially in countries around northern Mediterranean area, such as Italy, Greece, the Balkans, Turkey, and Eastern Europe. Pasta filata is an Italian word meaning "stretched curd" or "spun paste", which referred to a specific step during its cheese making (plasticization and stretching) (Kindstedt et *al.*, 2004). During Pasta Filata manufacturing, the acidified curds are soak in hot water or salt brine until a specific plastic consistency is achieved. Subsequently, the hot curds are manually or mechanically kneaded and stretched to obtain a cheese with a homogenous structure fiber-like. Finally, the curds are molded into different shapes (Salvadori del Prato, 1998).

Some of Pasta filata cheese are soft or semisoft varieties, which are consumed fresh (Mozzarella di Bufala Campana P.D.O., Fior di latte) or after a short ripening, while others are semihard and hard, which are characterized of a long ripening before being consumed (Caciocavallo Silano P.D.O., Caciocavallo Pugliese) (Kindstedt et *al.*, 2004). Fior di Latte and Mozzarella cheese P.D.O. are high-moisture Mozzarella cheeses produced from cows' milk and raw whole water-buffalo milk, respectively. Generally, these types of cheese is sold fresh and consumed in a few days after manufacture. They represent some of the most highly valued unripened « pasta filata » cheese known in Italy (Coppola et *al.*, 1988, 2006). Caciocavallo Silano P.D.O. made from cows' milk is one of the most famous Italian varieties with ripening period of at least 30 days (Gobbetti et *al.*, 2002; *G.U. n. 196 del 21.08.93*). Caciocavallo Pugliese is another type of Pasta filata cheese that may be on the market after just 2 weeks of ripening or 2–4 months or longer (>12 months) if made as grated cheese (Gobbetti et *al.*, 2002).

#### 1.7.1. Provolone del Monaco P.D.O. cheese

Provolone del Monaco is a Pasta filata cheese (spun paste) made exclusively from cows' raw milk without adding starters and is ripened for at least six months. This type of cheese is traditionally produced in the Lattari mountains area (Campania region, Italy).

The area of breeding cows, production and aging is bordered by the towns of Agerola, Casola di Napoli, Castellamare di Stabia, Gragnano, Lettere, Massa Lubrense, Meta, Piano di Sorrento, Pimonte, Sant'Agnello, Sorrento, Santa Maria La Carità and Vico Equense. The typical name Provolone del Monaco dates back to ancient times, and is closely linked to the way in which this cheese was transported in the past for sale at the market of Naples. The cheeses were transported on the backs of mules to the beaches and were loaded onto rowing boats from the farmers. The farmers covered with great coat similar to the monks dress during transport by boat. Once the cheese arrived in Naples, people who worked in the market near the port began to call the carrier, Monaco (Monaco is an Italian word which means monk), and its cheese, Provolone del Monaco (the literal translation is Monk's provolone).

Provolone del Monaco is produced from mixture of milk originated from cattle native genetic type (TGA) Agerolese enrolled in the population register (20%) and cattle from different breeds (Holstein Friesian, Alpine Brown, Simmental Red, Jersey, Podolica and Métis locals) (80%). Nowadays, Agerolese, a native of Naples province, can only be found in towns of Agerola and Gragnano. This race is derived from crosses of cattle Holstein Friesian, Jersey and Alpine Brown with the local indigenous population and is considered endangered by the Food and Agriculture Organization (FAO). Agerolese has low milk production but high in quality due to the clean environment surrounding Lattari mountains. The breeding of cattle on the slopes of the mountains Lattari dates back in 264 BC. when the Picentini, who are the first inhabitants of the mountains, transformed the space into farmland and began the breeding of cattle especially producing milk for dairy.

The product is a P.D.O and it must have the following characteristics:

a) a curing period of at least 180 days (six months), with a maximum yield of 9 kg per hectolitre of processed milk;

b) a form of a slightly elongated melon or pear without a head with a minimum weight of 2.5 kg and a maximum of 8 kg; a thin crust of yellowish colour with slightly darker tones, almost smooth with 6 faces divided from longitudinal furrows corresponding to the strings of "raffia" used for binding;

c) a paste, cream colour with a yellowish tone, elastic, compact, uniform and without flaking, soft and with typical holes (like "bird's eyes") of variable diameter up to 5 mm, with possible presence of sporadic holes of diameter greater, up to 12 mm, more abundant toward the centre of mass;

d) a fat content in the dry matter of not less than 40.5%;

e) a sweet and buttery flavor and a light and pleasant spicy taste;

f) environments ripening cheese which are able to produce flavors enhanced by the length of maturation. After 7-8 months the provolone will tend to further yellowing, thickening the crust and assuming a gradually more spicy flavor and a more consistent appearance of the pasta, though still soft enough and always free of flaking.

## 2. Materials and Methods

### 2.1. Cheese making process

Raw milk for the production of "Provolone del Monaco" must come from cows that have not exceeded the maximum limit of 231 days of lactation. The production of "Provolone del Monaco" is permitted throughout the year, exclusively from milk collected after each milking stall without refrigeration or daily with refrigeration in the barn. The milk processing must take place within 48 hours of milking and storage at the cheese must be made no later than 24 hours in a refrigerated stainless steel tanks.

16 q of raw bovine milk were used for producing a lot of Provolone del Monaco in this study. According to the production specification, 12.8 q (80%) and 3.2 q (20%) of raw bovine milk derived exclusively from the morning milking of cows belong to different breeds (Holstein Friesian, Alpine Brown, Simmental Red, Jersey, Podolica and Métis locals) and cattle native genetic type (Agerolese).

The raw milk was stored in different tanks at a temperature of +4° C upon arrival. Subsequently, the milk was transferred by self priming pumps from both tanks to a small boiler where milk was mixed and heated at 39-40° C (or subjected to treatment having equivalent effect) (Fig. 5 and 6).

**Fig. 5. Stored in different tanks at 4 °C**

**Fig. 6. Mixing of milks Treatment less than 40°C**

Milk was placed by pump to a second big boiler. The coagulation of milk by rennet was obtained after 35-40 minutes (max 60 minutes) by adding kid rennet paste (at least 50% of kid rennet) and liquid calf in combination at tmperature 34-42 °C (20g of each rennet per litre of milk) (Fig. 7 and 8).

**Fig. 7. Adding kid rennet paste and liquid calf**

**Fig. 8. Coagulation (35 minutes)**

When the curd reached the desired consistency, it was broken to get very small lumps the size of a hazelnut at first and later a grain of corn. It was left to rest for about 20 minutes. The curd was heated to a temperature of about 48-52 °C (~40 minutes), and left to rest up to a maximum of 30 minutes while maintaining the temperature at around 45 °C (Fig. 9, 10 and 11).

**Fig. 9. Curd cutting to hazelnut size Left to rest for about 20 minutes**

**Fig. 10. Cooking at 48-52°C for 40 minutes**

**Fig. 11. Left to rest for 30 minutes maintaining a temperature above 48°C**

Subsequently, the whey was removed and the curd remained in the boiler with part of the whey were drained through small holes on the end of it (Fig. 12 and 13).

**Fig. 12. Whey removing**

**Fig. 13. Whey draining**
**The curd still in the boiler with part of the whey for about an hour**

The curd was transferred to a work plan where it was pressed by hands, cut with knife, wrapped in flaxen cloth and placed in perforated steel baskets for draining (Fig. 14, 15, 16 and 17).

**Fig. 14. Displacement of the curd from the boiler to a work plan**

**Fig. 15. Pressed by hand**

**Fig. 16. Cut with a knife**

**Fig. 17. Whey draining by squeezing curd blocks in flaxen cloth**

After 4-5 hours, the curd was removed from flaxen cloth and left on the table at room temperature for 12-14 hours (curd acidification). The curd was cut into strips of varying sizes in the following morning. The elasticity and strength of the curd was evaluated prior to cutting by spinning trials in a hot water. In addition to that, pH measurement was also conducted prior to curd cutting (Fig. 18, 19, 20 and 21).

**Fig. 18. Left in flaxen cloth for 4-5 hours**

**Fig. 19. Freed of flaxen cloth**

**Fig. 20. Curd acidification on a table at room temperature for 12–14 hours**

**Fig 21. Stretching in water at 80–90 °C for 30 minutes**

Stretching in hot water at 85-95°C could be made manually or mechanically; pasta filata was broken mechanically in smaller pieces which were moulded to obtain the shape and the required size (Fig. 22, 23, 24 and 25).

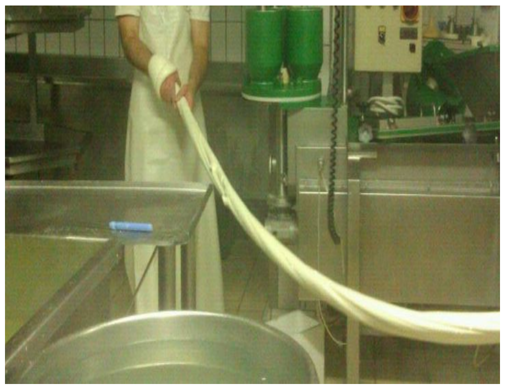


**Fig. 22. Stretching manually**

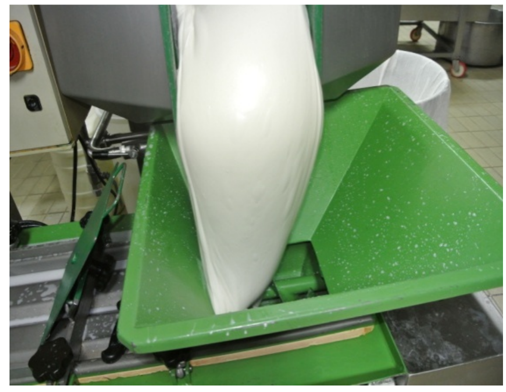


**Fig. 23. Stretching mechanically**

**Fig. 24. Splitting mechanically**

**Fig. 25. Molding manually in pieces of 3–5 kg**

The cheeses were subjected to a pre-firming at room temperature for 20 minutes; they were tied (first binding) and placed in a bath for a firming at 10 °C for 4-5 hours. The first binding was removed before salting in brine; the salting step lasted 8-18 hours per each kilogram of product (Fig. 26, 27, 28, and 29).

**Fig. 26. First Firming at room temperature**

**Fig. 27. Binding**

**Fig. 28. Second Firming at 10 °C for 4-5 hours**

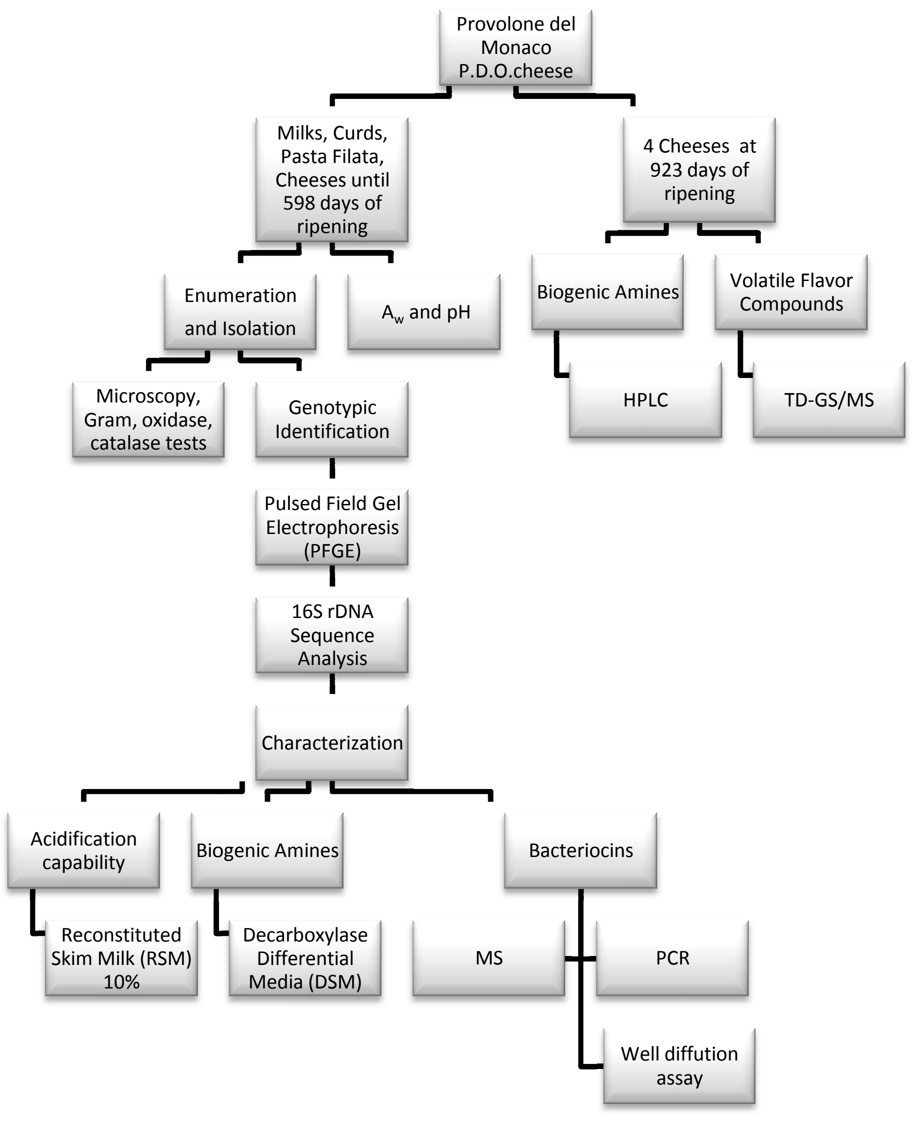
**Fig. 29. Salting in brine**

After manufacturing, the cheeses obtained from 2.8 kg each were tied (second binding) hung on special racks and were left to mature for lactic-proteolytic maturation before drying at room temperature from 10 to 20 days at room temperature. Subsequently, the cheeses were set to ripen between 8 to 15 °C for at

least six months and subjected at this time exclusively for washing and cleaning of moulds and possible oiling (extra-virgin olive oil P.D.O. Sorrentine Peninsula). Provolone del Monaco cheeses could have a minimum weight of 2.5 kg and a maximum of 8 kg after a long term of ripening (Fig. 30).

**Fig. 30. Drying at room temperature for 10-20 days**
**Ripening 8-15 °C for at least six months**

## 2.2. Experimental Layout



### 2.3. Sampling and Enumeration

Samples were collected throughout the technological process from the following various conditions: milk in the cheese vat (M1 and M2 milk produced by different breeds and Agerolese, respectively), milk heated to temperatures lower than 40 °C (MT), from two different locations of the curd after cooking at 48°C for 40 minutes (C1) and from the curd at the end of ripening at room temperature (C2), the cheese after stretching in hot water (PF, pasta filata) and during drying and ripening period (of more than one year) with an interval of about 40 - 50 days. Three cheese after 1, 5, 20 days of drying (Fp0, Fp1, Fp2) and 11 cheeses after 49, 81, 123, 207, 257, 314, 368, 404, 444, 494 and 598 days of ripening respectively (from Fp3 to Fp13). All analysed cheese samples were from the same production batch within one factory.

Milks samples were shaked before sampling, while cheese samples were obtained by incision in clove cutting from the centre of the cheese in order to obtain a homogenous sample representative of the whole cheese, and after removal of the crust. A 25 g sample from the interior of each cheese was taken aseptically and added to 225 ml of sterile Sodium Citrate's solution and homogenized using a stomacher (Stomacher 400 circulator pbi international) for 2 minutes twice. Ten-fold serial dilutions in Ringer's solution were prepared for microbial enumeration in duplicate.

The following media, plate technique, and incubation conditions were used:

✓ Kanamicyn Aesculin Azide (KAA) agar (Oxoid) modifited (Audicana et *al.*, 1995) inoculated by spreading plate method and incubated in aerobiosis at 37°C for 1-2 days;

✓ M17 agar (Oxoid) with 1% of lactose (LM17), inoculated by the pour-plate technique, incubated in anaerobiosis (Anaerogen kit, Oxoid) at 30°C for 3 days;

✓ M17 agar (Oxoid) with 1% of lactose (LM17), inoculated by the pour-plate technique and incubated in anaerobiosis (Anaerogen kit, Oxoid) at 44 °C for 3 days;

- ✓ Rogosa agar (Oxoid) acidified with acetic acid to pH 5.4, inoculated by the pour-plate technique and incubated in anaerobiosis (Anaerogen kit, Oxoid) at 30°C for 5 days;
- ✓ Rogosa agar (Oxoid) acidified with acetic acid to pH 5.4, inoculated by the pour-plate technique and incubated in anaerobiosis (Anaerogen kit, Oxoid) at 44 °C for 3 days.

### 2.4. Water Activity ($A_w$) and pH

Water Activity ($A_w$) and pH were evaluated using the ro-tronic Higro-lab PBI-international and a Metrohm 691 pH meter, respectively.

### 2.5. Isolation of LAB

10 colonies were randomly picked from countable plates of KAA Agar modified (Audicana et *al.*, 1995) (black colonies with grey halo); from LM17 at 30°C and 44°C, and Rogosa at 30 °C and 44°C (white discoid colonies). Each isolate was purified by repeated streaking on MRS or LM17 and incubating at 30 °C or 37 °C for 1-2 days. All isolates were characterized by Gram staining, catalase and oxidase activity. Pure cultures were maintained as frozen stocks at -79°C in LM17 or MRS broth and 20% sterile glycerol until genotypic characterisation of isolates was undertaken. When required, cultures were activated by one or two consecutive transfers to LM17 or MRS broth incubated over night at 37 or 30°C, respectively.

### 2.6. Genotypic identification of LAB

Isolates were chosen from following samples for genotypic identification: M1, M2, MT, C1, C2, PF, Fp2, Fp3, Fp4, Fp6, Fp7, Fp9, Fp11, Fp12 and Fp13 in order to have a representative number of all samples.

#### 2.6.1. Pulsed Field Gel Electrophoresis (PFGE)

Pulsed Field Gel Electrophoresis was performed as described previously by Simpson et *al.,* (2002, 2003). Each culture broth was inoculated at 1% and grown overnight in LM17 or MRS broth containing 20 mM threonine, 1.0 ml of refreshed culture broth was pipetted into sterile eppie and centrifuged on max for 3-5 minutes. The supernantant was discarded and pellet was suspended in 1.0 ml of 10 mM Tris-HCl, 1 M NaCl, pH 7.6 and re-centrifuged. The pellet was resuspended in 200 µl of the same buffer (10 mM Tris-HCl, 1 M NaCl, pH 7.6) and mixed gently with a micropipette. An equal volume (200 µl) of 2 % low melting point agarose in 0.125 M EDTA (pH 7.6) was added to the cell suspension, mixed and poured in mold plugs leaving to solidify in duplicate moulds for each isolate at room temperature. The plugs were put into a petri dish and incubate with 1 ml of EC buffer (1 M NaCl, 6 mM Tris-HCl, 100 mM EDTA, 1% sarkosyl, pH 7.6), containing 10 mg/ml lysozyme and 20 units/ml mutanolysin, incubated overnight in a bath at 37°C (or 55°C). The lysozyme solution was removed with a micropipette and replaced with 1 ml of the proteinase K solution (0.5 mg/ml in 0.5 M EDTA, 1% sarkosyl, pH 8.0) and plugs were incubated overnight at 55°C with gentle shaking. The petri dish's wells were emptied by proteinase K solution and the plugs were washed twice in 1 ml of 1 mM PhenylMethaneSulfonyl Fluoride solution (PMSF) in TE 10/1 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and left for 1 hour at 37°C with gentle shaking. The plugs were taken from the petri dish gently with a small spatula and placed into eppendorf tube containing 1 ml of TE 10/100 (10 mM Tris-HCl, 100mM EDTA), the plugs were left at 4°C until required. Each plug was taken from eppie and put on clean glass slide where it was cut in duplicate or triplicate slices (1-2 mm thickness) using a clean coverslip and put gently into plastic petri dishes. Plugs were replaced in their eppendorf tubes and stored at 4°C until required for a maximum of 6 months. The slices were washed in 1 ml of TE 10/0.1 (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) three times (for 30 minutes each) at room temperature with gentle shaking and then

they were transferred in eppendorf tubes containing 100 µl of Cut Smart buffer and incubated at 4°C for at least 30 minutes. In each tube containing slices, it was added 0.3-0.5 of restriction enzyme (Asc I, Sma I or Apa I) and incubated overnight for 24 hours at 37°C (Asc I) or 55°C (Sma I or Apa I) with gentle shaking. Asc I was used mostly for cutting DNA belongs to rod shape isolates. Sma I was added as restriction enzyme for cocci shape bacteria. Apa I was utilised only when other two restriction enzymes did not work/cut (the restriction was partial). The day after, the reaction was stopped adding 0.5 ml of 0.5 M EDTA pH 8.0 and stored at 4°C. Following digestion, the slices were put on a PFGE comb, were positioned at the far end of the tooth of the comb and then left to dry. The slices and Low Range PFG Markers were loaded into the wells of a 200 ml 1% PFGE grade agarose gel previously dissolved in hot in 0.5X Tris-borate buffer (4 L: 200 ml 10X stock + 3800 ml water) and cooled at 50°C. When the gel was polymerized, comb was removed and the gel was placed in a horizontal electrophoresis system containing enough 0.5X Tris-borate buffer to the cover agarose gel. The gels were run in 0.5X Tris-borate buffer using a CHEF-DR® II PFGE apparatus at 6V/cm, 14°C for 16 hours with the pulse ramped from 1 to 20 seconds. The gels were stained with Ethidium bromide (0.5 µg/ml = 25 µl in 500 ml, 1 drop per each 50 ml of distilled water) at room temperature for 1 hour and then destained in water twice for 30 minutes each. The gels were photographed using Alpha Innotech - AlphaImager HP camera and the picture was captured by Alpha Imaging System. Profiles were compared using BioNumerics software 6.6. All profiles that showed a similarity greater than 70% were grouped.

### 2.6.2. 16S rDNA Sequence Analysis

One profile was chosen among profiles belong to the same group (similarity > 70%) like representative one. One strain corresponding to that representative profile was grown overnight in LM17 or MRS broth; 1.0 ml of refreshed culture broth was pipetted into sterile eppie and centrifuged for max 3-5 minutes. The supernatant was discarded and the pellet was washed with 300 µl sterile Phosphate Buffered Saline (PBS) and re-centrifuged. The pellet was suspended in 200 µl of 10% Sodium Dodecyl Sulphate (SDS). Extraction of genomic DNA and 16S rDNA sequencing were carried out by an external laboratory (Eurofins Genomics, Germany). The primers used were forward 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse 1492r (5'-GGTTACCTTGTTACGACTT-3'). Sequencing results obtained with

forward and reverse primers were matched using DNASTAR - Lasergene Version 8.0.2. The sequences were determined and compared with known 16S rDNA gene sequences in the National Center for Biotechnology Information database (NCBI) (www.ncbi.nlm.nih.gov) by using the Basic Local Alignment Search Tool (BLAST) algorithm. BLAST is a set of programs for fast approximate comparison of biological sequences, such as the amino-acid sequences of different proteins or the nucleotides of DNA sequences. BLAST is useful for the comparison between a query sequence and a library or database of sequences, in order to identify library sequences that resemble the query sequence above a certain threshold. In BLASTN, both the database and the query are nucleotide sequences (Altschul et *al.*, 1997).

### 2.7. Characterization of selected LAB isolates

The isolates discriminated by PFGE and sequenced for 16S rDNA were subjected to various experiments to evaluate their ability to acidify in skim milk, biogenic amines (tyramine and histamine) and bacteriocins production.

#### 2.7.1. Acidification capability

Strains, activated in MRS or M17 broth (at 30 or 37 °C for 24 hours), were centrifuged for max 3-5 minutes, and the pellets were washed with sterile PBS and re-suspended in the same medium. The cell suspension (1% v/v) was inoculated in duplicate in tubes containing 10 ml of Reconstituted Skim Milk (RSM) 10% (pH = 6.5) and incubated at 30 or 37 °C overnight. Then, pH of inoculated RSM was read after incubation using a pH meter (Metter Toledo). The acidification in tube at 1% was a first screening to evaluate acidification ability and especially to reduce strains' number. All strains which acidified RSM 10% at least 1 unit were chosen to evaluate the acidification ability using the Cinac system (ASM Systea). Strains were activated by transfer in LM17 or MRS and the cell suspension (2% v/v) was inoculated in 10 ml of RSM 10% in tube and incubated at 30 or 37 °C overnight and then, skim milk with strain grown was mixed by vortex and was inoculated (2% v/v) in a bottle containing 200 ml of RSM 10%. Bottles, performed in duplicate, were incubated in Cinac heating bath (Corrieu et *al.*, 1988) at 30 or 37 °C for 16-18 hours and in each bottle was inserted a probe to monitor and record ΔpH and Δtemperature every minute.

### 2.7.2. Biogenic Amines using Decarboxylase Selective Media (DSM)

Screening methods for identify the BA-producer strains were based on the use of differential media containing a pH indicator such as bromocresol purple (Bover-Cid et *al.*, 1999). However, these methods require the isolation and growth of the producer microorganism, thus are laborious and time-consuming (Linares et *al.*, 2011). Isolates were activated in broth and were streaked on Decarboxylase Differential Media (DSM); this medium is selective for decarboxylase-positive LAB, as described by Bover-Cid and Holzapfel (1999). DSM contains (g/L): tryptone, 5.0; yeast extract, 5.0; meat extract, 5.0; NaCl, 2.5; glucose, 0,5; Tween 80, 1.0; $K_2HPO_4$, 2.0; Tri-ammonium citrate, 2.0; $CaCO_3$, 0.1; $MgSO_4.7H_2O$, 0.2; $MnSO_4.4H_2O$, 0.05; $FeSO_2.7H_2O$, 0.04; thiamine, 0.01; pyridoxal-5-phosphate, 0.05; bromocresol purple, 0.06; agar, 22.0 and one of the precursor amino acids (l-tirosine or l-histidine), 10.0. Media were called TDSM and HDSM for the presence of l-tirosine or l-histidine, respectively. The pH was adjusted to 5.3 for the medium containing tyrosine and 5.0 for the medium containing histidine. Decarboxylase activity for histidine DSM was indicated by the appearance of a deep blue/purple discoloration of the medium surrounding each colony. This is due to histamine production and rise in pH which resulted in a colour change in the presence of bromocresol purple. In tyrosine DSM, plates are not translucent but appear cloudy due to the low solubility of tyrosine salts.

### 2.7.3. Bacteriocins

#### 2.7.3.a. Well diffusion assay

The potential producing strain was grown to stationary phase in a suitable liquid medium they were grown at isolation temperature overnight. *Listeria innocua* LMG 13568 was used as reference sensitive microorganism and it was taken from stock, grown up in BHI broth, streaked in BHI agar and then 2-3 subcultures were grown up in BHI broth, every step was done at 37°C overnight. *Lactococcus lactis* DPC 3147 was used as a positive control, grown in LM17 broth at 30°C overnight; while un-inoculated broth was used as a negative control. 1 ml of the producing strains and *L. lactis* DPC 3147 were centrifuged 3000 rpm at 4 °C for 5 minutes (in bench top) and supernatant was removed to a fresh tube. The supernatant was re-centrifuged and put in a clean tube to assure a minimum bacteria cells was in the supernatant. The pH of supernatant was adjusted to ~ 7.00 using 4N NaOH to be sure that the zones of inhibition did not due to acid pH and followed by filtration through a 0.2 µm cellulose acetate filter. 1 ml of *Listeria innocua* LMG 13568 was added in 200 ml of tempered of BHI agar and mixed gently; 20 ml of seeded culture was poured plate Petri dishes and allowed to cool. The broad end of Pasteur pipette was flamed in alcohol, cooled and used to make wells in BHI agar. 50 µl of the cell free supernatant (pH ~ 7.00) of test strains, positive and negative controls were added to the wells in the agar; the plates were incubated at 37°C overnight. The zones of inhibition in the agar were visually observed and measured.

#### 2.7.3.b. Effect of proteinase K, pH and heat treatment on bacteriocin stability

In the event of culture producing zones of inhibition, well diffusion assay was repeated and antimicrobials' sensitivity to proteolytic enzymes was studied by adding 3 µl of proteinase K (10 mg/ml) near the wells and then incubated at 37 °C overnight. The zones of inhibition within the agar were disappeared in that precise point if the antimicrobial was a protein in nature. The effects of pH on producing-bacteriocin strains were tested by adjusting the pH values of cell-free supernatants from 2 to 10 (at increments of 2 pH unit) with sterile 1 mol/l NaOH or 1 mol/l HCl. After 30 and 90 minutes of incubation at 37 °C, the pH samples were readjusted to

neutral and tested for antimicrobial activity. To test heat sensitivity, neutralized cell free supernatants were heated at 60 °C or 100 °C, for 10, 30, 60 or 90 minutes, respectively. Antimicrobial activity was also assayed using the well diffusion assay.

#### 2.7.3.c. Bacteriocin identification by Mass Spectrometry (MS)

A full loop of colonies was mixed with 50 µl of 70% propan-2-ol 0.1% Trifluoroacetic Acid (TFA). The suspension was vortexed and cells spun down in a bench top centrifuge at 14000 rpm for 30 seconds. A 0.5-µl aliquot of matrix solution Alpha-Cyano-4-Hydroxy Cinnamic Acid (CHCA), 10 mg/ml in 50% acetonitrile- 0.1 % (v/v) TFA acid was deposited onto the target and left for 1-2 minutes before being removed. The residual solution remaining was allowed air-dry and the sample solution was deposited onto the pre-coated sample spot. 0.5-µl of matrix solution was added to the deposited sample and allowed air-dry. The sample was subsequently analysed in positive-ion linear mode using Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS). Mass spectrometry was performed with an Axima Performance MALDI TOF/TOF mass spectrometer (Shimadzu Biotech, Manchester, UK). After getting a first spectrum, another steps were made to improve the detection of active peptide single fractions. One litre of TY broth culture was grown overnight at 37°C in TY broth which had been passed through a column containing Amberlite XAD to reduce the peptide load. The culture was centrifuged at 8000 rpm for 20 minutes and cells separated from supernatant. Cells were mixed with 250 ml of 70% isopropan-2-ol, 0.1% TFA (IPA) and stirred at room temperature for 3 hours. The resulting cell suspension was centrifuged as described above and the cell supernatant retained for further purification. The IPA was removed from the cell suspension and it was applied to a 2 g, 12 ml Strata-X C18 SPE column (Phenomenex, Cheshire, UK) pre-equilibrated with methanol and water. The column was washed with 12 ml of 30% ethanol and antimicrobial activity eluted with 70% IPA. Activity was assessed on *Listeria innocua* indicator plates and it was tested the cells supernatant, the C18 SPE column FT, 30% ethanol and activity in 70% IPA eluent. The 70% IPA eluent was assessed using MALDI-TOF MS. The IPA was removed from the C18 SPE IPA eluent and applied to a Proteo Jupiter semi preparative (10 x 250 mm, 4µ, 90Å) RP-HPLC column (Dionex ICS 3000 HPLC fitted with a UV detector, Phenomenex, Cheshire, UK) running a 25-45% acetonitrile, 0.1% TFA gradient over 40 minutes. Eluent was

monitored at 214 nm and fractions were collected at 1 minute intervals. Only active fractions were assessed again using MALDI-TOF MS.

#### 2.7.3.d. Bacteriocin identification by PCR

A PCR test was performed to see whether structural genes of presumptive enterocin A from *E. faecium* was present in the bacteriocinogenic isolates. DNA was extracted by UltraClean® Microbial DNA Isolation Kit (MOBIO Laboratories Inc., USA). All the PCR reactions were carried out using 1x KAPA2G Robust HotStart ReadyMix (Kapa Biosystems, USA), 0.3 mM of each primer (forward primer was 5-GGTACCACTCATAGTGGAAA-3 and reverse primer was 5-CCCTGGAATTGCTCCACCTAA-3), 50 ng of DNA and PCR grade water to a final volume of 25 ml per reaction. The reaction conditions were 5 minutes denaturation at 95°C, followed by 30 cycles of 30 seconds at 95°C, 30 seconds at 58°C, and 30 seconds at 72°C; this was followed by 5 minutes at 72°C and a cool down to 4°C. Agarose gel electrophoresis was performed in 1x Tris–acetate EDTA buffer (Merck) (10x TAE containing 48.5 g Tris–acetate, 11.4 ml glacial acetic acid and 20 ml 0.5M EDTA pH 8.0), with 0.5 µg/ml of Ethidium Bromide (Sigma). The wells in gel were loaded with amplified DNA and KAPA Universal DNA Ladder (Kapa Biosystems, USA). Amplified DNA was separated by 1.0% (w/v) agarose gel electrophoresis. Gel was run at 100 V for 40 minutes and was visualised by UV light using a Alpha Innotech transilluminator. The gel picture was photographed by AlphaImager HP camera and captured by Alpha Imaging System.

### 2.8. Biogenic Amines and Volatile Flavor Compounds in aged Provolone P.D.O. cheese

Four Provolone del Monaco cheeses were collected and sent to Teagasc research center, Fermoy Co. Cork (Ireland) for High Performance Liquid Chromatography (HPLC) analysis and Thermal Desorption Gas Chromatography Mass Spectrometry (TD-GC/MS). Those cheeses were sampled at 923$^{rd}$ day of ripening and were stored in vacuum plastic package at 4° C. All these cheeses belonged to the same batch production of Provolone del Monaco P.D.O. cheeses already analysed microbiologically. HPLC analysis and TD-GC/MS were done to investigate the amount of Biogenic Amines and identify volatile flavor compounds, respectively.

#### 2.8.1. Biogenic Amines by HPLC

HPLC analysis, as described previously by Özoğul, (2004), comprehends:

a. preparation of solutions;
b. preparation of amine standards;
c. BA extraction from cheese;
d. BA derivatization;
e. HPLC setup.

#### 2.8.1.a. Preparation of solutions

Firstly, 0.9N $H_2SO_4$ was prepared by adding 24.9 ml of 96% $H_2SO_4$ to distilled water. Then, 0.013N $H_2SO_4$ was prepared by adding 13 ml of 0.9N $H_2SO_4$ to 887 ml of distilled water. Benzoyl chloride was prepared mixing 98 ml of Acetonitrile and 2 ml of Benzoyl chloride. All of these solutions were prepared in the fume hood. Saturated NaCl solution was obtained by adding excess NaCl to MilliQ water and was warmed until no more salt would have dissolved.

#### 2.8.1.b. Preparation of amine standards

All amine standards at a stock concentration of 10 mg/ml were prepared by taking into account the salt concentration of the amine standards. For example, putricine dihydrochloride, 1M 161.07 g/L but 73 g of that molecule is salt, which

means that the standard is 54.678% pure. Therefore, in order to prepare a 10 mg/ml solution, 18.288 mg/ml was weighed out and so on it was made for all the standards. The amine standards solution were stored in glass bottles at 4°C.

The highest standard concentration Mix 5 was prepared by adding:

- ✓ Cadaverine (10 mg/ml) 0.2 ml;
- ✓ Histamine (10 mg/ml) 2 ml;
- ✓ Putrescine (10 mg/ml) 0.1 ml;
- ✓ Tyramine (10 mg/ml) 1.0 ml;
- ✓ Tryptamine (10 mg/ml) 0.4 ml;

all amine standards were put in to 10 ml volumetric and making up to the mark with MilliQ water.

**2.8.1.c. BA extraction from cheese**

5g of cheese per each one were weight in duplicate and were homogenized using a stomacher for 10 minutes with 20 ml of 0.013 N $H_2SO_4$. The mixture was put into 50 ml falcon tubes. Another 20 ml of 0.013 N $H_2SO_4$ were added for cleaning up the stomacher bag from the samples residues and were stomachered again for 2 minutes and poured into the same 50 ml falcon tube. Tubes were centrifuged for 15 minutes using a speed 5.000 rpm at 4°C and after that the supernatant from each tube was put into a 50 ml volumetric flask. Samples supernatant was brought to 50 ml volume using 0.013N $H_2SO_4$, then 10 ml aliquot were filtered into steriline tubes using a 0.2 µm disposable nylon membrane filter and 10 ml syringe. BA derivatization was made the day after storing steriline tubes at -20°C.

**2.8.1.d. BA derivatization**

1 ml of each of each standards mixes was transferred into glass test tubes and 1 ml of sample in duplicate was transferred into glass test tubes pipetting out all sample. 1 ml of 2N NaOH was added to all tubes. All following steps were performed in the fume hood. 1 ml of 2% Benzoyl Chloride was added; all tubes were vortexed twice for ca. 30 seconds and left to react for 15 minutes. The reaction was stopped by adding 2 ml of saturated NaCl and then all tubes were mixed quickly. 2 ml of Diethyl Ether were added to each test tube and gently shake. The sample in

each tube seemingly to divide into two layers: BA in Diethyl Ether solution on the top while water on the bottom. The top layer was transferred by plastic Pasteur pipette to a second clean test tube without taking any water drop; 80% of BA was extracted in this step. Other 2 ml of Diethyl Ether were added to the first test tube and transferred after gently shaking to the second test tube; 80% of the remaining 20% BA were extracted at this step). Diethyl Ether was evaporated using a stream of Nitrogen at 45°C for around 20 minutes. 1 ml of Acetonitrile was added and then all test tubes were mixed by vortex to dissolve the biogenic amines. Standards and samples were poured in HPLC vials, loaded into HPLC carousel and were run immediately overnight.

#### 2.8.1.e. HPLC set up

Buffer A was 100% Acetronile and buffer B was 100% Milli Q water. The biogenic amine standard and samples were separated and quantified on a Luna C18 RF 5µm, 100 Å column 250x4.6 mm (Phenomenex, Queens Avenue, UK) at initial flow rate of 1.6 ml/min for 30 minutes and using the following gradients:

- ✓ 0 - 1 minute 1.6 ml/min 40%A + 60%B;
- ✓ 1 - 10 minutes 1.8 ml/min 50%A + 50%B;
- ✓ 10 - 20 minutes 2.0 ml/min 60%A + 40%B;
- ✓ 20 - 25 minutes 2.0 ml/min 70%A + 30%B;
- ✓ 25 - 26 minutes 1.6 ml/min 40%A + 60%B;
- ✓ 26 - 30 minutes 1.6 ml/min 40%A + 60%B.

The new column was first washed through MilliQ water then equilibrated with the eluent 40%Acetonitrile/60%MilliQ water for at least 20 minutes at 1.6 ml/min before any standards or samples were run.

### 2.8.2. Volatile Flavor Compounds by TD-GC/MS

10 of grated cheese was placed in an inert stainless steel cup holder on the Markes Micro Chamber/Thermal Extractor (u-CTE). The sample was heated to 40°C and pre incubated for 10 minutes. Nitrogen was purged through the sample at 50 ml/min for 20 minutes and collected on a Markes Tenax/Carbograph sorbent tube, which had previously been conditioned. This tube was subsequently placed in a Markes Unity 2 Thermal Desorption system which contained a materials emissions cold trap. The tube was predesorbed for 2 minutes at 20 ml/min helium. Tube desorption onto the cold trap was carried out using helium at 50 ml/min, for 5 mins at 150°C followed by 5 minutes at 300°C. Pre trap purge for 2 minutes at 50 ml/min using helium, then trap desorption was achieved starting at 30°C which was increased to 300°C at 24°C/min then held for 5 minutes with a split ratio of 2:1. Volatiles were directly sent to heated transfer line (160°C) directly connected to a HP Innowax column (60x0.25x0.5) in an Agilent 7890GC. The temperature of the column oven was set at 40°C, heated to 50°C at 2°C/ min, then to 120°C at 40°C/min and held for 1 minute, followed by heated to 250°C at 10°C/min and held for 12 minutes, yielding at total GC run time of 48.5 minutes. The carrier was helium at a constant flow of 1.2 ml/min. The detector was an Agilent 5975C single quadrupole mass spectrometer. The ion source temperature and interface temperature were set at 150°C and 250°C, respectively with the MS mode in electronic ionization (-70v) with the mass range between 33 and 350 amu. Compounds were identified using mass spectra comparisons to the NIST 2011 mass spectral library, linear retention indices (LRI) were determined using the van den Dool and Kratz procedure with a series of alkanes from C6 – C18, and from an internal data base with known target and qualifier ions for each compound. An auto-tune of the GCMS was carried out prior to the analysis to ensure optimal GC/MS performance. A set of external standards was also run at the start and end of the sample set and abundances were compared to known amounts to ensure that both the thermal desorption extraction and MS detection was performing within specification.

## 3. Results

### 3.1. Enumeration, $A_w$ and pH

The microbiota present in milks, curds, pasta filata (spune paste) during manufacturing and Provolone del Monaco cheeses throughout ripening process was enumerated by cultivating on different media with the intention of selecting different groups of Lactic Acid Bacteria. Figure 31 shows the microbial performance on different media used. Counts determined on LM17 were higher (red and blue curves) than Rogosa (green and yellow curves) and Kanamycin (black curve) media in the milk and throughout the whole cheese-making process. Microbial counts on Kanamicyn medium showed a development trend comparable to others (Rogosa and LM17) even if always characterized by lower levels.

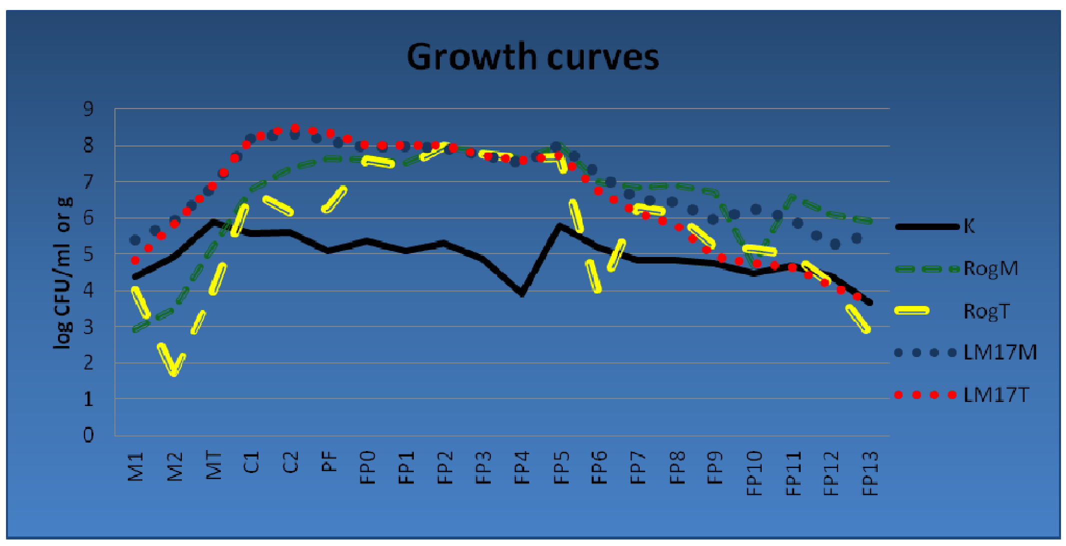



**Fig. 31.** The growth curves of all the samples analysed

Performance (log CFU/ ml or g) of rod and coccus presumptive lactic acid bacteria in:
Fresh Milk from Holstein Friesian, Alpine Brown, Simmental Red, Jersey, Podolica and Métis locals breeds (M1); Fresh Milk from Agerolese breed (M2); Milk after heating Treatment < 40°C (MT); Curd before acidification (C1); Curd after acidification (C2); pasta filata in hot water at 80-90°C (PF); Formaggio Provolone after salting brine (FP0) and at different time of drying or ripening (FP1, 2, 3, 4, 5, 6, 7, 8, 9,....).
Counts determined on Kanamycin medium (K), on Rogosa media (RogM and RogT incubated at 30 and 44°C, respectively) and on LM17 media (LM17M and LM17T incubated at 30 and 44°C, respectively).

The microbial counts in different samples is shown in Table 8. Counts of milk (M1) obtained from different cattle breeds had slightly lower levels than those of milk (M2) from local Agerolese breed. There was an exception for the presumptive thermophilic lactobacilli, higher values were found in the first (M1) than in the second (M2) sample (4.02 log CFU/ml in confront to 1.69 log CFU/ml). Population levels in milk (MT) after heating treatment (not higher than 40°C)

showed an increased for all media; probably due to the fact that this temperature is normally used to facilitates growth. Curd acidification, pasta filata (spune paste) stretching in hot water (80–90 °C) and salting in brine of the young cheese did not seem to considerably affect the microbial contents; LAB kept values ranging from 5,07 log CFU/g to 8,35 log CFU/g in these samples (C1, C2, PF, FP0 and FP1). At 20th day of drying the values are roughly constant in all media. Counts decreased during maturation period until 81 days and then they started to increase reaching highest level at 123th day, it happens before cheese is ready to be sold (one hundred and eighty days or about six months). A progressive reduction of all the counts was observed since 207th day until the end of ripening period.

**Table 8**. The microbial counts in different samples

| Day | Sample | K | RogM | RogT | LM17M | LM17T |
|---|---|---|---|---|---|---|
| | | log CFU/ml o g | | | | |
| 0 | M1 | 4,37 | 2,93 | 4,02 | 5,38 | 4,84 |
| 0 | M2 | 4,93 | 3,48 | 1,69 | 5,91 | 5,83 |
| 0 | MT | 5,88 | 5,19 | 3,96 | 6,86 | 6,89 |
| 0 | C1 | 5,56 | 6,77 | 6,75 | 8,2 | 8,2 |
| 0 | C2 | 5,59 | 7,37 | 6,17 | 8,33 | 8,51 |
| 0 | PF | 5,09 | 7,65 | 6,26 | 8,12 | 8,35 |
| 1 | FP0 | 5,35 | 7,6 | 7,62 | 7,93 | 8,01 |
| 5 | FP1 | 5,07 | 7,51 | 7,42 | 7,96 | 8,01 |
| 20 | FP2 | 5,3 | 7,94 | 7,96 | 7,91 | 8 |
| 49 | FP3 | 4,88 | 7,83 | 7,77 | 7,74 | 7,72 |
| 81 | FP4 | 3,9 | 7,59 | 7,64 | 7,52 | 7,56 |
| 123 | FP5 | 5,8 | 8,01 | 7,67 | 8,02 | 7,72 |
| 207 | FP6 | 5,16 | 7 | 3,89 | 7,22 | 6,75 |
| 257 | FP7 | 4,85 | 6,83 | 6,28 | 6,51 | 6,13 |
| 314 | FP8 | 4,85 | 6,9 | 6,14 | 6,43 | 5,83 |
| 368 | FP9 | 4,75 | 6,7 | 5,19 | 5,93 | 4,93 |
| 404 | FP10 | 4,48 | 4,69 | 5,13 | 6,26 | 4,73 |
| 444 | FP11 | 4,69 | 6,6 | 4,98 | 5,98 | 4,66 |
| 494 | FP12 | 4,4 | 6,1 | 4,2 | 5,25 | 4,12 |
| 598 | FP13 | 3,68 | 5,9 | 2,8 | 5,53 | 3,71 |

The results of water activity and pH are shown in Figures 32 and 33. High $A_w$ was observed during manufacturing process and reached 0.90 in sample after salting (Fp0); this value was the lowest in the first 27 hours. An increasing $A_w$ value was observed after 5 days (0.98) and then $A_w$ values progressively decreased reaching lowest $A_w$ (0.85) at the end of sampling. The lowest pH resulted in the curd at the end of acidification (C2) and in the pasta filata (PF). Then it increased in cheese

before and after salting in brine. The pH since 20th day until the end of ripening was within 5.7-6 pH range.

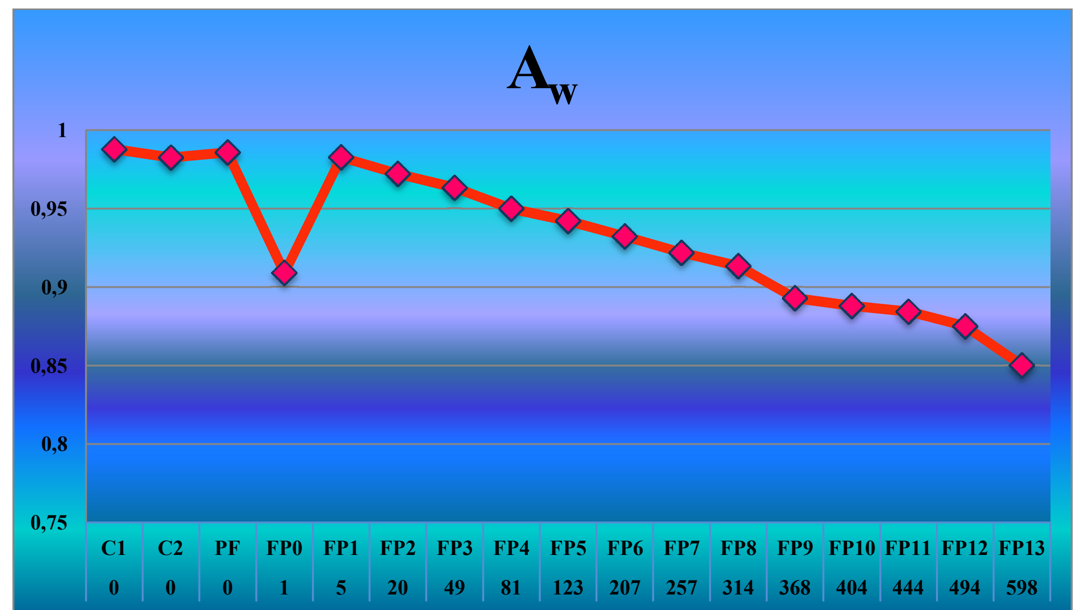



**Fig. 32.** $A_w$

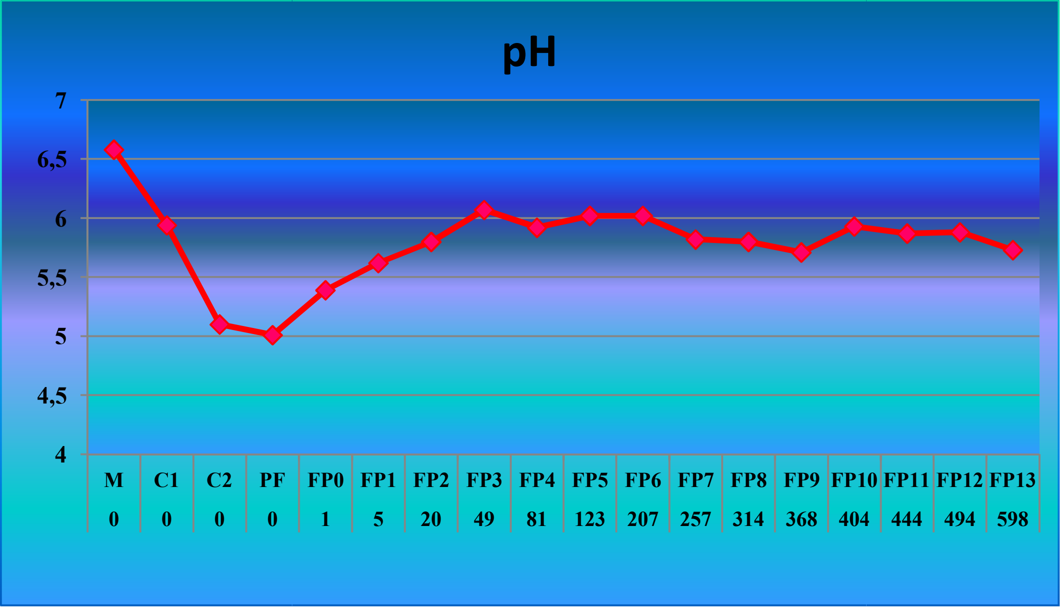



**Fig. 33.** pH

### 3.2. Genotypic identification of LAB

A total of 371 presumptive LAB isolates were activated by transfers to LM17 or MRS. Gram-positive, catalase negative, oxidase-negative isolates with rod or cocci shapes were considered presumptive LAB and selected for further examination. Pure colonies chosen were subjected to Pulsed Field Gel Electrophoresis (PFGE). Gel images were generated by PFGE with the following restriction enzymes: Asc I, Sma I and Apa I. Three fingerprint experiments were created (each one per restriction enzyme). For each experiment, BioNumerics comparison among all profiles were made. Using the 70% similarity level established by Fitzsimons et *al.,* (2001), the restriction enzyme Asc I generated 75 different restriction enzyme digestion profiles, Sma I generated 23 different profiles and Apa I generated 7 ones. Asc I generated 75 different restriction enzyme digestion profiles: 33 beloged to *casei/paracasei* group, 29 belonged to *Lb. delbrueckii*, 5 to *Lb. fermentum,* 5 to *Lb. rhamnosus,* 1 to *Lb. hilgardii,* 1 to *plantarum/pentosus* group and 1 to *Lb. buchneri* (Fig. 34 and 35).

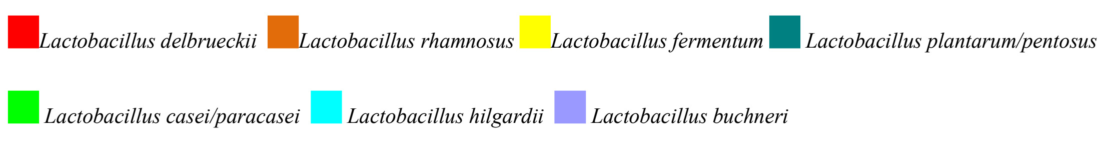
Pearson correlation (Opt:1.50%) [0.0%-100.0%]
PFGE-AscI

PFGE-AscI

40 60 80 100

Fp06 - LbT 03
Fp09 - LbT 08
Fp04 - LbT 03
Fp04 - LbM 10
Fp04 - LcT 13
Fp06 - LbT 09
Fp06 - LbT 05
Fp06 - LbT 08
Fp04 - LcT 02 (sm.c.)
Fp06 - LcT 01
Fp04 - LbT 01
Fp06 - LbM 04
Fp06 - LbT 02
Fp02 - LbM 06
Fp06 - LbT 01
Fp04 - LbM 04 (m.c.)
Fp03 - LbM 03
Fp02 - LbM 10
Fp04 - LbM 01
Fp06 - LbT 04
Fp09 - LbT 05
Fp04 - LbM 09
Fp06 - LbT 11
Fp04 - LbT 02
Fp04 - LbT 11
Fp04 - LcT 11
Fp04 - LbT 05
Fp02 - LbT 02
Fp04 - LcT 04
Fp04 - LbT 04
Fp04 - LbT 06
Fp04 - LbT 13
Fp09 - LbT 01
Fp04 - LcT 01 (r.c.)
Fp06 - LbM 01
Fp04 - LcT 12
Fp06 - LbM 02

**Fig. 34.** The dendrogam revealed diversity in PFGE patterns after digestion with Asc I (part n. 1)

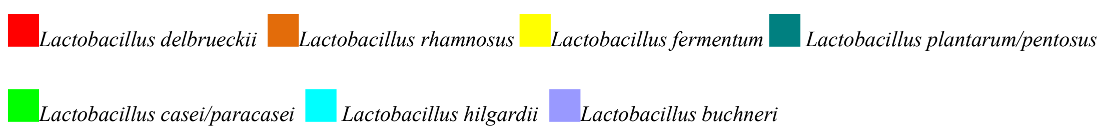



(continues)

(continues)

Fp09 - LbT 10
Fp11 - LbM 01 (w.c.)
Fp09 - LbT 04
Fp07 - LbM 02
Fp07 - LbM 03
Fp02 - LbT 05
Fp13 - LbM 02
Fp02 - LbM 07
Fp12 - LbM 04
Fp03 - LbM 02
Fp12 - LbM 02
Fp04 - LbM 08
Fp04 - LcM 11
Fp11 - LbM 02 (m.c.)
Fp04 - LbM 07
Fp09 - LcM 03
Fp06 - LcM 09
PF - LbM 02
Fp04 - LbM 02
MT - LbM 01
Fp02 - LbM 03
Fp06 - LbM 09
Fp09 - LbM 04
Fp04 - LcM 09
Fp04 - LbM 13
Fp09 - LbM 10
Fp02 - LbM 05
Fp02 - LbM 08
Fp13 - LbM 04
Fp09 - LbM 06
Fp09 - LbM 08
Fp09 - LbM 02
Fp11 - LbM 07
Fp11 - LbM 06
Fp06 - LbM 05
M02 - LbM 01
Fp06 - LbM 08
Fp06 - LbM 03

**Fig. 35.** The dendrogam revealed diversity in PFGE patterns after digestion with Asc I (part n. 2)



Sma I generated 23 different restriction enzyme digestion profiles: 3 belonged to *S. macedonicus,* 4 to *S. lutetiensis,* 3 to *L. lactis,* 1 to *S. thermophilus,* 7 to *E. faecium,* 3 to *E. fecalis* and 2 to *E. durans* (Fig. 36).

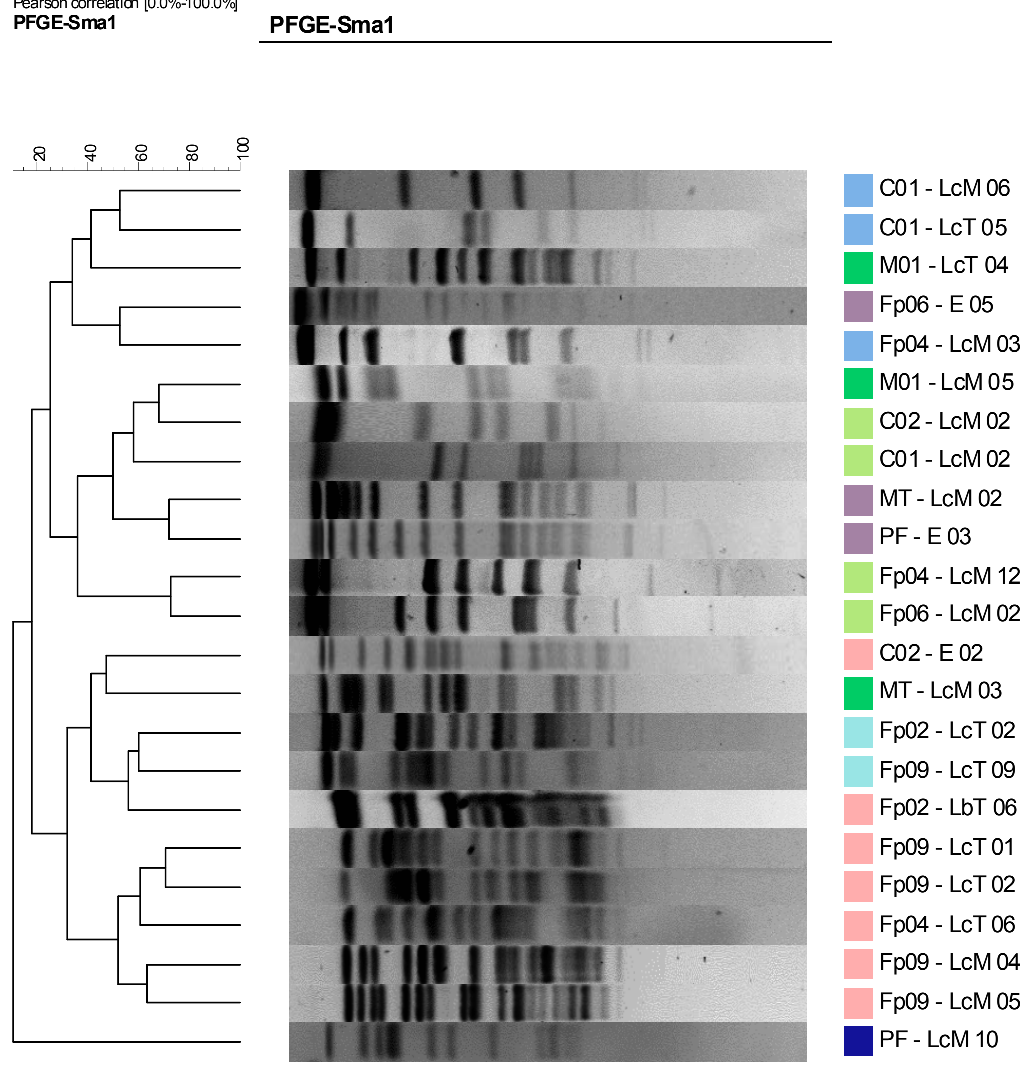
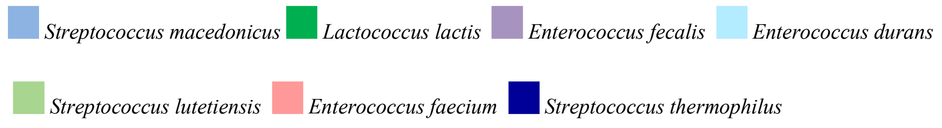



**Fig. 36.** The dendrogam revealed diversity in PFGE patterns after digestion with Sma I



Apa I generated 7 different restriction enzyme digestion profiles: 3 belonged to *Lb. helveticus,* 1 to *L. lactis,* 1 to *S. lutetiensis* and 2 to *S. macedonicus* (Fig. 37).

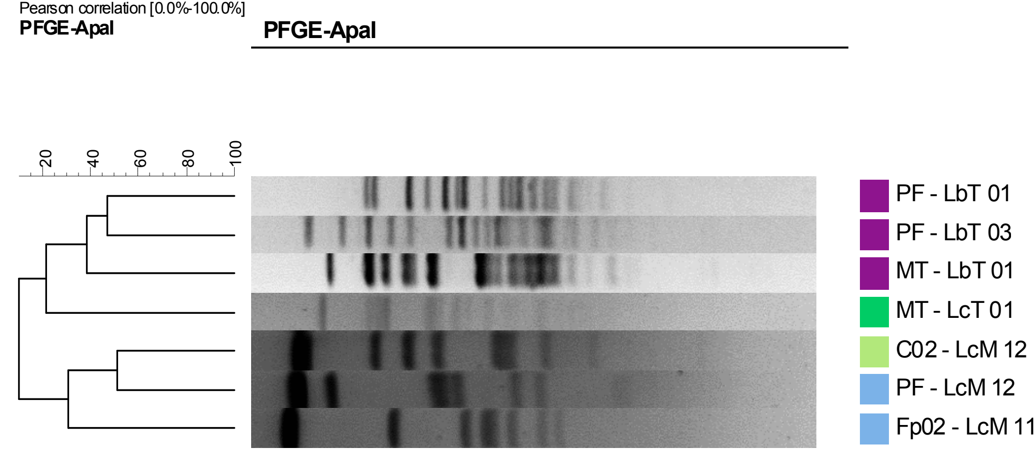



**Fig. 37.** The dendrogam revealed diversity in PFGE patterns after digestion with ApaI

A total of 105 different PFGE types were distinguished; different strains were taken and send in external laboratory (Eurofins Genomics, Germany) for 16S rDNA Sequence Analysis. The assembling of 16S rDNA sequences forward and reverse were made and the assembled 16S rDNA was compared with known 16S rDNA gene sequences in the NCBI database. A BLAST search of the 16S rRNA gene sequences obtained was then performed at NCBI revealing high similarity values (99-100%) to a number of sequences in the GenBank database. The GenBank database sometimes gave two different species as a result of sequencing 16S rRNA. The two species showed same score and high percentage similarity as compare to the GenBank reference strain (Fig. 38 a, 38 b and 38 c).

| | Description | Max score | Total score | Query cover | E value | Ident | Accession |
|---|---|---|---|---|---|---|---|
| ☐ | Lactobacillus paracasei 16S ribosomal RNA, complete sequence | 2385 | 2385 | 100% | 0.0 | 100% | NR_121787.1 |
| ☐ | Lactobacillus casei ATCC 334 strain ATCC 334 16S ribosomal RNA, complete sequence | 2385 | 2385 | 100% | 0.0 | 100% | NR_075032.1 |
| ☐ | Lactobacillus paracasei subsp. tolerans strain NBRC 15906 16S ribosomal RNA gene, partial sequence | 2385 | 2385 | 100% | 0.0 | 100% | NR_041054.1 |
| ☐ | Lactobacillus paracasei strain R094 16S ribosomal RNA gene, partial sequence | 2385 | 2385 | 100% | 0.0 | 100% | NR_025880.1 |

**Fig. 38. a**

| | Description | Max score | Total score | Query cover | E value | Ident | Accession |
|---|---|---|---|---|---|---|---|
| ☐ | Lactobacillus plantarum WCFS1 strain WCFS1 16S ribosomal RNA, complete sequence | 2551 | 2551 | 100% | 0.0 | 100% | NR_075041.1 |
| ☐ | Lactobacillus plantarum strain CIP 103151 16S ribosomal RNA gene, partial sequence | 2551 | 2551 | 100% | 0.0 | 100% | NR_104573.1 |
| ☐ | Lactobacillus plantarum strain JCM 1149 16S ribosomal RNA gene, partial sequence | 2551 | 2551 | 100% | 0.0 | 100% | NR_117813.1 |
| ☐ | Lactobacillus plantarum strain NRRL B-14768 16S ribosomal RNA gene, partial sequence | 2551 | 2551 | 100% | 0.0 | 100% | NR_042394.1 |
| ☐ | Lactobacillus pentosus strain 124-2 16S ribosomal RNA gene, partial sequence | 2551 | 2551 | 100% | 0.0 | 100% | NR_029133.1 |

**Fig. 38. b**

| | Description | Max score | Total score | Query cover | E value | Ident | Accession |
|---|---|---|---|---|---|---|---|
| ☐ | Streptococcus lutetiensis 16S ribosomal RNA, complete sequence | 2340 | 2340 | 100% | 0.0 | 100% | NR_121743.1 |
| ☐ | Streptococcus infantarius subsp. infantarius CJ18 strain CJ18 16S ribosomal RNA, complete sequence | 2340 | 2340 | 100% | 0.0 | 100% | NR_102799.1 |
| ☐ | Streptococcus lutetiensis strain CIP 106849 16S ribosomal RNA gene, partial sequence | 2340 | 2340 | 100% | 0.0 | 100% | NR_115719.1 |
| ☐ | Streptococcus lutetiensis strain HDP90246 16S ribosomal RNA gene, partial sequence | 2340 | 2340 | 100% | 0.0 | 100% | NR_037096.1 |
| ☐ | Streptococcus lutetiensis strain NEM 782 16S ribosomal RNA gene, partial sequence | 2340 | 2340 | 100% | 0.0 | 100% | NR_042051.1 |

**Fig. 38. c**

Examples of two species with same similarity and score

The distribution of the LAB species isolated from milk, curds, pasta filata and different cheese samples and their isolation frequencies are shown in table 9. In this study, it was found that the dominant population among isolates was lactobacilli. The predominant species in almost all cheese samples was *Lactobacillus casei/paracasei* (19,4%) followed by *Lactobacillus delbrueckii* (12,7%). *Lactobacillus casei/paracasei* not only represented the majority of the isolates, especially during cheese ripening, but it was during manufacturing process in milk and pasta filata. *Lactobacillus rhamnosus* (6,2%) isolates were present from the beginning of the ripening up to one year. Other lactobacilli were detected in different samples such as, *Lactobacillus hilgardii* (3,8%) *Lactobacillus fermentum* (3%) and *Lactobacillus helveticus* (1,6%). They were in milk and also in aged cheese samples. Only one isolate of 175 lactobacilli was assigned to the species *Lactobacillus bucknerii* (0,3%) and *Lactobacillus plantarum/pentosus* (0,3%). It is important to highlights that the media used for lactobacilli (Rogosa agar) were highly selective for these groups. In contrast, microorganisms detected in LM17 agar belong to several different genera; among them there were once again *Lactobacillus* genus and many microbial colonies of the *Streptococcus*, *Lactococcus* and *Enterococcus* genera. *Streptococcus macedonicus* (19.1%) among cocci was the dominant species followed by *Streptoccocus lutetiensis/infantarius* (10,8%). The first one was in all phases of the cheese manufacture and until 81 days in cheese; the second one was very represented quantitatively with a lot of isolates in curd and present until one year. Only one *Streptoccocus thermophilus* (0,3%) isolate was detected in pasta filata sample. *Lactococcus lactis* (5,1%) was exclusively in the milk and in the curd samples, and it was never detected during cheese ripening. Kanamicyn agar showed to be very selective medium for bacteria belong to *Enterococcus* genus, especially for *Enterococcus facalis* (13,2%) which was always present in all analysed samples. *Enterococcus faecium* (3,5%) and *Enteroccus durans* (0,8%) were isolated mostly from LM17 during ripening time.

**Table 9.** Genotypic identification of the LAB isolates using 16S rDNA Sequence Analysis and distribution of these species in all samples

| Samples | Milk | | | Curd | | Pasta filata | Cheese Ripening time (days) | | | | | | | | | Isolates | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Genotypic Identification | M1 | M2 | MT | C1 | C2 | PF | Fp2 | Fp3 | Fp4 | Fp6 | Fp7 | Fp9 | Fp11 | Fp12 | Fp13 | | |
| | | | | | | | 20 | 49 | 81 | 207 | 257 | 368 | 444 | 494 | 598 | n* | %** |
| *Lb. casei/paracasei* | 2 | 5 | | | | 11 | 6 | 1 | 9 | 7 | 2 | 13 | 8 | 1 | 6 | 71 | 19,4 |
| *Lb. delbrueckii* | | | | | | | 6 | | 20 | 15 | | 7 | | | | 48 | 12,7 |
| *Lb. rhamnosus* | | | | | | | 2 | 1 | 5 | 8 | 3 | 4 | | | | 23 | 6,2 |
| *Lb. hilgardii* | | | 7 | | | | | | | | | | 4 | | 3 | 14 | 3,8 |
| *Lb. fermentum* | 5 | | | | | | 2 | | 2 | | | 1 | | 1 | | 11 | 3,0 |
| *Lb. helveticus* | | | 2 | | | 4 | | | | | | | | | | 6 | 1,6 |
| *Lb. bucknerii* | | | | | | | | | | | | | | | 1 | 1 | 0,3 |
| *Lb. plantarum/pentosus* | | | | | | | | 1 | | | | | | | | 1 | 0,3 |
| *S. macedonicus* | 1 | 11 | 2 | 4 | 7 | 22 | 19 | | 5 | | | | | | | 71 | 19,1 |
| *S. lutetiensis/infantarius* | | | | 15 | 15 | | | | 2 | 7 | | 1 | | | | 40 | 10,8 |
| *S. thermophilus* | | | | | | 1 | | | | | | | | | | 1 | 0,3 |
| *L. lactis* | 11 | | 7 | 1 | | | | | | | | | | | | 19 | 5,1 |
| *E. fecalis* | 1 | 5 | 9 | 1 | 4 | 5 | 5 | | 6 | 5 | | 8 | | | | 49 | 13,2 |
| *E. faecium* | | | | | 1 | | 1 | | 1 | | | 10 | | | | 13 | 3,5 |
| *E. durans* | | | | | | | 2 | | | | | 1 | | | | 3 | 0,8 |
| Total | 20 | 21 | 27 | 21 | 27 | 43 | 43 | 3 | 50 | 42 | 5 | 45 | 12 | 2 | 10 | 371 | 100,0 |

*Isolation number (n) and **percentages (%) are reported, too

### 3.3. Characterization of selected LAB isolates

The capability of the selected isolates to acidify the skim milk in the shortest possible time was evaluated in this part of study. This is a useful feature if the organisms isolated can be used as starter cultures.

Biogenic amines and bacteriocins produced by LAB have long been the subject of study. The identification of some bacteriocinogenic and aminobiogenic strains provides useful information concerning food safety.

#### 3.3.1. Acidification capability

The acidifying of LAB generally recognized as safe and potentially utilized as starters or flavor-producing adjuncts were investigated. A first screening was made evaluating LAB acidification in tubes containing 10 ml of RSM 10% after incubation overnight. Only 46 strains acidified at least 1 unit and they were monitored by using the Cinac system. Three classes were defined (Roushdy, 1999): class I included strains that lowered the pH to $> 6.0$, while class II and class III comprised strains that reached $6 < pH < 5.5$ and $pH \leq 5.5$, respectively. After 8 h of incubation fermentation, 17 strains, which were classified as fast acid producers, fell into class III: 12 of these were *Lb. delbrueckii*, 2 strains belonged to *S. macedonicus*, while the other 3 belonged to *S. thermophilus*, *L. lactis* and *S. lutetiensis*. Although the acid production were initially slow for the remaining selected strains, 22 of them was enhanced after 16 hours, whereas the other remaining 6 strains after approximately 2 hours, 28 further strains fell into class III. Table 10. All strains screened in tubes containing 10 ml of RSM 10% fell into class I and II group (data not shown).

**Table 10.** Acidification capability of selected LAB isolates

| Species | Strain | 8 h | 16 h | 18 h | Group |
|---|---|---|---|---|---|
| *Lb. delbrueckii* | Fp04 - LbT 06 | 4,49 | 3,85 | 3,8 | III |
| | Fp04 - LcT 04 | 4,58 | 3,86 | 3,8 | III |
| | Fp04 - LbT 12 | 4,58 | 3,74 | 3,68 | III |
| | Fp04 - LbT 13 | 4,64 | 3,82 | 3,78 | III |
| | Fp04 - LbT 05 | 4,59 | 3,92 | 3,87 | III |
| | Fp06 - LcT 10 | 4,75 | 3,98 | 3,9 | III |
| | Fp04- LcT 04 | 5,06 | 4,02 | 3,93 | III |
| | Fp02 - LbT 02 | 5,14 | 4,11 | 4,03 | III |
| | Fp06 - LbT 04 | 5,21 | 4,28 | 4,08 | III |
| | Fp04 - LcT 13 | 5,42 | 5,1 | 5,01 | III |
| | Fp 09 - LbT 01 | 5,43 | 4,62 | 4,46 | III |
| | Fp04 - LbT 11 | 5,47 | 4,26 | 4,12 | III |
| | Fp06 - LbT 01 | 5,86 | 4,25 | 4,05 | III |
| | Fp04 - LbT 03 | 5,88 | 4,28 | 4,03 | III |
| | Fp04 - LcT 01 (r.c.) | 5,91 | 4,68 | 4,49 | III |
| | Fp04 - LbT 02 | 5,99 | 4,74 | 4,56 | III |
| *Lb. casei/paracasei* | Fp12 - LbM 2 | 5,9 | 5,21 | 5,11 | III |
| | Fp02 - LbM 08 | 5,95 | 5,23 | 5,03 | III |
| | Fp09 - LbM 10 | 5,96 | 5,22 | 5,07 | III |
| | Fp06 - LbM 05 | 5,97 | 5,28 | 5,1 | III |
| | Fp02 - LbM 07 | 6,06 | 5,41 | 5,26 | III |
| | Fp02 - LbM 05 | 6,06 | 5,61 | 5,54 | III |
| | Fp07 - LbM 02 | 6,07 | 5,56 | 5,41 | III |
| | Fp11 - LbM 07 | 6,12 | 5,74 | 5,63 | III |
| | Fp02 - LbT 05 | 6,13 | 5,54 | 5,33 | III |
| | Fp13 - LbM 08 | 6,15 | 5,68 | 5,54 | III |
| *Lb. rhamnosus* | Fp06 - LbT 04 | 5,69 | 4,61 | 4,42 | III |
| | Fp06 - LbT 11 | 5,79 | 5 | 4,83 | III |
| | Fp06 - LcM 07 | 5,83 | 4,95 | 4,79 | III |
| *Lb. helveticus* | PF - LbT 03 | 5,86 | 4,92 | 4,56 | III |
| **RSM 10%** | Control - | 6,5 | 6,49 | 6,37 | |
| *Lc. lactis* | MT- LcM 03 | 5,42 | 4,86 | 4,77 | III |
| | MT - LcT 08 | 5,74 | 5,14 | 5,03 | III |
| | M01 - LcT 04 | 5,78 | 5,19 | 5,1 | III |
| | M01 - E 03 | 5,82 | 5,22 | 5,12 | III |
| | M01 - LcT 02 | 6,22 | 5,81 | 5,6 | III |
| *S. thermophilus* | PF - LcM 10 | 5 | 4,46 | 4,38 | III |
| *S. macedonicus* | Fp02 -LcM 11 | 5 | 4,51 | 4,44 | III |
| | PF - LcT 01 | 5,4 | 4,81 | 4,74 | III |
| | PF - LcM 12 | 5,69 | 4,75 | 4,65 | III |
| | C01 - LcT 05 | 5,74 | 5,05 | 4,91 | III |
| | PF - LcT 05 | 5,77 | 5,22 | 5,13 | III |
| | MT - LcT 06 | 5,81 | 5,12 | 4,97 | III |
| *S. lutetiensis/infantarius* | Fp06 - LcT 06 | 5,42 | 4,58 | 4,48 | III |
| | C01 - LcM 01 | 5,52 | 5,04 | 4,93 | III |
| | C02 - LcM 05 | 5,59 | 5,05 | 4,93 | III |

The data are expressed as mean values

### 3.3.2. Biogenic Amines using DSM

Identified strains were screened on histidine and tyrosine DSM media and only tyramine-producing strains were found. The tyramine-producing strains that were identified were *Lactobacillus casei/paracasei* (1 strain) *Enterococcus faecium* (5 strains), *Enterococcus fecalis* (6 strains) and *Enterococcus durans* (1 strain). The screening was subsequently repeated on all enterococci and not only on those sequenced. It was observed that all those isolated from kanamycin were (*E. fecalis* and *E. faecium*) tyramine producing (Fig. 39).

**Fig. 39.** *Enterococcus fecalis*

### 3.3.3. Bacteriocins

#### 3.3.3.a. Well diffusion assay, effect of proteinase K, pH and heat treatment on bacteriocin stability

Sequenced strains were screened for antibacterial activity by the agar well diffusion assay. Supernatants of LAB strains were tested against *Listeria innocua* and only two strains showed anti-Listeria activity. 16S rDNA sequence analysis identified these strains as *Lactococcus lactis* and *Enterococcus faecium.*

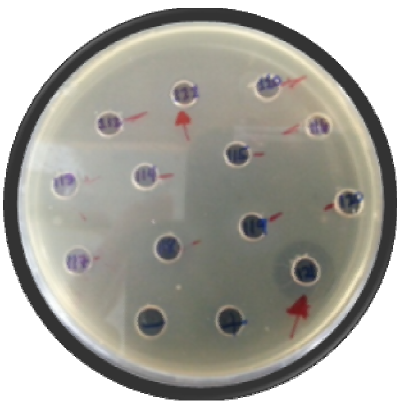


**Fig. 40.**

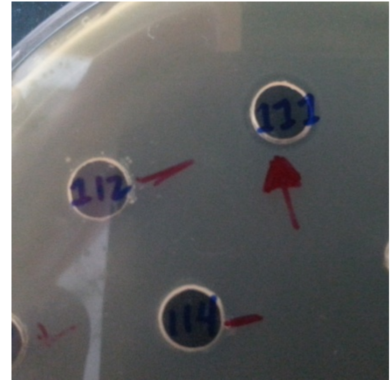


**Fig.41** *Lactococcus lactis* (1-2mm) **Fig.42.** *Enterococcus faecium* (5-6 mm)

The zones of inhibition in the agar were visually observed and measured.

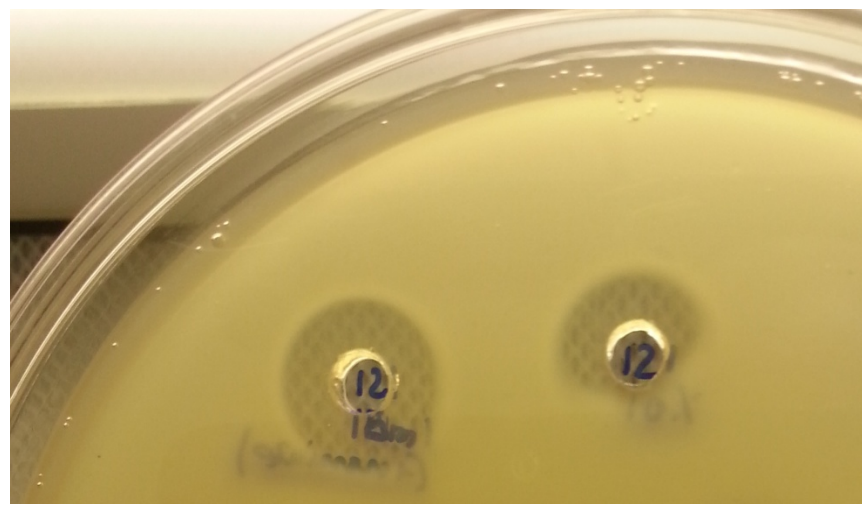



**Fig. 43**. Antimicrobial activity exhibited by *E. faecium* by using proteinase K

The antimicrobial activity exhibited by *E. faecium* (Fig. 43) and *L. lactis* was completely inactivated by using 3 µl of proteinase K (10 mg/ml) near the wells.

**Table 11.** Antimicrobial spectrum of *Enterococcus faecium* and *Lactooccus lactis* isolated from Provolone del Monaco P.D.O. cheese

<table>
<tr><th rowspan="3">Strain</th><th rowspan="3">Bacteriocin</th><th colspan="11">Inhibitory activity (mm)</th></tr>
<tr><th colspan="11">pH (37° C)</th></tr>
<tr><td>7</td><td colspan="2">2</td><td colspan="2">4</td><td colspan="2">6</td><td colspan="2">8</td><td colspan="2">10</td></tr>
<tr><td></td><td></td><td>o.n.*</td><td>30'</td><td>90'</td><td>30'</td><td>90'</td><td>30'</td><td>90'</td><td>30'</td><td>90'</td><td>30'</td><td>90'</td></tr>
<tr><td>E. faecium</td><td>Enterocin A</td><td>5-6</td><td>5-6</td><td>5-6</td><td>5-6</td><td>5-6</td><td>5-6</td><td>5-6</td><td>5-6</td><td>5-6</td><td>5-6</td><td>5-6</td></tr>
<tr><td>L. lactis</td><td>Nisin A</td><td>1-2</td><td>inact. **</td><td>inact. **</td><td>1-2</td><td>1-2</td><td>1-2</td><td>1-2</td><td>1-2</td><td>1-2</td><td>1-2</td><td>1-2</td></tr>
<tr><td rowspan="3"></td><td rowspan="3"></td><td colspan="9">Temperature (pH 7)</td><td colspan="2" rowspan="3">Protein. K</td></tr>
<tr><td>37°C</td><td colspan="4">60°C</td><td colspan="4">100°C</td></tr>
<tr><td>o.n. *</td><td>10'</td><td>30'</td><td>60'</td><td>90'</td><td>10'</td><td>30'</td><td>60'</td><td>90'</td></tr>
<tr><td>E. faecium</td><td>Enterocin A</td><td>5-6</td><td>5-6</td><td>5-6</td><td>4-5</td><td>4-5</td><td>5-6</td><td>4-5</td><td>1-2</td><td>inact.</td><td colspan="2">inact.</td></tr>
<tr><td>L. lactis</td><td>Nisin A</td><td>1-2</td><td>1<</td><td>inact. **</td><td>inact. **</td><td>inact. **</td><td>1<</td><td>inact. **</td><td>inact. **</td><td>inact. **</td><td colspan="2">inact. **</td></tr>
</table>

*o.n., over night
**inact., Bacteriocin inactivation after treatment, no presence of halo

The antimicrobial substances produced by *E. faecium* was effective within the 2-10 pH range, whereas inhibition activity exhibited by *L. lactis* was present within the 4-10 pH range and absent at pH 2 after 30 and 90 minues of incubation at 37°C. The thermo-stability of bacteriocins produced by *E. faecium* and *L. lactis* was also investigated. It was found that treatment of the extracellular extracts of *E. faecium* bacteriocin-producing strain at 60 °C for 30 minutes and 100 °C for 10 minutes did

not result in any loss of antimicrobial activity. A slight antimicrobial activity reduction of *E. faecium* was observed at 60 °C for 60 and 90 minutes, and also at 100 °C for 30 minutes, while a strong reduction of the zones of inhibition was observed at 100 °C for 60 minutes. However, bacteriocin produced by *Enterococcus* was completely inactivated after treatment at 100 °C for 90 minutes. The thermo-stability of bacteriocins produced by *L. lactis* was still present but reduced at 60 °C and 100 °C after 10 minutes and was not presence using both temperature after 30 minutes (Table 11).

### 3.3.3.b. Bacteriocin identification by MS

For *Lactococcus lactis*, the MALDI-TOF results indicated that the molecular weight of the purified bacteriocins was approximately 3354 Da and it was found to be similar to Nisin A (Fig. 44).

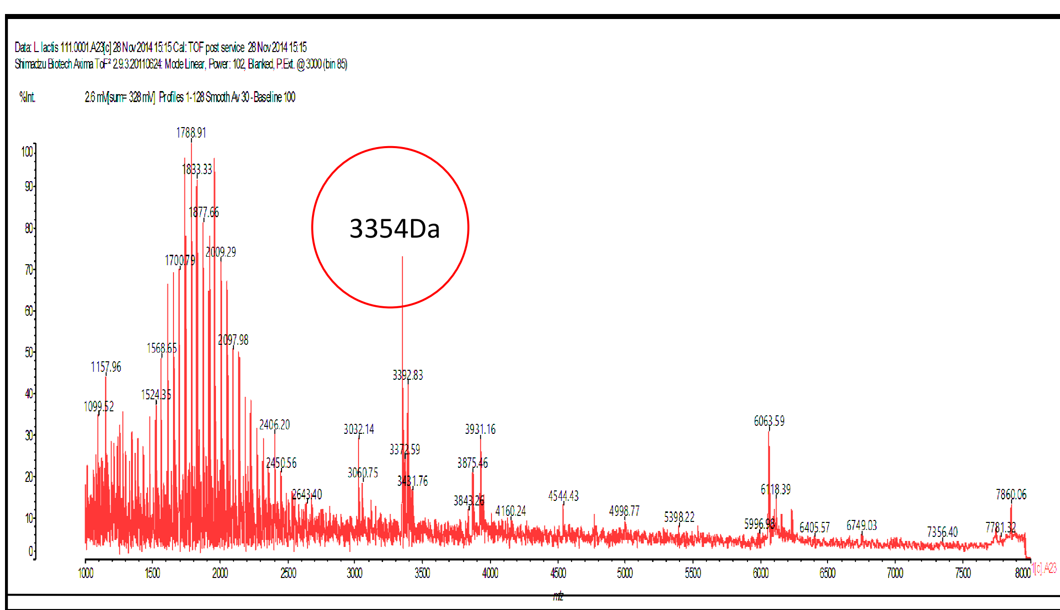



**Fig. 44.** MALDI-TOF mass spectrophotometry of *Lactococcus lactis* bacteriocin

For *Enterococcus faecium*, the spectrum showed different peaks and it was supposed the presence of more than one bacteriocin produced (Fig. 45).

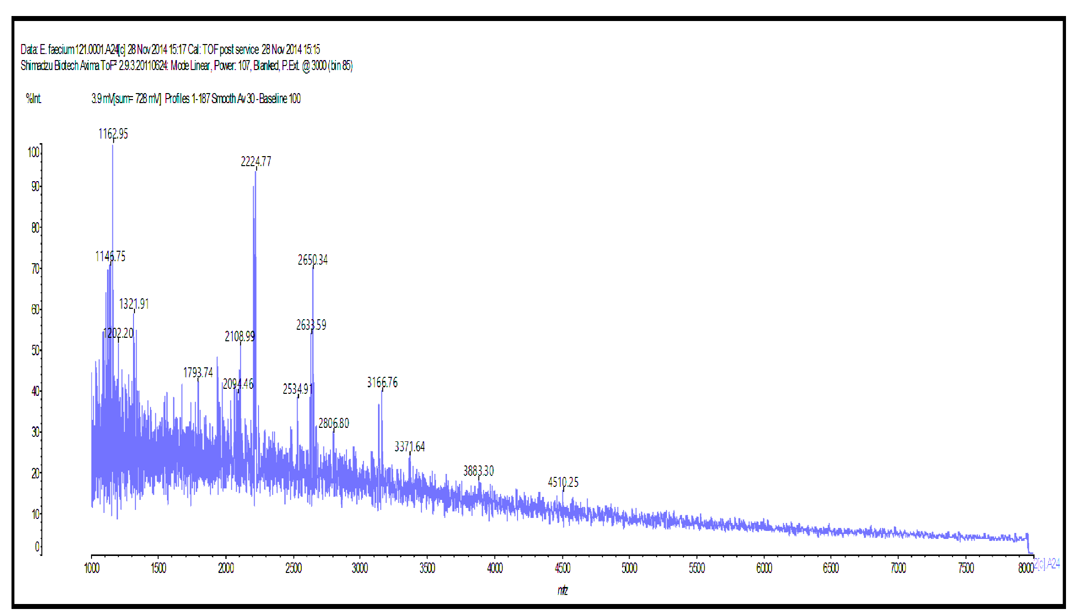



**Fig. 45.** MALDI-TOF mass spectrophotometry of *Enterococcus faecium* bacteriocins

The bacteriocins from *Enterococcus faecium,* were purified and eluted with 70% IPA as describe in Material and Methods. Activity was assessed on *Listeria innocua* indicator plates. The cell supernatant was active, the C18 SPE column FT and 30% ethanol was not acidic as expected and activity was eluted in the 70% IPA eluent (shown in Figure 46). The 70% IPA eluent was assessed using MALDI TOF Mass Spectrometry in order to determinate weight of the purified bacteriocins.

**Fig. 46**

For *Enterococcus faecium,* the spectrum showed a number of potential bacteriocin masses (Fig. 47).

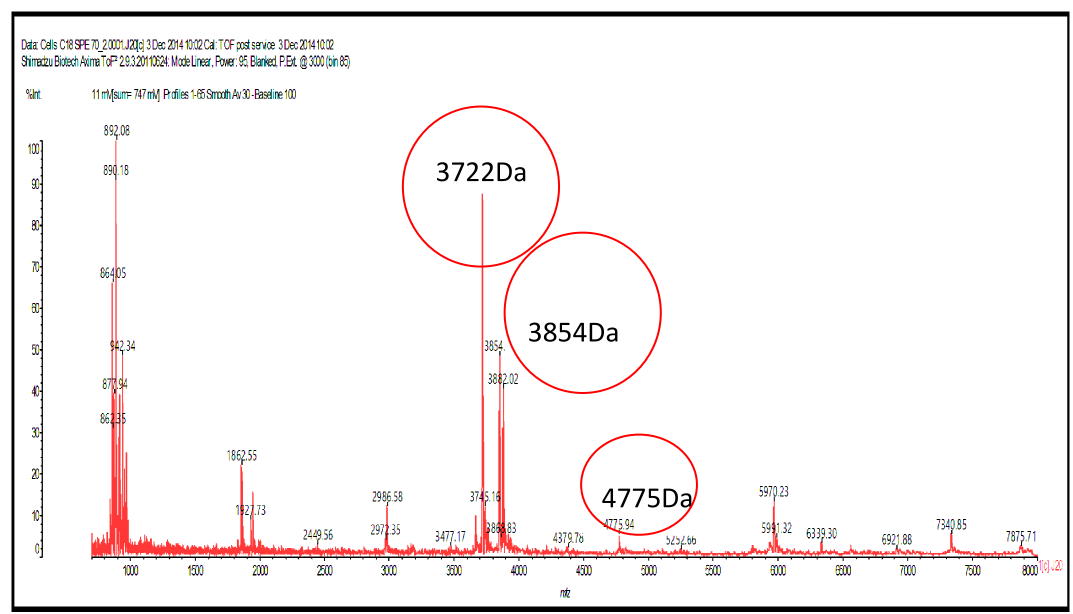



**Fig. 47** MALDI-TOF mass spectrophotometry of the 70% IPA eluent

A reverse-phase HPLC was used to separate the different bacteriocins instead to reduce the number of analytics (Fig. 48).

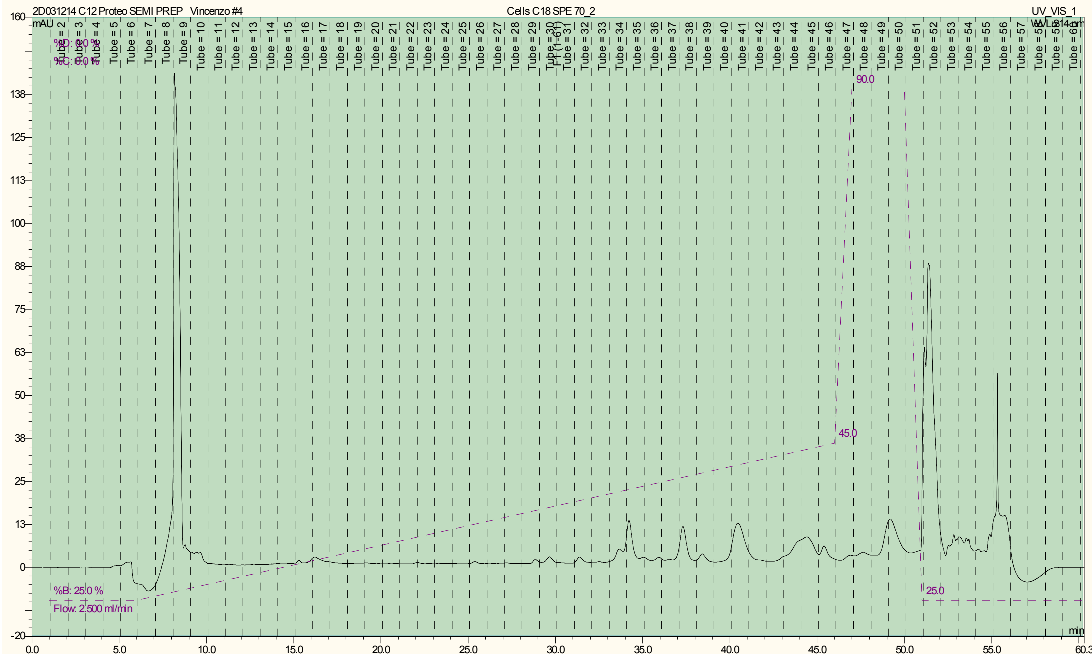


**Fig. 48.** HPLC Chromatogram

Different fractions were collected and assayed for bacteriocins activity. Positive results were found in the fractions number 29 and 30 (Fig. 49).

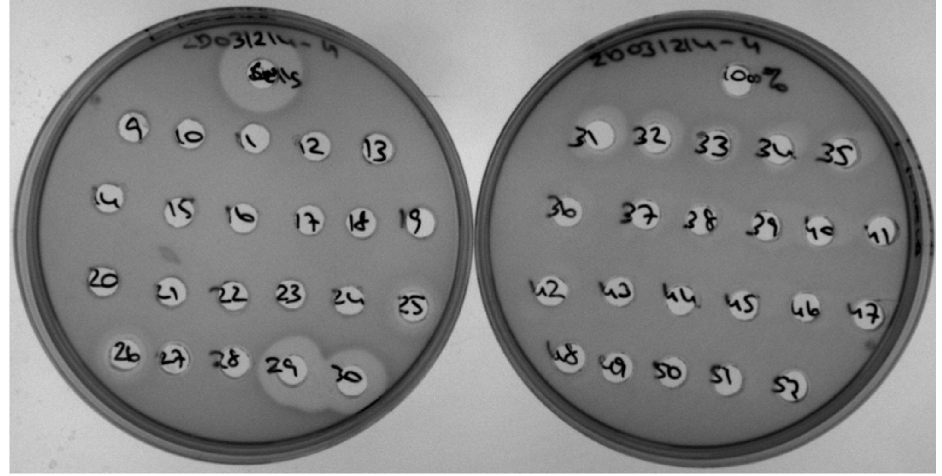



**Fig. 49.** *L. innocua* indicator plates show that activity elutes in fractions 29 and 30

The MS spectrum for fraction 29 shows a mass of 4829 Da which could correspond to the mass of enterocin A (4832 Da) (Aymerich et *al*,. 1996) (Fig. 50).

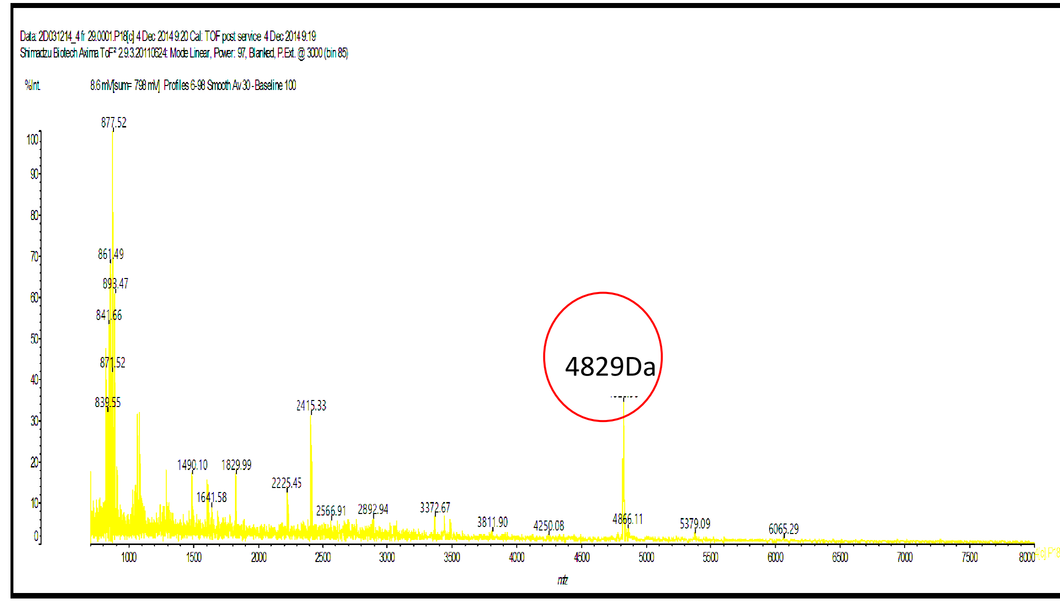



**Fig. 50** MALDI-TOF mass spectrophotometry of purified fractions

### 3.3.3.c. Bacteriocin identification by PCR

After agarose gel electrophoresis, a PCR fragment with a size of 138 bp was amplified from genomic DNA of *E. faecium*. The fragment obtained was that expected for enterocin A (Moreno et *al.*, 2003) (Fig. 51).

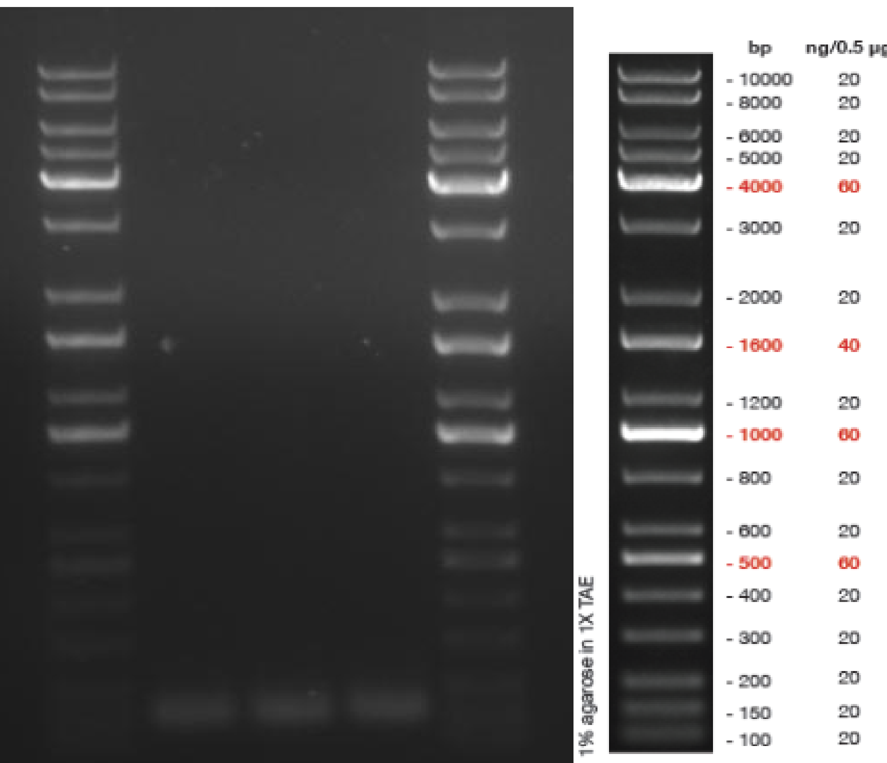



**Fig. 51.** PCR result for the detection of enterocin A

### 3.4. Biogenic Amines and Volatile Flavor Compounds

Three (N.1, N3, and N.4) of the four cheeses analysed (Fig. 52, 54 and 55) had the same typical sensorial characteristics of Provolone del Monaco P.D.O. cheese, while only one (N.2) showed the defect of medium-sized eyes (Fig.53). They were subjected by HPLC and TD-GC/MS analysis in order to investigate the amount of Biogenic Amines and identify volatile flavor compounds, respectively.

**Fig. 52.** Cheese N.1

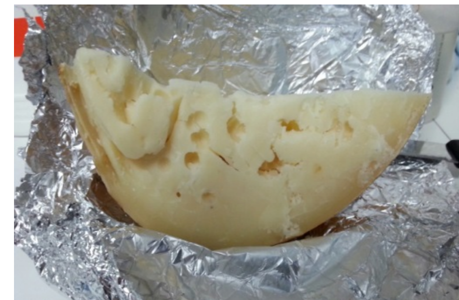


**Fig.53.** Cheese N.2

**Fig.54.** Cheese N.3

**Fig. 55.** Cheese N.4

#### 3.4.1. Biogenic Amines by HPLC

Four cheeses were analysed to detect Biogenic Amines, in particular cadaverine (CD), histamine (HI), putrescine (PU), tyramine (TY) and tryptamine (TR). BAs were detected in almost all four samples with the exception of putrescine. The concentration were very similar for samples N.1, N.3 and N.4, while sample N.2 showed highest average amine contents. Tyramine content was the most abundant BAs in three similar samples reaching minimum and maximum values of 104,89 and 153,42 mg/kg, respectively; tyramine value in cheese N.2 was 297,45 mg/kg. Cadaverine and putrescine in the three similar cheeses reached maximum values of 19,71 and 1,49 mg/kg, respectively. Cadaverine and putrescine showed highest concentration of 560,48 and 458,92 mg/kg in cheese N.2. Histamine value was lowest among BA in sample N.2 at level of 66,19 mg/kg and it was found in a range of 24,33 and 37,46 mg/kg in other samples. Tryptamine was detected in provolone

N.2 with a concentration of 88,62 mg/kg and in remaining three cheeses within 8,99-13,90 mg/kg concentration range (Table 12).

**Table 12. BA** concentration (mg/kg) determined by HPLC

| Sample | CD (mg/kg ) | HI (mg/kg ) | PU (mg/kg ) | TY (mg/kg ) | TR (mg/kg ) | Total BA (mg/kg ) |
|---|---|---|---|---|---|---|
| N.1 | 19,71 | 24,33 | 1,49 | 153,42 | 13,90 | 212,85 |
| N.2 | 560,48 | 66,19 | 458,92 | 297,45 | 88,62 | 1471,67 |
| N.3 | 12,49 | 26,47 | 1,00 | 104,89 | 8,99 | 153,84 |
| N.4 | 15,99 | 37,46 | 0,00 | 105,40 | 9,49 | 168,34 |

### 3.4.2. Volatile Flavor Compounds by TD-GS/MS

32 volatile compounds were positively identified and semi-quantified, 12 esters, 7 acids, 7 ketones, 3 aldehydes, 2 benzene, and 1 alcohol. As evident from Table 13, only 17 of the volatiles are found in all 4 samples, which indicates that the level of discrimination between the samples as a group is high. This is also evident by the principal component percentages; 40 (PC1) + 54 (PC2) = 94% (Fig. 56). In all samples, especially in N.2 and N.4, were found a very unusual high number of esters, some of which such as diethyl ether, propyl propionate, propyl butanoate, ethyl heptanoate and methyl octanoate are not typically found in cheese. Such high levels of esters would lead to very complex fruity flavor. Cheese samples N.1 and N.3 were the most similar with N.3 very strongly associated with ketones (acetone, 8-nonen-2-one, 2-nonanone and 2-octanone). N.1 was a little more strongly associated with acetic acid and with other ketones (2-pentanone and 2-heptanone). Sample N.2 was quite different to all other samples and strongly associated with esters such as n-propyl acetate, ethyl acetate, ethyl propionate, propyl propanoate, propyl butanoate and propyl hexanoate, but also with the secondary ketone, 2-butanone and secondary alcohol, 2-butanol. Sample N.4 was very different to all the other samples, with a strong association with a number of wide number of compounds; esters (ethyl butanoate, ethyl hexanoate, ethyl heptanoate, ethyl octanoate, methyl octanoate), acids (butanoic acid, hexanoic acid, heptanoic acid, octanoic acid, decanoic acid) plus benzene and styrene.

**Table 13.** Volatile compounds detected by TD-GC/MS analysis

| Name | Compound | N.1 | N.2 | N.3 | N.4 |
|---|---|---|---|---|---|
| **Esters** | Diethyl ether | 74.708 | 69.237 | 68.549 | 91.019 |
| | n-Propyl acetate | 0 | 10.120.749 | 0 | 0 |
| | Ethyl Acetate | 0 | 4.193.207 | 0 | 0 |
| | Propanoic acid, ethyl ester (ethyl propionate) | 0 | 8.476.718 | 0 | 0 |
| | Propanoic acid, propyl ester (propyl propionate) | 0 | 4.293.635 | 0 | 0 |
| | Butanoic acid, ethyl ester (ethyl butanoate) | 6.138.787 | 6.329.777 | 6.037.970 | 10.709.813 |
| | Butanoic acid, propyl ester (propyl butanoate) | 164.858 | 2.258.073 | 0 | 0 |
| | Hexanoic acid, ethyl ester (ethyl hexanoate) | 314.681 | 496.186 | 323.937 | 924.340 |
| | Hexanoic acid, propyl ester (propyl hexanoate) | 0 | 1.027.824 | 0 | 0 |
| | Heptanoic acid, ethyl ester (ethyl heptanoate) | 0 | 0 | 0 | 75.386 |
| | Octanoic acid, ethyl ester (ethyl octanoate) | 754.794 | 2.022.289 | 496.372 | 2.241.737 |
| | Octanoic acid, methyl ester (methyl octanoate) | 0 | 0 | 0 | 1.676.808 |
| **Ketones** | Acetone | 13.203.490 | 0 | 25.881.441 | 0 |
| | 2-Butanone | 20.180.722 | 425.400.629 | 6.205.135 | 0 |
| | 2-Pentanone | 283.706.173 | 51.818.897 | 209.265.631 | 70.262.380 |
| | 2-Heptanone | 14.047.514 | 3.663.350 | 12.097.391 | 11.317.289 |
| | 8-Nonen-2-one | 819.067 | 251.369 | 920.554 | 334.804 |
| | 2-Octanone | 1.111.001 | 0 | 10.312.924 | 1.113.069 |
| | 2-Nonanone | 14.378.397 | 4.075.686 | 19.490.311 | 9.146.247 |
| **Aldehydes** | Butanal, 2-methyl- | 899.688 | 433.261 | 971.865 | 851.693 |
| | Butanal, 3-methyl- | 2.329.346 | 755.784 | 2.839.778 | 2.530.571 |
| | Propanal, 2-methyl- | 731.176 | 876.705 | 1.003.066 | 284.411 |
| **Alcohol** | 2-Butanol | 9.645.877 | 1.057.246.878 | 1.086.324 | 0 |
| **Acids** | Acetic acid | 405.314.797 | 301.806.748 | 246.881.140 | 165.748.592 |
| | Propanoic acid | 139.634.586 | 292.964.711 | 0 | 0 |
| | Butanoic acid | 416.037.005 | 253.097.917 | 576.821.812 | 714.173.665 |
| | Hexanoic acid | 86.414.879 | 100.773.951 | 176.066.449 | 365.005.827 |
| | Heptanoic acid | 0 | 0 | 0 | 2.169.791 |
| | Octanoic acid | 7.579.775 | 10.583.646 | 18.275.018 | 37.501.326 |
| | Decanoic acid | 677.206 | 641.555 | 1.323.384 | 2.414.301 |
| **Benzenes** | Benzene | 1.056.683 | 1.277.470 | 1.553.389 | 2.959.096 |
| | Styrene | 180.162 | 0 | n/d* | 997.330 |

***n/d:** outside range of standards
Units are arbitrary peak areas or abundance values

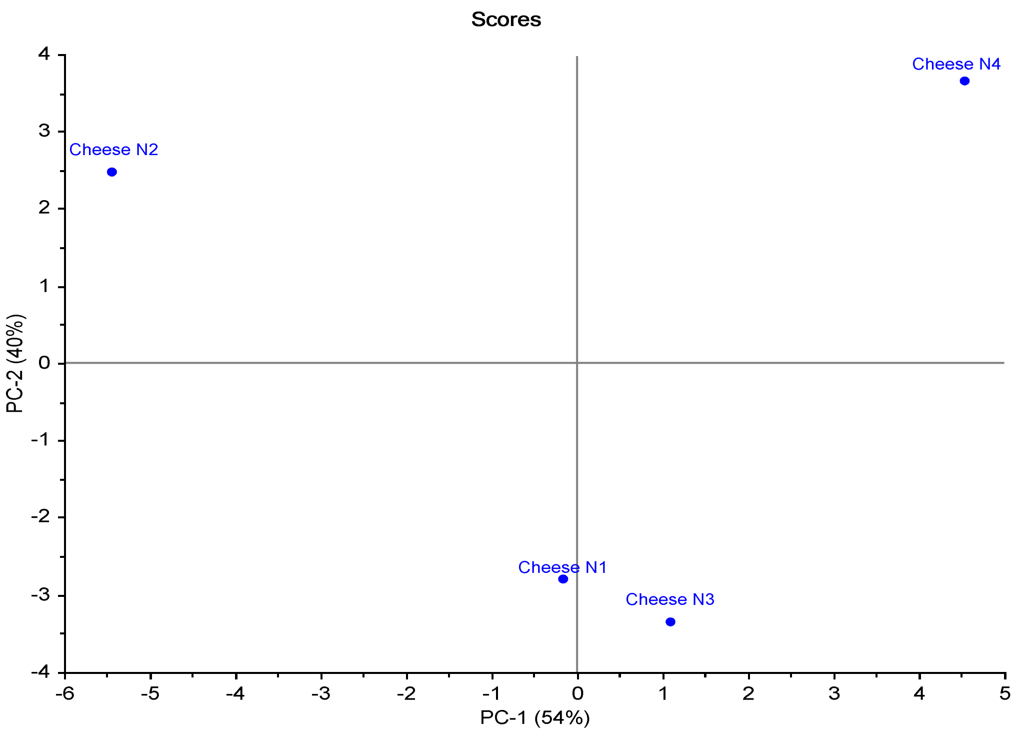



**Fig. 56.** PCA Analysis – Sample Discrimination

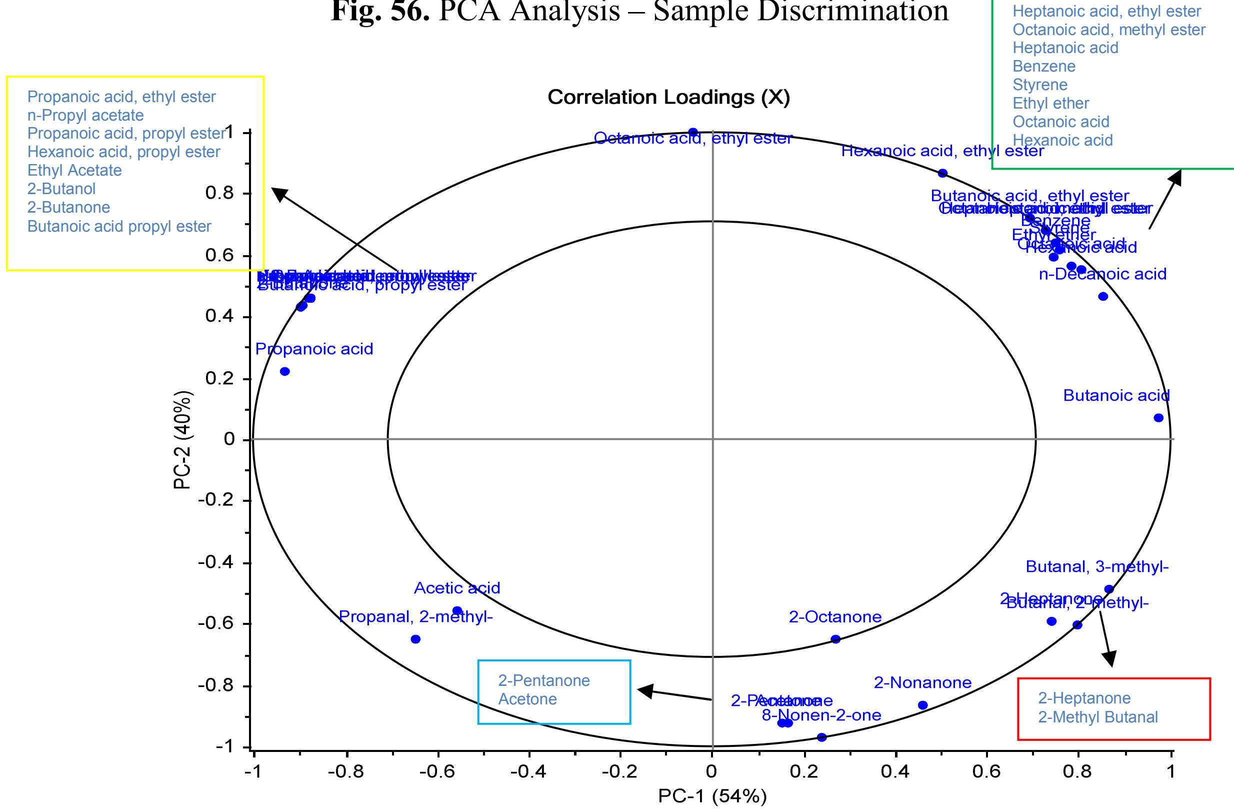



**Fig. 57.** PCA Analysis – Volatile Discrimination

**Table 14.** Association of volatile flavor compounds with odour

| Name | Compound | CAS | Odour Description |
|---|---|---|---|
| **Esters** | Diethyl ether | 60-29-7 | Sweet, mineral spirits |
| | n-Propyl acetate | 109-60-4 | Pineapple, bananna |
| | Ethyl Acetate | 141-78-6 | Pineapple, fruity, fruity gum, apples |
| | Propanoic acid, ethyl ester (ethyl propionate) | 105-37-3 | Pineapple, solvent |
| | Propanoic acid, propyl ester (propyl propionate) | 106-36-5 | Pungent sweet, pineapple |
| | Butanoic acid, ethyl ester (ethyl butanoate) | 105-54-4 | Fruity, buttery, ripe fruit, green, apple, pineapple, banana, sweet |
| | Butanoic acid, propyl ester (propyl butanoate) | 105-66-8 | Sweet, fruity, apricot, bubble gum |
| | Hexanoic acid, ethyl ester (ethyl hexanoate) | 123-66-0 | Fruity, malty, young cheese, mouldy, apple, green, orange, pineapple, banana |
| | Hexanoic acid, propyl ester (propyl hexanoate) | 626-77-7 | Pineapple, blackberry |
| | Heptanoic acid, ethyl ester (ethyl heptanoate) | 106-30-9 | Fruity, pineapple, sweet, banana, berry, cognac and slightly green |
| | Octanoic acid, ethyl ester (ethyl octanoate) | 106-32-1 | Fruity, apple-like, green, fatty, orange, winey, pineapple, apricot |
| | Octanoic acid, methyl ester (methyl octanoate) | 111-11-5 | Waxy, green, sweet, orange,vegetable, herbal |
| **Ketones** | Acetone | 67-64-1 | Wood pulp, hay |
| | 2-Butanone | 78-93-3 | Sour milk |
| | 2-Pentanone | 107-87-9 | Orange peel, sweet, fruity |
| | 2-Heptanone | 110-43-0 | Blue cheese, spicy, roquefort |
| | 8-Nonen-2-one | 5009-32-5 | Cooked |
| | 2-Octanone | 111-13-7 | Fruity, earthy |
| | 2-Nonanone | 821-55-6 | Malty, fruity, hot milk, smoked cheese |
| **Aldehydes** | Butanal, 2-methyl- | 96-17-3 | Malty, dark choclate |
| | Butanal, 3-methyl- | 590-86-3 | Malty, powerful, cheese, green, dark chocolate |
| | Propanal, 2-methyl- | 78-84-2 | Banana, malty, chocolate-like |
| **Alcohol** | 2-Butanol | 78-92-2 | Fruity |
| **Acids** | Acetic acid | 64-19-7 | Vinegar, peppers, green, fruity floral, sour |
| | Propanoic acid | 79-09-4 | Pungent, sour milk, cheese, gas, burnt, cloves, fruity |
| | Butanoic acid | 107-92-6 | Sweaty, butter, cheese, strong, acid, facel, rancid, dirty sock |
| | Hexanoic acid | 142-62-1 | Sweaty, cheesey, sharp, goaty, bad breath, |
| | Heptanoic acid | 111-14-8 | Fatty, rancid |
| | Octanoic acid | 124-07-2 | Cheesey, rancid, pungent, sweat |
| | Decanoic acid | 334-48-5 | Waxy, warm, stale, butter, sour, fruit, grassy, fatty, cheese, milk, aged Cheddar |
| **Benzenes** | Benzene | 71-43-2 | Sweet |
| | Styrene | 100-42-5 | Pungent, plastic-like, ethereal |

**CAS (RNs)**: Chemical Abstracts Service Registry Numbers

# 4. Discussion

Provolone del Monaco P.D.O. is a pasta filata cheese (spun paste) manufactured exclusively with cows' raw milk without the addition of starter cultures, ripened for at least six months and traditionally produced in the Lattari mountains area (Campania region, Italy).

The uniqueness of raw-milk cheeses made without commercial starter addition has led to improved efforts for the identification and characterization of strains in dairy products (Mannu et *al.,* 2000). These raw milk cheeses have a wild lactic acid flora, which includes mesophilic lactobacilli (De Angelis et *al.*, 2001), enterococci (Giraffa, 2003) and lactococci (Mannu et *al,.* 2000). In this study, microbial enumeration was made using Rogosa, KAA and LM17 media. A great number of putative LAB bacteria to 598 days of cheese ripening was found using these media. The cheese samples used for analyses in this study was from the same batch production. There are two main problems to identify these bacteria; firstly, they often have same nutritional requirements and, secondly they are able to grow with very similar environmental conditions (Vandamme et *al.*, 1996).

Media used for LAB are often defined selective, but according to some others they are not always have strong action (Randazzo et *al.*, 2002; Ercolini et *al.*, 2003). For example, enterococci and lactobacilli isolates from Provolone del Monaco P.D.O. cheese were both found on LM17. The incubation temperature used in the study did not led to discriminate strongly mesophilic and thermophilic LAB species, except for the thermophilic *Lb. delbrueckii* which grew mostly at 44 °C. Although some author used the temperature parameter to differentiate mesophilic from thermophilic (Torres-Llanez et *al.*, 2006); considering the results obtained, it could be right to broaden the temperature range.

This first selection using the conventional microbiology was followed by PFGE, a molecular typing method considered to be the gold standard among molecular techniques. PFGE has been used widely and successfully to identify and subtypes LAB food associated (Klein, et *al.*, 1998; Giraffa et *al.*, 2000; Domig, et *al.*, 2003; Coppola, et *al.*, 2006). PFGE technique followed by 16S rRNA amplified

sequencing has been considered as the gold standard for bacterial identification (Cocolin et *al.*, 2007).

This study shows clearly that the environment contributes to the biodiversity of the flora in cheese. The presence of wild bacterial populations plays pivotal and critical role in the development of the unique characteristics of each cheese. Indigenous LAB selected by the cheese making environment characterizes all production steps and have an important function in ripening process. Therefore, the main objective of this study was to obtain information about the different microbial groups involved in Provolone del Monaco P.D.O. production.

Microbiological counts obtained from Provolone del Monaco PDO samples highlighted a significant presence of all microbial groups targeted with values comparable to those reported from other authors in Italian Pasta filata cheeses (Coppola et *al.*, 2006; Aponte et *al.*, 2008). LAB reached the highest levels in the curd after 123 days of ripening. They started to strongly decrease in aged cheeses as a result of autolysis and microbial competition events (Fox et *al.*, 2004).

*L. lactis subsp. lactis* was the species isolated only in milk and curd, and thus responsible for acid production. Lowest pH was read in the curd at the end of acidification. High density of wild *L. lactis subsp. lactis* as predominant species during manufacturing was isolated in many European artisanal cheeses (Cogan et *al.*, 1997). Lactococci have the ability to produce enough acid during cheese manufacture resulting in a significant reduce of pH (Beresford et al., 2001). Lactococci produce lactic acid from lactose followed by slowly consumption in ripening stages. This reduction activity happens for the NSLAB action and it could be explained, in Provolone del Monaco P.D.O. samples analysed, by the significant presence of lactobacilli. According to Garcıá Fontán et *al.*, (2001), the high variability, especially among lactobacilli, could be due to the heterogeneity of raw milk provided from two farms with different cow breeds and by cheese making environment. Many other LAB are usually identified during cheese ripening, which help especially the growth of heterofermentative lactobacilli; they belong to the group called "secondary microflora" and contribute to flavor development (Beresford et *al.*, 2001). Among the different lactobacilli isolated, *Lb. casei/paracasei* and *Lb. delbrueckii* species were found from ripened Provolone del Monaco P.D.O. cheese

samples with the highest frequencies. *Lb. casei/paracasei* was also isolated from Provolone del Monaco milk and according to other studies, *Lb. paracasei* is often detected from traditional fermented milk and other fermented foods (Mathara et *al.* 2004). Although its predominance is documented in cheese, such as Cheddar (Fitzsimons et *al.,* 1999), Salers (Duthoit et *al.*, 2003), Caciocavallo Pugliese (De Pasquale et *al.,* 2014) and a traditional Turkish Kargi tulum cheese (Kunduhoglu et *al.*, 2014). The fact that *Lb. paracasei* is widespread in cheese seems due to its presence in raw milk and mesophilic attitude (Depouilly et *al.*, 2004). *Lb. casei/paracasei* not only represented the majority but it was present in the last ripened Provolone del Monaco. It is often observed that when this adventitious bacterium is dominant on all the other lactobacilli in cheese, it become predominant by the end of ripening (Ostlie et *al.* 2004; Poznanski et *al.,* 2004). PFGE analysis of *Lb. casei/paracasei* isolates from different samples revealed numerous profiles with similarity < 70%. Their patterns were also all different within the same sample. Thus, diversity at the intraspecies level was large and varied for *Lactobacillus delbrueckii*, too. A previous review has discussed the importance of preserving this type of traditional artisan cheese, usually made from raw cow's milk, because of their high microbial biodiversity and in particular high species richness of "wild" LAB with diverse metabolic activities and of great potential as dairy starters or even probiotic agents (Montel et *al.*, 2014).

*Lb. delbrueckii* was the second most-isolated species, but it occurred nearly exclusively in ripened cheese. *Lb. delbrueckii* have been previously detected as dominant species in Grana Padano whey and cheese by Massoni et *al.* (1982). It has been also identified in Akawi cheese (Ayyash et *al*., 2012). Although *Lb. delbueckii* have been isolated from ripened Provolone del Monaco P.D.O. cheese, they are considered thermophilic starter cultures (Parente et *al.*, 2004) and can be often found (or used) independently from of the temperatures which reached during the cheese manufacture (Beresford et *al.*, 2001; Limsowi et *al.*, 1996; Parente et *al.*, 1997). Natural or selected starters belonging to *Lb. delbrueckii* species are often used with other strains because of their peculiar metabolic and technological properties, especially for fermented dairy products and cheese (Hammes et *al.*, 1991).

*Lb. rhamnosus* occurred only during ripening cheese of almost one year, while Aponte et *al.*, (2008) found it significantly throughout the Provolone del

Monaco production process. *L. rhamnosus* was the only species isolated and identified until 8 months of ripening in a Piedmont hard cheese (Bautista-Gallego et *al.*, 2014). Its presence in cheese is very interesting because it is recognized as a good probiotic microorganism (Tuo et *al.*, 2013). *L. rhamnosus* strain GG, previously isolated from healthy human intestine, has most of the properties usually associated to a good probiotic strain, such as excellent survival in the stomach and small intestine and transient colonization of the gastrointestinal tract, because of its adhesion capacity to intestinal cells (Saxelin et *al.*, 2010). *L. rhamnosus* was also isolated in Parmigiano Reggiano (Bove et *al.*, 2011) and Grana Padano (Pogačić et *al.*, 2013).

*L. helveticus* was detected only in milk and pasta filata samples with moderate incidence, was highlighted by enzyme restriction APAI. This species can show probiotic potentials very similar to those of *L. acidophilus, L. rhamnosus, and Bifidobacterium animalis subsp. lactis* (Taverniti et *al.*, 2012). *L. helveticus* showed to be very important during Swiss-type cheese fermentation as an acid-tolerant starter culture (Hammes et *al.*, 2009; Stiles et *al.*, 1997), and may contribute to the flavor of Provolone del Monaco P.D.O. The presence of *Lb. helveticus* at the first steps of cheese making has been already described by other authors (Fortina et *al.*, 1998; Gatti et *al.*, 2003). *L. helveticus* can grow adapting to different conditions especially for its ability to ferment many carbohydrates and to metabolize different proteins (Dimitrov et *al.*, 2005). This species has been identified in Provolone (Giraffa et *al.*, 1998), Parmigiano Reggiano cheeses (Gatti et *al.*, 2003) and in Grana Padano (Pogačić et *al.*, 2013).

*Lb. hilgardii* was isolated in milk and also in aged Provolone del Monaco samples. This specie was present in the traditional Turkish "Civil" cheese (Şengül, 2006). *Lb. fermentum* emerged in milk and especially during ripening period, maybe selected by the modified cheese environment according to Aponte et *al.,* (2008). One isolate was assigned to the species *Lb. bucknerii* and *Lb. plantarum/pentosus*. *Lb. fermentum, Lb. bucknerii and Lb. plantarum/ pentosus* were also identified in traditional Turkish Kargi tulum cheese made from raw milk. The contribution to the ripening process of other *Lactobacillus* species, such as *Lb. hilgardii*, *Lb. plantarum/pentosus* and *Lb. bucknerii* isolated in smaller amounts, remains

unknown. *Lb. plantarum* showed to enhance flavor intensity when was used as adjunct cultures during cheese making (Antonsson et *al.*, 2003).

The dominant cocci-shaped LAB were the thermophilic *S. macedonicus* and *S. lutetiensis/infantarius. S. macedonicus* is very interesting presence in cheese; it was found for the first time in Greek Kasseri cheese (Tsakalidou, et *al.,* 1998) and then in many Italian cheeses (Pacini et *al.*, 2006). *S. macedonicus* has a lipolytic and proteolytic activity influencing sensory property of cheeses (Lombardi et *al.*, 2004). *S. lutetiensis* was isolated from calf intestinal tracts (Busconi et *al.*, 2008) and associated to bovine mastitis. (Schabauer et *al.*, 2014). These association may justify its presence in dairy products. *Streptococcus lutetiensis* and *S. infantarius subsp. infantarius* were also found in Turkish cheese and in African camel milk, respectively (Hande, 2012; Jans et *al*, 2013). *S. infantarius subsp. infantarius* is a member of the S. bovis/S. equinus complex (SBSEC) (Schlege et *al.*, 2003a). Some species belong to SBSEC are associated with human and animal infections (Herrera et *al.*, 2009), which means providing a health risk for consumers.

Only one isolate of *S. thermophilus* was found in pasta filata. Regarding to this result, two consideration can be made: first, that the stretching of the pasta filata occurred in hot water at 85-95°C, second, the temperature cheese ripening has never exceeded 15 °C as compare to the optimal growth temperature range (40- 45°C) for this species (Zirnstein et *al.*, 2004). In addition, this species might be outcompeted by *S. infantarius subsp. infantarius strains*, a potential pathogen (Corredoira et *al.*, 2008), according to Jans et *al.*, (2012) who showed evidence that an African variant was able to adapt to the dairy niche during its evolution.

*E. faecalis* species was isolated with high percentage from milk and curd samples as well as aged cheese. *E. faecium* and *E. durans* were detected only during ripening period. With regard to the biodiversity seen in Provolone del Monaco cheese, it is widely reported that enterococci constitute a notable part of LAB population in artisan raw milk cheeses (Giraffa, 2003). Although their presence in raw milk and during manufacturing steps is due mainly to low hygienic conditions (Garcia Fontan et *al.*, 2001), their isolation from curd and ripened cheeses could be related to low pH, tolerance against high-salt content, adaptability to a wide range of different substrates growth conditions (Giraffa, 2003) as well as their intense

lipolytic activity (Mucchetti et *al.*, 2006). As previously shown, the peculiar cheese-making technique used for Provolone del Monaco P.D.O cheese production, which includes heating of the curd and stretching in hot water, could also contribute to the selection of these thermally resistant micro-organisms. Despite their remarkable abundance in artisanal dairy products (Cogan et *al.*, 1997), the use of these micro-organisms as starter cultures or adjuncts in the dairy industry is still debated due to their role as potential pathogens and / or reservoirs of transferable antibiotic-resistance genes (Giraffa et *al.*, 1997).

Furthermore, sequenced strains and isolates from kanamycin identified as *E. fecalis* and *E. faecium* were screened for histidine (*hdc* activity) and tyrosine (*tdc* activity) decarboxylase activity using improved agar growth media TDAM and HDAM. These differential media are a cheap, easy, and fast conventional technique to select strains possessing amino acid decarboxylase activity (Marcobal *et al.*, 2006a). There are few species identified as tyramine producing using this technique. The identified species are *Lactobacillus casei/paracasei* (1 strain) *Enterococcus faecium* (5 strains), *Enterococcus fecalis* (6 strains) and *Enterococcus durans* (1 strain). Enterococci with *tdc* activity were present in the raw milk, during manufacturing and ripening; this means that the potential biogenic amines production was constantly found in all samples. However, the detection of BA producing bacteria by conventional culture techniques is often tedious and unreliable, exhibiting various drawbacks such as lack of speed, appearance of false positive / negative results, low sensibility, requirements for costly and sophisticated equipment or that only one BA is detected (Landeta et *al.,* 2007).

One strain of *E. faecium* tyramine producing showed also an enterocin A against *Listeria innocua.* The molecular weight of enterocin A has previously been reported as 4.8 kDa (Aymerich et *al.*, 1996). Specific primers for enterocin A confirmed its identification by MALDI-TOF. Enterocin A showed resistant within pH range 2-10 and a high thermo-stability. One strain of *L. lactis* was found Nisin A producing which had a molecular weight of 3.3 kDa using MALDI-TOF (Zendo et *al.*, 2003). Nisin A was active within pH range 4-10 and inactive after heating treatment for 30 minutes. The use of Nisin is allowed like additive in food because it is produced by GRAS microorganisms and widely used as a food-preservative in certain dairy products (Deegan et *al.*, 2006; Delves-Broughton et *al.*, 1996).

However, many other LAB bacteriocins producing are lack of GRAS status, thus are broadly explored for hypothetical application in food preservation. The use of Enterocin A, *Enterococcus* bacteriocin is not permitted as additive in food although many of Enterococci species (no GRAS recognized) represent non-pathogenic flora found in many dairy products (Morandi *et al.* 2006).

On the basis of acid production, the isolates were divided into three groups and only those in the one group that lowered the pH to 5.5 after 8 h fermentation were classified as fast acid producers. *Lb. delbrueckii*, *S. macedonicus*, *S. thermophilus*, *L. lactis* and *S. lutetiensis* were identified as fast acid producers in skim milk. As the ability to lower pH during cheese ripening is essential in cheese starter strains (Fox at *al.*, 1990), these identified strains can be used as a natural starter cultures during cheese manufacturing.

In this study, Provolone del Monaco P.D.O cheeses, aged for more than 2 years, were also analysed for their amount of Biogenic Amines and volatile flavor compounds using HPLC and TD-GC/MS, respectively. These cheese belonged to the same batch production of the others subjected to microbial analysis. Three of the four had the same typical sensorial characteristics of Provolone del Monaco P.D.O., while only one, N.2, showed the defect of medium-sized eyes. The sample N.2 resulted with highest level of singles (CD, HI, PU, TY, TR) and total Biogenic Amines compared with the other samples. In cheese N.2, CD and PU were very high level (>450 mg/kg) following by TY (297 mg/kg), TR and HI (67-88 mg/kg) and total BAs was up to 1400 mg/kg. Other cheese had approximately similar BAs concentrations. In these cheese, TY had the highest value (>100 mg/kg) and total BAs were >150 mg/kg in all three cheeses. Some limits have been fixed only for histamine in fresh fish (200 mg/kg) and fish products obtained by enzymatic maturation in brine (400 mg/kg) (EC N. 1019/2013); while no upper limit of BAs levels of cheese has been legally recommended. Other foods have only been recommended or suggested an upper limit of 100 mg/Kg of histamine; whereas a limit of between 100 and 800 mg/Kg and of 30 mg/Kg has been recommended for tyramine or β-phenylethylamine, respectively (Ten Brink et *al.*, 1990; Halász et *al.*, 1994). Moreover some authors have suggested 900 mg/kg as a sum of HI, TY, PU and CD (Shalaby, 1996). Comparing to these suggested limits, cheese N.2 showed upper values especially for histamine, tyramine and total Biogenic Amines

concentrations. It is important to consider that at unacceptable levels of biogenic amines corresponds to a non-acceptability of the cheese for the obvious defect. The other three cheeses were acceptable with regard to the total biogenic amine content, while tyramine exceeded the minimum limit suggested. The analysed Provolone del Monaco cheeses were ripened for more than 2 years. Several authors have been already reported that the time of ripening is considered as a critical factor for the BAs accumulation (Bunkovà et *al.*, 2010; Lapa-Guimaraes et *al.*, 2004; Novella-Rodrìguez et *al.*, 2002; Ordonez et *al.*, 1997; Schneller et *al.*, 1997). It has been well known that during cheese aging, the proteolysis increases the release of amino acids from casein, which can be subsequently decarboxylated by bacterial enzymes giving rise to the accumulation of BAs. A major role in the accumulation of BA was attributed to NSLAB, but the release of peptidase after lysis SLAB seems to be essential to provide precursor amino acids (Valsamaki et *al.*, 2000). However, other authors observed a higher BA accumulation in cheeses obtained from raw milk than in similar products from pasteurized milk (Schneller et *al.*, 1997). This also indicates that the heat treatment of milk play a role in the BAs formation. Many decarboxylating bacteria cannot survive pasteurization. As the use of milk of high microbiological quality is a prerequisite to prevent amine formation (Gennaro et *al.*, 2003; Lanciotti et *al.*, 2007), it is assume that pasteurization can also be crucial to reduce the growth of decarboxylase-positive microorganisms. Cheese made from raw milk like Provolone del Monaco cheese P.D.O. have higher content of BAs than those made from pasteurized milk (Gennaro et *al.*, 2003; Ordonez et *al.*, 1997; Schneller et *al.*, 1997; Novella-Rodrìguez et *al.*, 2002). All the analysed cheeses were characterized by a high amount of TY as according to Joosten (1988), Petridis et *al*. (1996), Bover-Cid et *al.* (1999), Montel et *al.* (1999) and Gardini et *al.* (2001), who observed that enterococci lead to accumulation of TY. The high level of tyramine in Provolone del Monaco P.D.O. is presumably due to the activity of thermo resistant enterococci, usual contaminants of raw milk. However, the heat resistance at more than 65˚C of some *Lactobacillus* species, particularly *Lb. casei/paracasei*, has been demonstrated (Jordan et *al.*, 1999). According to Spizzirri et *al.,* (2013), who found high levels of BA in Parmigiano Reggiano and Grana Padano P.D.O. cheeses, hard-ripened cheeses made from cow's raw milk are the products with high BA content. Besides the long ripening period, it should be underlined that the biogenic amines formation in cheese is linked by temperature

(mainly if >18 °C), pH (>5) and low salt content as demonstrated by different authors (Ten Brink et *al.*, 1990; Joosten, 1988). Due to the considerable presence of BA in cheeses with long ripening, and the well known effects of unhealthy intake of large amounts of some BA, the high consumption of aged cheeses should be reduced, especially for sensitive individuals. Additionally, study on investigating the critical control points to reduce the accumulation BA in this type of cheese should be done.

Regarding volatile flavor compounds analysis, in all samples was found a very unusual high number of esters. Esters are common volatile compounds in cheese, but their mechanism of synthesis in it is still largely unknown. It is known that are responsible for fruity flavors which is considered a defect of the cheese or the opposite, a good flavor (Liu et *al.*, 2004). Sample N.2 was associated to many esters according to Thierry et *al.*, (2005). They first studied the ability of one strain of *Propionibacterium freudenreichii* (PAB) to produce volatile compounds in cheese, by comparing the profiles of volatiles of experimental Swiss-type cheeses. Ethyl propionate and n-propyl acetate were more abundant in sample N.2. This evidence can explain the presence of medium size eyes in cheese N.2, a defect for Provolone del Monaco P.D.O. cheese and as previously reported in Emmental cheese (Thierry et *al.*, 2004). Ethyl butonoate, ethyl hexanoate and ethyl heptanoate were present in other samples; they were synthesised, always in Swiss cheese, mainly during cold storage of the cheese. In Provolone del Moncaco cheese P.D.O. could be linked to the storage in cold room before GS/MS analysis (Thierry et *al.*, 2005). Ester compounds are the result of an extensive lipolysis, which is also characteristic of Provolone and other Italian varieties such as Grana Padano, Parmigiano-Reggiano and Romano. The high lipolytic activity is due to the lipoprotein lipase (LPL), which is of more significance in raw milk cheese than in cheese made from pasteurized milk, since its activity is not reduced by pasteurization (Collins et *al.*, 2003). Besides, the Provolone cheese is made with rennet paste, which contains the lipase and pregastric esterase. These enzymes are responsible for extensive lipolysis, resulting in the characteristic ‘‘piccante’’ flavor of these varieties (McSweeney et *al.*, 2000; Collins et *al.*, 2003). The esters are produced mainly by pregastric esterase from rennet paste (Collins et *al.*, 2003), but also could be produced by microbial esterase activity (Castillo et *al.*, 1999). Samples N.1 and N.3 were in general very similar and associated mostly with ketones. High levels of methyl ketones are common in mold-

ripened cheeses and also in hard cheese such as Parmigiano Reggiano (Qian et *al.*, 2002). Ketones are the products of unsaturated fatty acids autoxidation. Some of these compounds, such as 2-pentanone, 2-heptanone and 2-nonanone, have been reported in Provolone cheese (Wirotama et *al.*, 1974, Liu et *al*., 2011). Secondary alcohols are derivative of the methyl ketones through enzymatic reduction (Molimard et *al*., 1996). Presence of some secondary alcohols such as 2-Butanol, has been reported in Provolone cheese (Wirotama et *al.*, 1974) as well. 2-Butanol was found in all samples. Branched aldehydes were detected in Provolone del Monaco P.D.O. cheeses, they were highest in sample N.4. Aldehydes found such as 2-methyl propanal, 2-methyl butanal and 3-methyl butanal, are significant flavor compounds in many food products (Smit et *al*, 2009). The 2-methyl propanal is derivative of valine through enzymatic reaction and through non-enzymatic chemical reaction with leucine as the substrate (Smit et *al*., 2005). This compound contributes to aromatic notes such as banana, malty, chocolate-like. The 3-methyl butanal is derivative of leucine catabolism by transamination followed by decarboxylation and it contributes to malty, powerful and cheese notes (Marilley et *al*., 2004; Smit et *al*., 2005). The 2-methyl butanal is derivative of isoleucine catabolism and contributes with aromatic notes such as dark chocolate and malt (Singh et *al*., 2003). The production of those aldehydes in Provolone del Monaco P.D.O. cheese could be related to the presence of *Lb. casei/paracasei* such has been reported by other authors (Thage et *al*., 2005; Peralta et *al.*, 2014). Butanoic, hexanoic and octanoic acids were present in all sample of Provolone del Monaco P.D.O. cheese and with highest levels in sample N.4. This result is in agreement with results previously reported (Ha et *al*., 1991; Liu et *al.*, 2011). Ha and Lindsay, (1991) found highest concentration for butanoic, hexanoic and octanoic acids among compounds detected in aged Italian Provolone cheese. Liu et *al.*, (2011) studied the effects on volatile compounds of radialized Provolone cheese and also reported very strong presence of those acids. Butanoic, hexanoic and octanoic acids are the compounds which give the characteristic flavor note to Provolone cheese (Woo et *al*., 1984). Iyer et *al.*, (1967) also observed that in Provolone cheese the short-chain free fatty acids were extremely high and the butyric acid was highest in concentration of all the free fatty acids. Benzene has been detected in Cheddar cheese by Urbach (1995), who suggests that it comes from fresh milk. Styrene is a common compound found in many plastic containers (Chiesa et *al.*, 2010) and it was in a typical Sicilian Pasta filata cheese (Ziino et *al.*, 2005). It did

not find in all samples although all cheese analysed were stored in vacuum plastic package. It’s difficult to be definitive but benzene and its derivatives are most likely contaminates from packaging or the environment (Lau et *al.*, 2000; Alloway et *al.*, 1997). However some benzene derivatives can be produced from amino acid catabolism, including styrene. Styrene can be produced from phenylalanine by molds (Molimard et *al.*, 1997).

## 5. Conclusions

The present study have investigated the presence of Lactic Acid Bacteria (LAB) in Provolone del Monaco P.D.O cheese. Among lactobacilli, casei/paracasei group dominated largely, up to the end of cheese ripening, followed by *Lb. delbrueckii*. Their diversity at the intra-species level was large and varied as was shown by Pulsed Field Gel Electrophoresis (PFGE). Many rod-shaped LAB were represented by *Lb. rhamnosus, Lb. fermentum, Lb. plantarum/pentosus*, *Lb. hilgardii, Lb. buchneri* and *Lb. helveticus.* High microbial biodiversity and in particular high species richness of "wild" LAB do understand the importance of preserving this type of P.D.O. cheese made from raw cow's milk. The dominant cocci-shaped LAB were the thermophilic *S. macedonicus* and *S. lutetiensis/infantarius.* They are already known mostly in cheese made with raw milk, but *S. lutetiensis/infantarius* is also associated with human and animal infections. Only one isolate of *S. thermophilus* was found in pasta filata, this can be due to low temperature (8-15 °C) used during cheese ripening. *E. fecalis*, *E. faecium* and *E. durans* were also present and represent a significant part of environmental LAB population. The tyramine-producing strains that were identified were *Lb. casei/paracasei* (1 strain) *E. faecium* (5 strains), *E. fecalis* (6 strains) and *E. durans* (1 strain). One strain of *E. faecium* tyramine producing showed bactericinogenic activity trough enterocin A. One strain of *L. lactis* was found Nisin A producing. Acidification capability test on all selected isolates showed *Lb. delbrueckii*, *S. macedonicus*, *S. thermophilus*, *L. lactis* and *S. lutetiensis* as fast acid producers; these identified strains can be a potential natural starter cultures to use during cheese making.

Provolone del Monaco P.D.O cheeses (at 593$^{rd}$ of ripening) were also investigated for their amount of Biogenic Amines (BA) and volatile flavor compounds using High Performance Liquid Chromatography (HPLC) and Thermal Desorption Gas Chromatography Mass Spectrometry (TD-GC/MS) techniques, respectively. All these cheeses belonged to the same batch production of cheeses already analysed microbiologically. It was found that three (N.1, N.3 and N.4) of the four cheeses had the same typical sensorial characteristics of Provolone del Monaco P.D.O. cheese, while only one (N.2) showed the defect of medium-sized eyes. HPLC analysis showed that BAs concentration was highest in sample N.2, while the other three had lowest and very similar concentrations. Many volatile compounds were

detected in these cheeses by TD-GS/MS. Cheese N.2 was found to have a different volatile flavor profile than the other cheeses. Samples were associated to at least one class of flavor compound. In particular, N.1 and N.3 were associated to ketones and characterized to a very complex flavor. N.2 was associated to a very high number of esters and fruity flavor. N.4 was correlated to acids and very strong flavors. All experiments done in this study have provided some new useful insights and information. Nevertheless, these results also confirms the need to investigate further the following aspects: first, it could be interesting to study the safety of all LAB isolated, including *Enterococcus* species, and also their antimicrobial activity against pathogens and other LAB. Second, it would be also interesting to study in detail the flavor producing capability of the LAB strains in a model system similar to a cheese environment.

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