APPLICATION OF CULTURE-DEPENDENT AND CULTURAL INDEPENDENT TECHNIQUES TO INVESTIGATE THE DYNAMICS OF MICROORGANISMS DURING INDUSTRIAL CHEESE MAKING OF GOUDA TYPE CHEESE

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Master thesis

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ABSTRACT

The aim of this work was to study the microbial dynamic in a Gouda type cheese applying a comparison between culture-dependent technique, plate count, and culture-independent technique as PCR-DGGE. The study was also focus on the analyse of the efficiency of the pasteurizer, and its cleaning steps.

The study was conducted analysing 4 batches for each of the two days of production, Monday and Friday. Between batch 16 and 17 there was a quick cleaning, and between batch 32 and batch 1, of the following day, there was a complete cleaning. For each batch samples of raw milk, pasteurized milk, starter, milk before rennet, cheese after pressing, cheese after brine were collected to understand the microbial development during the cheese making-process.

Plate count was conducted with selective media, M17, for lactococci; MRSV, (MRS with vancomycin), to select Leuconostoc; LBS, for lactobacilli. The PCR-DGGE was conducted both with universal primer for V1 and V3 of 16S DNA, and with specific primer for lactobacilli, Leuconostoc/Pediococci, and selective for Lactococci/Enterococci and Streptococci. The gradient 35-55% of urea-formamide was used during DGGE (100% denaturant corresponding to 7M urea and 40% [v/v] formamide was used). Identification of the DGGE bands was possible by the comparison among different migration distance of pure strains, loaded in the gel, like a marker and by the sequencing of the band.

The monitoring of total lactic acid bacteria (LAB) and non-starter lactic acid bacteria (NSLAB), from the dairy samples and starter cultures the presence of *Lactococcus (Lc.) lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*, as the dominant species of LAB was shown, while *Lc. Raffinolactis, Lactobacillus (Lb.) casei* and *Lb. paracasei* could be considered NSLAB.

Studying the pasteurization step, *Anoxybacillus flavithermus, Aeribacillus pallidus/ Geobacillus Pallidus* were identified, which may produce, heat resistant spores and these may also create biofilm on the steel pasteuriser surface. It was supposed that the cleaning in at middle of the production day was not sufficient to eliminate this bacillus. At the other hand it was possible to conclude that the pasteurized treatment was effective. *Streptococcus dysgalactiae* subsp. *dysgalactiae*, may underline the presence of mastitis in the milk.

However the combination of temperature and time in the pasteurizer, was able to kill this species which was not found after pasteurisation.

The technique, which was applied in this study, could not permit the quantification of the amount of microorganism with precision. In fact thanks to the comparison between culture dependent and culture independent techniques, it was possible to understand the proportion and the balance between the species of LAB, indicating which were predominant.

TABLE OF CONTEN

1. Introduction

1.1. General aspect of cheese production

Cheese is one of the most ancient food in the history. Even though the development of technology, the cheese making steps are still the same as in traditional production. In the world a lot of cheese varieties exist, most of them are unified by two steps: growth of lactic acid bacteria (LAB) and coagulation by rennet or acid precipitation of the casein. In fact one of the main factors studied to improve the quality of cheese is focused on the starter and its activity in the cheese.

In the Figure 1.1 A hypothetical cheese production flow-sheet is showed.

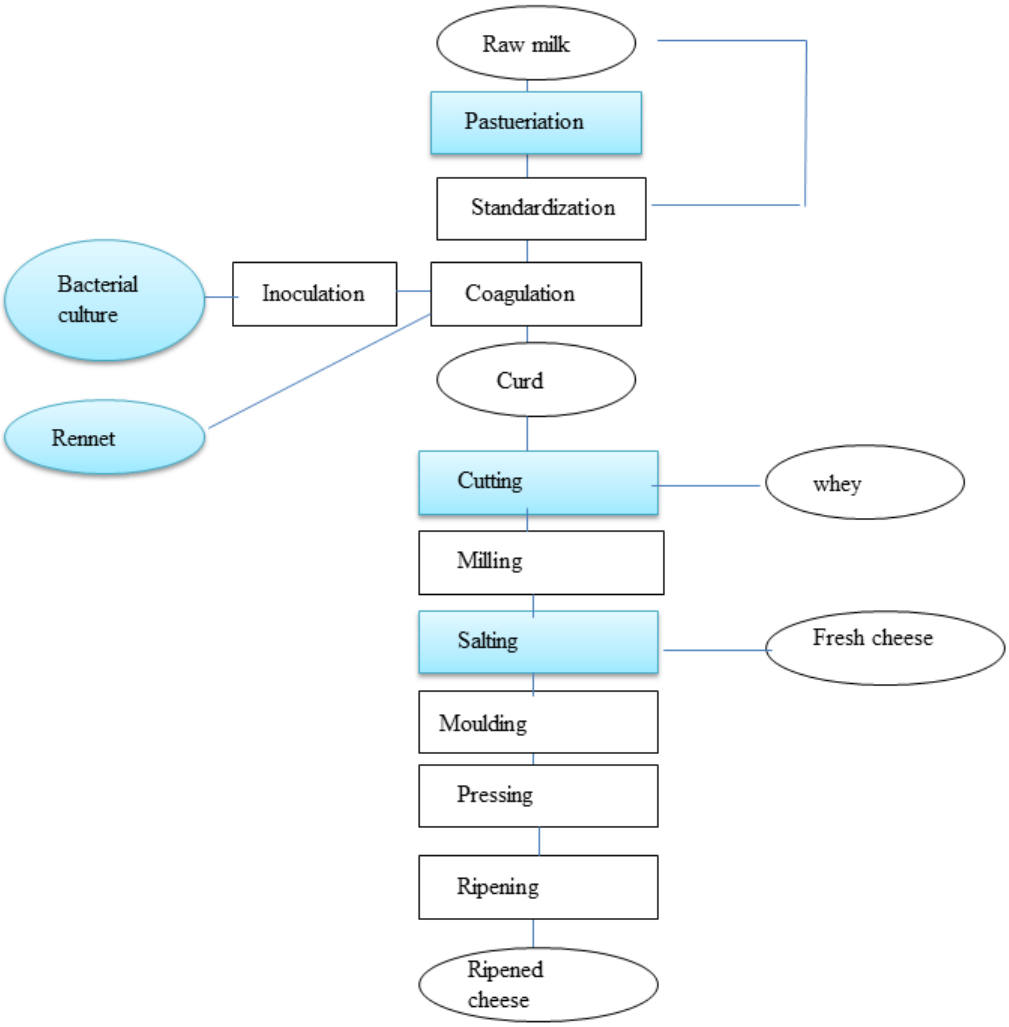


Figure 1.1 Example of cheese production flow-sheet.

(http://www.lsbu.ac.uk/biology/enztech/proteases.html).

In this flow-sheet some important steps are underlined. The first is pasteurization. It is important but not made in all cheese makings. In Southern Europe, especially in the Alp region a lot of cheeses are made from raw milk. But in the North of Europe and USA pasteurization is ordinary practice. Usually the heat treatment is high temperature short time (HTST) and pathogens are killed.

Adding of starter (SLAB) is nowadays common practice in most cheese varieties, but with various mix of starters and technologies. The principal activity of the SLAB is to convert lactose to lactic acid, through a homofermantative metabolism or heterofermentative metabolism, to produce the characteristic flavour and create the texture, that particular cheese and prevent the growth of involuntary microorganism for the pH 5.2-5.3 (Fox et al., 2004).

The third fundamental and common step is the coagulation made by the addition of rennet, a mixture containing a specific enzyme, which are active to proteins. (Fox et al., 2004).

In the next step the cutting, stirring, cooking, salting or pressing of the curd, most part of whey is expelled (syneresis). At the last step cheese is salted by three different techniques: mixing salt with the dry and milled curd, rubbing into mould dry salt several times, dipping the cheese into brine, a solution of water and salt where the concentration of salt depend on the type of cheese (Fox et al., 2004).

1.2. Gouda cheese

Gouda cheese is name from the city of Gouda in the Netherlands and is one of the most famous produced cheese varieties. Gouda cheese is a semi hard cheese made from bovine milk. This type of cheese ripens for 1-20 months during which the flavour changes from mild to strong. It can be consumed at several stages of maturity, determinated by the flavour preferences of the consumers and other economic factors (Van Hoorde, Verstraete et al. 2008). Gouda cheese is compared with Edam, with a difference in the fat content Edam has 40% fat in Dry Matter and Gouda 48%, (Fox, et al., 2004). In Norway Gouda cheese is represented by “Norvegia” and Synnøve Gulost. There is different types of Norvegia produced: Classic Norvegia, low fat content Norvegia with 16% of fat, ecological Norvegia, Very matured Norvegia (9 month), and Extra Matured Norvegia (15month).

1.3. Main characteristic of Gouda

Gouda is one of the main representatives of the class of semi-hard cheese and can be characterized "by the use of fresh pasteurized cow's milk, the milk is normally partly skimmed, milk clotting by means of rennet (usually extracted from calves' stomachs), the use of mixed-strain starters (MSS) consisting of mesophilic lactococci and leuconostocs, producing CO_2 , the water content of the fat-free cheese below 63%, pressing of the cheese to obtain a closed rind, acidification mainly in the curd blocks after separation of the whey during pressing, salting in brine after pressing, absence of an essential surface flora, being at least somewhat matured (for 4 weeks) and it having undergone significant proteolysis, the fat content in the dry matter ranges from 40 to over 50%, water content in the fat-free cheese ranges from 53-63%, salt content in the cheese water is around 1.5%, pH in the ripened phase may be anywhere from 5.5-5.6, maturation may take from 2 weeks to 2 years under drying conditions", (Fox et al., 2004).

1.4. Gouda cheese making

In figure 1.3 the flow-sheet of Gouda cheese making is showed.

1.4.1. Milk treatments

The quality of milk is one of the most important hazard points in the cheese production. The composition of milk has to be defined: the fat, casein and lactose content. The wrong fat content could create off-flavour in the final product. Thanks to centrifugation it is possible to divide the fat part from the skin milk and remove the surplus, then part of the fat will be added into the milk to standardize the cheese milk.

Milk in the udder of healthy animals should be essentially sterile, milking and storage are opportunities for contamination. Milk cooled to 15-21°C is dominated by mesophilic micro-organisms, particularly *Lactococcus(Lc)* and *Enterobacter* species, (McKinnon et al., 1990).

By milk storage at 4°C, the microbiological growth is retarded, but psychotropic bacteria, such as *Pseudomonas*, *Flavobacterium* and *Acinetobacter* grow and dominate the flora. *Streptococcus Thermophilus*, faecal streptococci, propionic acid bacteria (PAB), *Clostridium Tyrobutyricum* do not growth, but they might survive (Fox et al., 2004).

Some of these microorganism might be a problem both for consume health and for the yield of cheese, texture and flavour. Figure 1.3 shows that there are two possible milk treatments to reduce the presence of undesirable microorganism: pasteurisation, and bactofugation. The condition of pasteurisation is high temperature short time HTST (15s, 73-74°C). *Listeria monocytogens, Bacillus, Clostridium, Enterobacteriacee,* PAB, are killed. The spore of *Bacillus, Clostridium, Enterobacteriacee* are not killed and may into the cheese. Moreover the pasteurization might give technological problem, in fact after some production cycle *Streptococcus thermophilus* and *Bacillus* could create biofilm inside the plant in particular on the metal surface. After pasteurization there is bactofugation, thanks to this physical treatment, the spores of thermoduric organism, such as *Clostridium Tyrobutyricum* are removed. (Fox, et al., 2004).

The bactofuge is a centrifuge, and is based on the principles that the bacterial spores have a higher density than bacteria. The bactofugation is able to reduce the number of spore drastically, even to about 2-3% of the number in raw milk. The last steps are the standardization of the fat content, and additives, usually nitrate (if is allowed), $CaCl_2$, and Colouring. (Fox, et al., 2004).

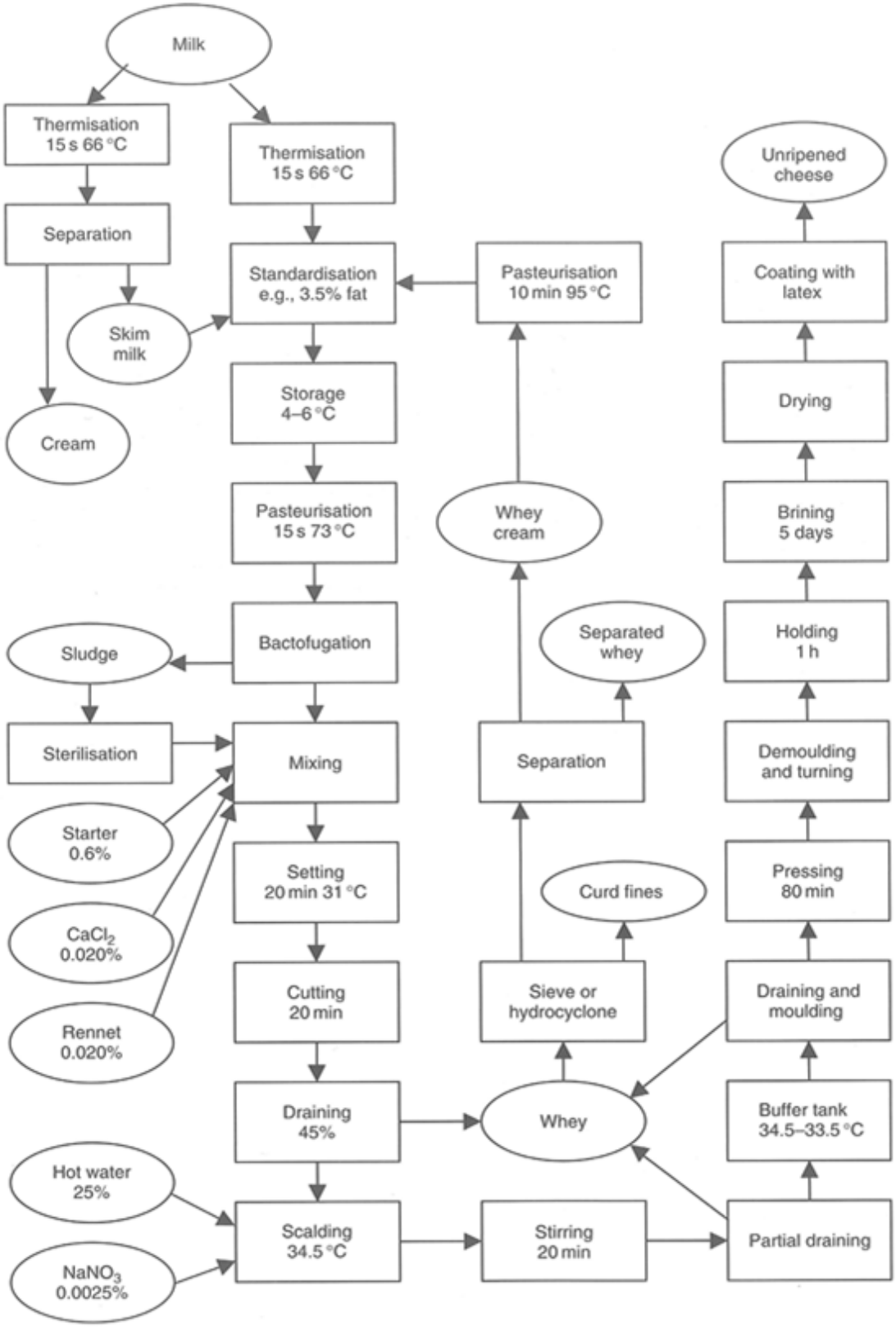


Figure 1.3 Gouda cheese flow-sheet (Fox et al., 2004).

1.4.2. Curd making

The aim of this step is to create the curd and start the syneresis. Rennet (0.020%), $CaCl_2$ (0.020%), Starter (0.6%) are added to the cheese. Rennet is an enzymatic solution containg mainly chymosin, a soluble aspartate proteinase and consequently an endopeptidase, which can split proteins, in this case the k-casein.

This enzyme is present in the stomach and it has optimal activity at about pH 4.7 on the bond between Phe and Met (residual 105 and 106). The end product of the lysis is para k-casein which remains on the casein micelle, and creates the gel. The macropeptide will be left with the whey.

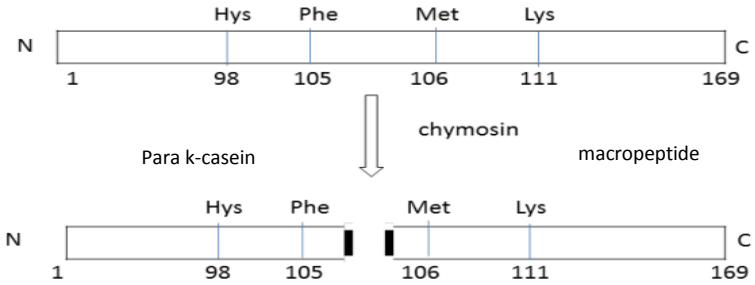


Figure 1.4 The activity of chymosin

This damage causes clotting of the milk and the formation of a gel network principally composed of casein and fat globules. At least 70% of k-casein has to be split, to obtain coagulation. The flocculation is due to Van der Waals attraction and Ca^{+2} activity, which diminish the electrostatic repulsion through the neutralization of the negative charges of the micelles. This is made possible by making bridges between them, which are made in addition to the bridges formed between the micelles with positive and negative charges. The rennet have a long phase before flocculating, but when flocculation starts, its rate increase quickly, usually the curd is obtained in 40 minutes. Important is the control of pH, in fact it has to be 6.50-6.55 in order to produce to have a slow formation of coagulum.

"Milk may also be coagulated by acidification and heating. Heating does not cause clotting but makes the serum proteins insoluble, and they associates to the caseins micelles. Acidification is instead carried out by lactic acid bacteria, which lowers the pH until the isoeletric point of casein, that is 4.5-4.7, at this value the protein coagulates, and it will then precipitate when the heating is applied". (Walstra et al., 2006).

The two technologies form different kind of coagulum. The coagulum of acidification is porous, brittle and unable to contract, typical for fresh cheeses such as cottage. Instead the coagulum for adding rennet is compact, flexible, elastic, waterproof and contractile.

The next step is the coagulum cutting. When the coagulum pieces have been cut sufficiently, the mass is stirred to drain off the desired amount of whey. In this step, it is very important the temperature control, because the temperature should be more than 38°C, this value speed up the syneresis, fusion curd, but will not kill the starters (mesophilic bacteria). The stirring should gradually to be more vigorous to prevent local fusion curd (Walstra et al., 2006).

1.4.3. Draining and moulding

"The coagulum can be cut easily and stirred without excessive loss of 'fines' in the whey and which shows rapid syneresis. Syneresis is enhanced by adding $CaCl_2$, adding more starter which causes a faster pH drop in the curd during the process" (Walstra et al., 2006).

After stirring the curd grains are allowed to settle. One of the negative consequences of this technique is the possible presence of the whey between the curd grains after drainage. At this stage until brining the most important parameter to check is the curd flow. "To ensure this the temperature of the curd block should be maintained high enough for good curd flow and to continue acidification and achieve rind formation during pressing. The mould is filled and still warm after cleaning, the temperature is approximately 20°C" (Fox, et al.,2004).

1.4.4. Pressing

To create a protection from the microorganisms, it's usual practice that the blocks of curd are put into moulds to obtain the typical shape and a closed rind. During brining and ripening, and to achieve salt penetration through the cheese surface (Fox, et al.,2004).

The main change happening in this step is the complete conversion of lactose to lactic acid. The starter bacteria slows down their activity, in particular due to the salt and low temperature.

1.4.5. Brining

Usually the pressing least 1 hour, afterwards there is the brining. The pH of cheese after pressing is to be around 5.9. The salt content of the young Gouda cheese is generally 4-5g/100g water in the cheese. Salt penetrates into the young cheese by diffusion, while water

moves out, and the curd loses weight. “There is more water where the salt has not arrived and less water closer to the brine, but at the end of ripening, water and salt should be more or less evenly distributed in the cheese” (Fox, et al., 2004)..This step is also important for the texture and to the taste of cheese because during salting, calcium is exchanged with sodium. (Fox, et al., 2004).

The brine usually contains 17-18% NaCl, but sometimes it is possible to use lower concentration of salt. This changing could be discriminated to the cheese quality due to the possible the growth of salt-tolerant lactobacilli, that may penetrate in the cheese to create flavour defect. The use of brine with a too low Ca^{2+} content could be the cause of the dissolution of cheese protein, which would cause a slimy rind (Fox, et al., 2004).

1.4.6. Rind treatment and curing

For Gouda and related cheese the presence of microorganism on the surface underline the fault of production. To prevent this, a plastic emulsion is put on the rind.

The plastic emulsion is characterized by a hydrophilic nature, and it is possible to add also fungicides, natamycin, an antibiotic produced by *Streptomyces natalensis*, which is active against moulds and yeast, or calcium sorbate. The using of these depends by the local law and strategy of dairy (Fox, et al., 2004).

“Generally the cheese is cured at 12-15°C and 85-88% RH” (Fox, et al., 2004). This step is one of the most delicate steps, because the curing has to be quick enough to do not permit the growth of undesirable microorganism, but there is the risk to break the plastic film, in this way yeast and moulds can grow. The other important parameter is the temperature, above this temperature the fat melt and cheese become greasy (Fox, et al., 2004).

The successive steps are: the application of the first coating on the cheese which is then stored and dried for 1 or 2 days, application of plastic for two or three times during the first 2 weeks. The surface of the cheese has to be sufficiently dry before each treatment. One important aspect is the insulation of the curing room in fact this have to be prevent the condensation of water on the walls, which bring the mould growth. Following the drying and treatment with paraffin (cheese wax). Before waxing the loaves has to be clean, present a dry surface, increase pH to prevent bacterial growth between wax and rind, with the formation of off-flavours and gas (Fox, et al., 2004).

1.5. The microbiology of Cheese

Cheese is one of the most complex foods, in fact this is made by the activity of various microorganisms. The source of microorganism is various and also the type of microorganism and their importance in the cheese manufacture.

At the first the potential source is raw milk with environment contaminants, such as storage or bad farming conditions. During the pasteurisation step the risk of the biofilm enhance the contamination. After the pasteurisation, the starters (SLAB), are added into the cheese (Marley, Crow 1993), It's possible to find also secondary microflora (NSLAB). After pasteurization the contaminants microorganism are very dangerous for healthy consumers, for the yield and quality of the cheese, one example is the presence of microorganism on the gouda surface, or the present of propionic acid bacteria (PAB), otherwise for Jerlberg and Leerdammer, in which the PAB are addicted that is an advantage. Normally the most dangerous contaminants of the cheese are the *moulds*, which may produce *mycotoxins*. (Fox, et al., 2004).

1.6. Starter lactic acid bacteria (SLAB)

One possible definition of a starter can be: it's a mix of microorganisms (bacteria and moulds), which are added to the raw materials or a semiprocessed product. The addiction is in the specific step of the process. The aim of addition is to start and control the microbiological activity.

Primary, starters are added because they produce lactic acid from lactose. But this is not the only reason for their addiction, in fact many of them also produce volatile compounds, like diacetyl from citrate and, produce also CO_2 , *Leuconostoc* is one of this microorganisms.
Moreover recently studies underline the competition between starter, pathogenic and spoilage microorganism, a cause of pH and Eh (Fox, et al., 2004). In this way they contribute to the microbial safety of cheese (Fox, et al., 2004).

Primary the starter is divided in mesophilic and thermophile starters. The first one produce lactic acid at temperatures under 40°C, while the second one begin the acidification when the temperature is around 38°C. Now the most common classification of a starter is based on the complexity of the microorganism.

In the table 1.1 is showed example of cheese types, starter culture and their function.

Table 1.1 Example of cheese types, starter culture and their function.

Cheese	Starters (LAB)	Starter type	Other (NSLAB)
Cottage, Quarg, Cream	LD	DSS, MSS	
Camembert, Brie	LD	DSS,MSS	*Geotrichum candidum, Penicillium camemberti*
Pizza cheese	O	DSS	
Roquefort, Stilton	LD	MSS,DSS	*Penicillium roqueforti,* staphylococci, yeasts
Gorgonzola	O	DSS	*Penicillium roqueforti*
Tilsit	O	NS, DSS	*Brevibacterium linens,* other coryneforms, staphylococci, yeasts
Cheddar	D	DSS	
Gouda, Edam	LD	MSS	
Emmenataler, Sbrinz	O	MSS	*Propionibacterium*
Gruyere	O	DSS	*Schermanii*
Parmiggiano-Reggiano	O	NS	

L-type Leuconostoc species as flavour producers; D- type S. lactis subsp, diacetylactis as flavour producer; LD-type Both leuconostocs and S. lactis subsp, diacetylactis as flavour producers; O-type No flavour producers presence. DSS Defined-strain starters; MSS Mixed-strain starters; NS natural starter

1.6.1. Starter formulation

Natural starter cultures are reproduced daily at the cheese plant. The disadvantages of this technique are that there is nothing to prevent contamination from raw milk, and the impossibility to define the strain in order to standardize the process. Two subtypes are recognized, whey and milk/starters depending on the media and the techniques used for their production.

Natural whey cultures are prepared by incubating some of the whey from the previous making, in selective conditions. One application of this technique is the manufacture of Parmigiano Reggiano and Grana Padano cheese, "whey is removed from the cheese vat at the end of cheese making at 48–52°C and is incubated overnight at controlled temperature (45°C), or in large containers in which the temperature decreases to 37–40°C, to a final pH as low as 3.3" (Limsowtin et al., 1996).

Natural milk cultures are used Southern and Northern Italy for traditional cheeses. "The selective pressure used for the development of the desired microflora includes

thermization/pasteurization of raw milk (62–65 °C for 10–15 min) followed by incubation at a high temperature (37–45 °C) until the desired treatable acidity is reached" (Fox, et al., 2004). The heat treatment is able to selective the microflora in the lattoinnesto, in fact these cultures are usually dominated by *St. thermophilus* but other species maybe present (Limsowtin et al., 1996); (Andrighetto et al., 2002).

Mixed-strain starters (MSS) is crated in the laboratory, selecting one or different strains, which characterized by particular activity. The reproduction of MSS, is made by several transfers in order to create the bulk starter. Nowadays the aim is to reduce the hazard of contamination. In according with this purpose the classic protocol has been replaced by the use of concentrated cultures to inoculate the bulk starter tank or cheese milk, (Fox, et al., 2004).

Defined-strain starters (DSS) are selective strains, which particular characteristics, many of them are phage-resistant. The positive aspect of this kind of technology is a standardize performance,(Fox, et al., 2004).

1.6.2. Type of starter

Liquid cultures are the selective culture, growth in a selective media to envelopment particular characteristic, or growth in milk or in whey. Usually to maintain the correct pH, it is added $CaCO_3$ and the culture are stored at a low temperature, 2-5°C. The stability is around 1 or 2 weeks and to obtain an active culture are needed several transfer, (Fox, et al., 2004).

Frozen cultures "frozen in liquid N_2. Mesophilic and Thermophilic cocci are more resistant than thermophilic lactobacillus and leuconostoc" (Fox, et al., 2004), but it's better create a mix to maintain the balance.

Freeze-dried cultures follow the same technology as the frozen cultures but afterwards the water is removed by sublimation under high vacuum. It is very common to use of antioxidants like ascorbic acid (Fox, et al., 2004).

1.6.3. Type of employment

Nowadays, it is possible to employ the starter with two different technologies:

Bulk starter: The starter has to be prepared before cheesemaking. The starter culture is put in the media, usually milk to growth the microorganism, and after growth of microorganisms (18-24h at 20°C) is added to the cheese after a pasteurisation 30 min at 95°C.

Direct Vat Set (DVS) or Direct Vat Inoculation (DVI): "The starter is sold to dairy in powder and add directly in the cheese. This technology present some advantages: a) It's better establishment a team technical experts and develop the necessary technology and protocols to produce a quality product and monitoring that; b) reduce the hazard of phage infection, the starter production area and cheese making area can be located in different far place; c) the no need of particular batch to prepare bulk starter reduce the step of cheese making and the possibility of cross contamination" (Mullan, W.M.A. 2006).

At the other hand the application of DVS can present some limits: "a) Not all starters respond well to the operations involved in concentrate production and/or storage, to damage the yield of product; b) use of although concentrate suppliers perform quality assurance on their products, starter suppliers generally offer only limited guarantees of concentrate quality; c) use of DVS cultures is expensive compared with bulk starter manufacture; d) In DVS cultures, and there is a lag period before the cultures commences growth and acid production" (Mullan, W.M.A. 2006).

The different between these types of starters is not only the preparation, but the use of them, in fact frozen after defrost usually put directly in the milk, while the freeze-dried culture before to put in the cheese need hydration step.

The most important critical point is the preparation of the bulk culture. "Three aspects of bulk culture preparation are worthy of detailed consideration: (a) the growth medium used; (b) medium sterilization and air filtration, and (c) the inoculation of the bulk tank" (Daly, 1983). The media should be phage free. The other two present the same problem about the possibility of environment contamination. The other critical points are temperature-time and pH, depending on the type of microorganism. "Usually an active mesophilic culture reaches the stationary phase in milk media in 6-8h at 30°C and in 16-18h at 18-21°C; the latter combination is obviously more suitable for overnight incubation. Thermophile cultures may reach the stationary phase in 6-8h at 37°C (Fox, et al., 2004).

1.6.4. Microorganism: mesophilic cultures

The microorganism, mesophilic cultures is possible divided in two groups: mesophilic cultures and thermophilus culture. The attention is focused on the mesophilic cultures, because in the Gouda cheese making use this kind of microorganisms. The mesophilic dairy

bacteria are: *Lactococcus (Lc.) lactis* subsp. *lactis*, *Lactococcus (Lc.) lactis* subsp. *lactis* biovar. *diacetylactis, Lactococcus(Lc.) lactis* subsp. *cremoris, Leuconostoc mesenteroides* subsp. *cremoris.* The acid producers (mainly *Lc. cremoris*) are always present and, depending on the identity of the flavour producing bacteria, these cultures are sometimes designated:

L *Leuconostoc* species as flavours producers

D *Leuconostoc lactis* biovar, *diacetylactis* as flavours producer

LD Both Leuconostocs and *Lactococcus. lactis* biovar *diacetylactis* as flavours producers

O No flavours producers presence

(Daly 1983).

Leuconostoc mesenteroides subsp. ***Cremoris*** **"**is a Gram-positive lactic acid bacteria (LAB) of great economic importance due to its ability to produce gas (CO2) from carbohydrates, flavour compounds (diacetyl, acetate and ethanol) in many dairy products, and dextran in saccharose-containing (dairy) products" (Hemme and Foucaud-Scheunemann, 2004). Some species of Leuconostoc (*Leuconostoc mesenteroides, Leuconostoc lactis*) are very important in the fermentative dairy industry, as they have traditionally been used in butter and cream manufacture, Edam and Gouda cheeses (Vedamuthu, 1994). One of the most important aspect about *Leuconostoc* is its vancomycin resistance. The vancomycin breaks the cell wall, in fact it is active against the D-alanina residue on the end of the peptide chains in the peptidoglycans, but in Leuconostoc and every bacteria which are characterized by vancomycin resistant, at the end of peptide chains of peptidoglycan there is last D-lactate and not D- alanina.

Lactococcus lactis subsp. ***lactis and Lactococcus lactis*** subsp**.** ***cremoris*** is gram positive, spherical or ovoid cell, appear in short chains, non-motile and does not form spores. It's classified like as homofermantative, produce only D-lactic acid. The production of lactic acid is important for cheese making process, acidification, and for development of flavour.

Some strain of *Lactococcus lactis* subsp. *lactis* produce also Nisin. Nisin is a bacteriocin. It is a natural antimicrobial agent with activity against a large number of Gram-positive bacteria, including humans pathogens such as *Listeria, Staphylococcus* and *Clostridium.* Nisin cannot be synthesis chemically.

Lactococcus lactis subsp**.** ***lactis*** biovar**.** ***diacetylactis*** **"**is a subspecies of *Lactococcus lactis subsp. lactis.* They have the same morphological characteristics, but different metabolism. In fact *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* converts citrate into α-acetolactate, key-flavour compound in the butter". (Haddad et al., 1997). In the Figure 1.6 the microscope imagines of microorganism starter are showed.

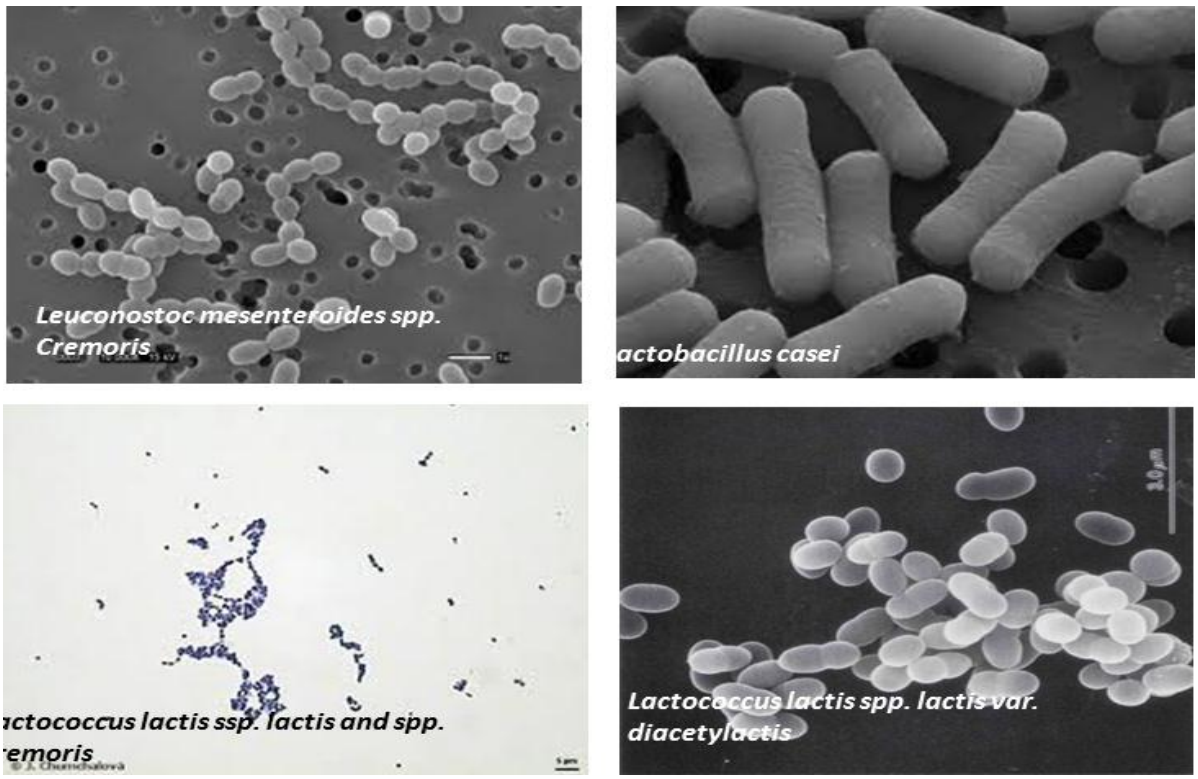


Figure 1.5 The microscope imagine of starter microorganism

1.6.5. The gouda cheese starter

The Gouda starter is a mixed-strain starter, which are selective for their activity in according to create taste, flavour, rate of acidification, characteristic texture and phage resistance. Usually, they are storage as inoculated milk in a frozen condition (Stadhouders and Leenders, 1984).

"The activity of the starter should be constant. Any change in activity can be an indication of a contamination of the starter with disturbing bacteriophages, a reduced activity of the starter (the storage conditions), the presence of antibiotic and/or disinfecting agents in the cheese milk, variations in the composition of the milk, " (Fox et al., 2004).

There are several critical points in the procedure for the manufacture as the possible contamination by air-born phage, and thanks to use the high efficiency particulate Air (HEPA) the risk is reduced. Furthermore, the good hygienic practice is important to avoid environmental contamination. The starter has to be storage for limited time (24h) below 5°C.

1.7.Non-Starter Lactic Acid Bacteria (NSLAB)

Non-starter lactic acid bacteria, by some authors called secondary microflora, are a significant proportion of the microbial population (Beresford et al., 2001).

Their activity is very important during ripening. They don't contribute to acid production, but may contribute directly to create a flavour blend and texture in the cheese. Moreover they are able to inhibit, indirectly, undesirable growth of NSLAB, which can be responsible for off-flavour production. (Marley, Crow 1993).

In any case the NSLAB need the condition to grow: "avaible energy substrate, right temperature, pH and time to allow the biochemical reactions. They grow at 2-53°C and are acid-tolerant with an optimal pH 5.5-6.2. They can be divided in: obligate homofermantative, *Lb. delbrucki*, and *Lb. helveticus*; facultative heterofermentative or obligate heterofermentative" (university lecture). The facultative heterofermenters are the most frequently such as *Lb. paracasei* and *Lb. plantarum*. Other species are *Lb. curvatus, Lb casei, Lb. brevis* and *Lb. rhamnosus.*

Lactobacillus casei "is gram positive, rod shaped, non-sporing, non-motile, anaerobic facultative. (Holzapfel et al., 2001). It's important to underline its optimal pH is 5.5, but at the end of acidification the pH of crud is 5.2- 5.3, grow as well. moreover nowadays its probiotics aspect is always highlighted" (Mishra and Prasad, 2005). It is classified like as facultative heterofermentative, convert sugar in particular lactose into lactic acid and produce also some alcohols, moreover it is oxygen tolerant, it is able to growth both with oxygen and without oxygen.

In the cheese it is most common to find *Pediococci, Leuconostoc* spp., *Enterococci,* and some species of *Lactobacillus,* only some species are able to survive in the cheese, for example in Gouda it is usually to find, *Lb. paracasei* (Østlie et al., 2004).

The importance of the secondary microflora is connected with their interactions with other microorganisms. *Lb. casei*, *Lb. rhamnosus* and *Lb. plantarum* may inhibit PAB and *Enterococci* in cheese as a result of competition for limiting nutrients (Lynch et al., 1996).

1.7.1. Leuconostoc

Leuconostoc ssp. produce diacetyl and acetoin from citrate and is very common to find in mixed strain L and DL cultures (Dellaglio et al. 1995). They are responsible of the eyes in the Dutch type-cheese, thanks to the production of CO_2. The application of the molecular techniques has made it possible to identify in the starter the presence of *Leuconostoc lactis* and three subspecies of *Leuconostoc mesenteroides*, (Morea et al., 1999, Server-Busson et al., 1999). *Leuconostoc* ssp could be consider both starter bacteria and NSLAB, depend on the kind of cheese. In fact its included in the starter and in NSLAB chapter,.

1.7.2. NSLAB in the gouda

"The NSLAB used in the manufacture of washed-curd cheeses, such as Gouda, commonly comprise citrate-utilising strains of *Leuconostoc* and *Lc. lactis spp. lactis var. diacetylactis"* (Stadhouders, Leenders,. 1984; Reiter, B. Moller-Madsen, 1963). *Leuconostoc* can produce bacteriocin active against the general NSLAB, but not against *Pediococcus*. (Martley, Crow, 1993).

1.8. Culture dependent technique

The culture dependent techniques are able to distinguish different species, using different but not able to identified the species. Moreover these techniques has the disadvantage of the long time for analyse, both for the preparation of the reagent and time to wait the answer. Even though it is able to count microorganism, in fact not every colture independent technique are able to quantify the microorganisms.

1.9. Molecular technique (culture-independent techniques)

1.9.1. introduction

Since 1993, when Kary Banks Mullis and Micheal Smith won the Nobel Prize for the discovery of the polymerase chain reaction (PCR) there was a development of molecular techniques. Thanks to this discovery it was possible to envelop the culture-independent

technique, in fact they were able to fill the gap between of culture-dependent techniques and the real conditions of bacteria growth, at different pH, temperature, and nutrients, media. Moreover the molecular techniques are more rapid and reliable.

“Applied molecular microbiology is a fast-moving area. One of the branches of this discipline is involved in the development of molecular methods for the identification and monitoring of microorganisms in natural ecosystems. Methods in molecular microbiology have become a valid support to traditional techniques” (Giovannoni et al., 1990, Ward et al., 1990, Muyzer et al., 1993, Ludwig and Schleifer, 1994, Amann et al., 1995). Molecular techniques are applied to search for the presence, number, and types of microorganism in the food industry. The most major applications are: “a) Identifying the bacterial flora of starter cultures and foods; b) Determining the total numbers of bacteria in food samples; c) Detecting particular strains and/ or biotypes in food products” (Cocolin L. et al., 2008). In table 1.2 “Summary of the most widely used culture-independent techniques and their applications to microbial ecology” is shown. (Cocolin L. et al., 2008)

Table 1.2 **"**Summary of the most widely used Culture-independent techniques and their applications to microbial ecology" (Cocolin L., Ercolini D., 2008). Abbreviation are explained in the bottom of the table

	Taxonomic Resolution	Applications to Microbial Ecology
PCR-BASED method		
PCR-DGGE/PCR-TGGE	Community members (genus/ species level)	Community fingerprinting; population dynamics
SSCP	Community members (genus/ species level)	Mutation analysis; community fingerprinting; population dynamics
T-RFLP	Community and population members (genus, species, strain level)	Community fingerprinting; dynamics between (species-dynamics) and within (strain-dynamics) populations
LH-PCR	Community members (genus/ species level)	Community fingerprinting; population dynamics
PCR-ARDRA	Community members (species level)	Automated assessment of microbial diversity within communities of isolated microorganisms
RISA/ITS-PCR	Particular community members (species groups level)	Community fingerprinting; population dynamics
AP-PCR	Population members (strain level)	Automated estimation of diversity (typing) within populations
AFLP	Community and population members (genus, species, and strain level)	Automated estimation of diversity within communities (species composition) and populations (typing)
In situ Methods		
FISH	Community members (species level)	Detection of viable (both cultivable and uncultivable) cells within communities; temporal and spatial distribution of microbes within ecosystems

Multiplex FISH	Community members (species level)	Similar to FISH; simultaneous investigation of complex communities (e.g. biofilms)
Fluorescence in situ PCR Community	Community members (species level)	Detection of viable, slowgrowing cells within communities; sensitive identification of target sequences with low copy number
Other methods		
Flow cytometry	Population members (strain level)	Selective enumeration of mixed microbial populations and sub-populations; physiological cell state analysis
Competitive PCR	Community members (species level)	Detection of cells into the VNC state
Quantitative hybridization	Community members (species level)	(Semi)quantitative population dynamics of physiologically-active microbial groups

PCR-DGGE/TGGE, PCR-Denaturing Gradient Gel Electrophoresis/Thermal Gradient Gel Electrophoresis; SSCP, Single Strand Conformation Polymorphism; T-RFLP, Terminal-Restriction Fragment Length Polymorphism; PCR-ARDRA, PCR-Amplification Ribosomal DNA Restriction Analysis; RISA/ITS-PCR, rRNA gene Internal Spacer Analysis/ Intergenic Transcribed Spacer-PCR; AP-PCR, Arbitrarily Primed-PCR; AFLP, adaptor fragment length polymorphism; FISH, Fluorescence in situ hybridization.

1.9.2. PCR-based Methods

The discovery of PCR was a revolution for the microbial ecology research, resulting in the development of several techniques of microbial community fingerprinting.

The most important and common PCR-based methods are PCR-DGGE, denaturing gel electrophoresis; PCR-TGGE, temperature gradient gel electrophoresis; RT-PCR, reverse transcription.

"RT-PCR are generally based on the amplification of only the variable region or the totality of the 16S rRNA gene, amplified fragments can also derive from total RNA extracted from food" (Cocolin L. et al., 2008).

In the PCR-denaturing gradient gel electrophoresis (PCR-DGGE) and PCR-temperature gradient gel electrophoresis (PCR-TGGE) the particular region of 16S DNA is amplified microorganism of the food matrix; followed by a separation of DNA fragments.

"Separation is the key-word of these techniques, in fact in both techniques a polyacrylamide gel containing a linear gradient of DNA denaturants (PCR-DGGE) or a linear temperature gradient (PCR-TGGE) are used" (Cocolin L. et al., 2008).Thanks to this gradient the fragment of DNA will show different behaviour, different position along the gel (Felske et al., 1998, Muyzer et al., 1993).

1.9.3. Advantage and disadvantage of culture-independent techniques

These culture independent techniques are quicker, reproducible and, focused than culture-dependent techniques. In fact they follow the genotyping approach, taking the stability of genomic DNA, and it's independent from cultural conditions. Thanks to these techniques it is possible to obtain a complete overview of product without the need to isolate.

This is possible because these techniques are based upon a "community DNA/RNA isolation approach. Although there are limitations to these methods, they can, nevertheless, be very useful once these limitations are taken into consideration" (Forney et al., 2004). The limitations include technical problem, for example one of the large limitations is to obtain representative amount of DNA from the food sample, or the use of the correct primer for the PCR and their condition.

In every experiment it is better to make a comparison between the cultural-depend technique and cultural-independent, phenotypic and genotypic methods, in order to have a complete "view" of product, and to find a technological improvement.

1.9.4. Molecular techniques in cheese study

The cultural independent techniques are applied in dairy products, because they overlap the gap of cultural dependent techniques. In particular the study focused on the cheese, because "it is one of the most microbiology complex foods, in fact it is characterized by the presence of several microorganisms, which play an important role in the manufacture and ripening" (Ndoye et al., 2011). Thanks to the use of culture-independent technique the study about cheese microbial ecosystem has been improved. In the figure 1.6 a flow diagram of the culture-dependent and culture-independent methods to study the population in the cheese microbiota is showed.

In cheese the PCR based methods present some pitfalls: In the cheese there are a lot of elements which could interfere during the DNA extraction. Other limits can be found in the PCR amplification in particular it's possible that the "primer mismatches at the annealing sites of the DNA template of some genotypes, or a lower rate of primer hybridization to certain templates due to differential denaturation of these templates" (Walsh et al., 1992, Suzuki and Giovannoni, 1996). The other limit is the heteroduplexes "that arise in later PCR cycles when primer concentration decrease and the concentration of PCR products is high enough to compete with the primers for annealing" (Kanagawa, 2003).

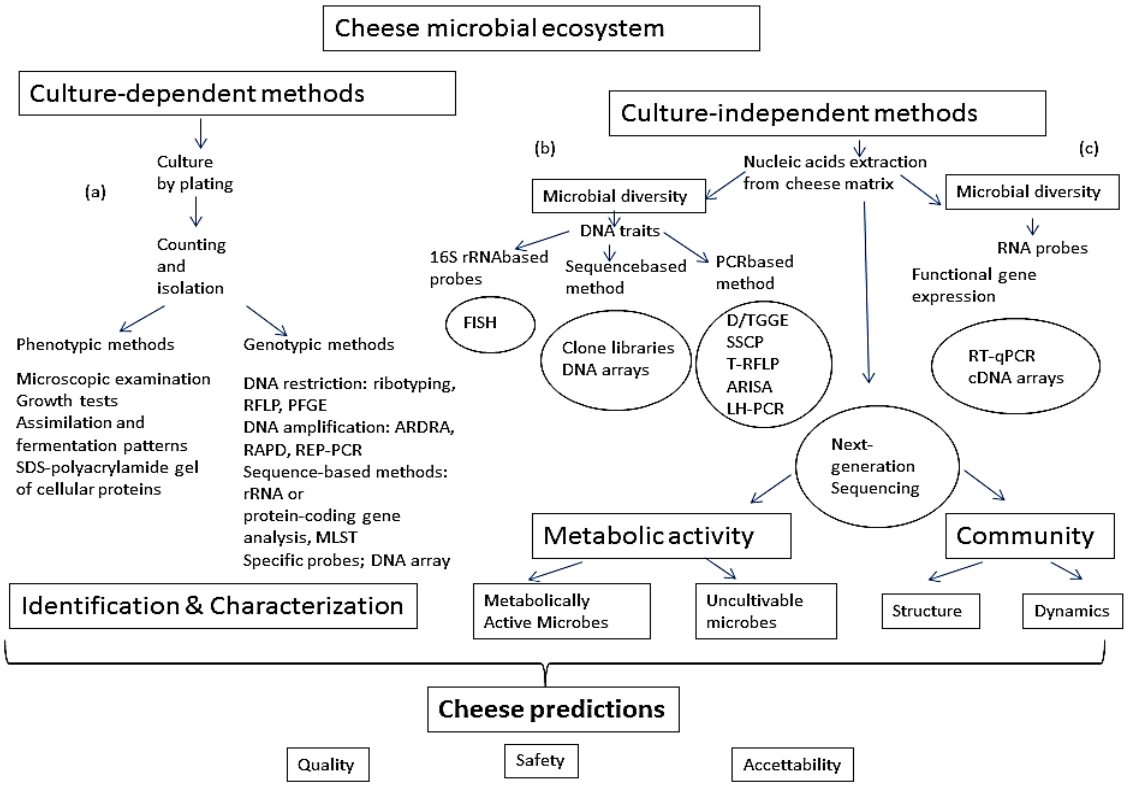


Figure 1.6 Flow diagram of the culture-dependent and culture-independent methods to study the community structure and activity of cheese microbiota.

"a) Culture-dependent methods. b, c) Culture-independent methods for microbial diversity (b) and microbial activity (c). RFLP restriction fragment length polymorphism, ribotyping, PFGE pulsed-field gel electrophoresis, ARDRA amplified ribosomal DNA restriction analysis, RAPD randomly amplified polymorphic DNA, REP-PCR repetitive extragenic palindromic PCR, ARISA automated rDNA internal spacer analysis, DGGE denaturing gradient gel electrophoresis, FISH fluorescence in situ hybridization, ISH in situ hybridization, LH-PCR length heterogeneity polymerase chain reaction, qPCR quantitative PCR, RT-PCR reverse transcription PCR, RTqPCR reverse transcription quantitative PCR, SSCP single-strand conformation polymorphism, SSH suppression subtractive hybridization, TGGE temperature gradient gel electrophoresis, T-RFLP terminal restriction fragment length polymorphism" (Ndoye et al., 2011).

1.10. PCR-DGGE

Denaturing Gradient Gel Electrophoretic (DGGE) is perhaps the most commonly used method among the cultural-independent techniques. The aim of this technique is the separation of PCR amplicons, of same length, but different sequences.

1.10.1. Technical aspect

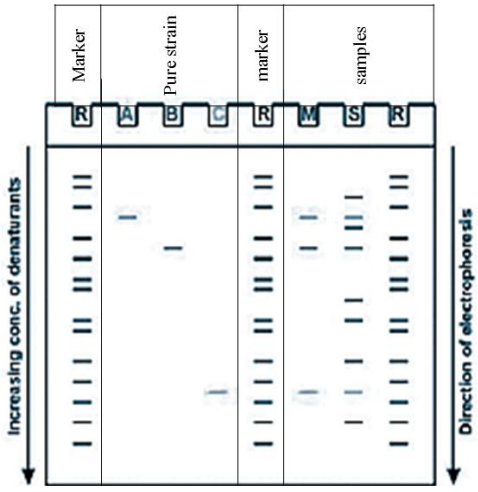
The complexity of the composition of the food matrix influenced the step of the DNA extraction. Aerobic or anaerobic storage, washing, transport, freezing, or refrigerating procedures may affect the development of the microbial species occurring in the food by increasing or reducing the number and the species to be detected (Ercolini, 2004).

The first step is **DNA extraction**, which is one of the most critical points, in fact the amount of DNA amount has to be in a quantity to permit the run in the gel. Sometimes the procedure is hampered by the organic part of the food, such as fat, protein or minerals. These could interfere with the polymerase enzymes that are used in the DNA extraction.

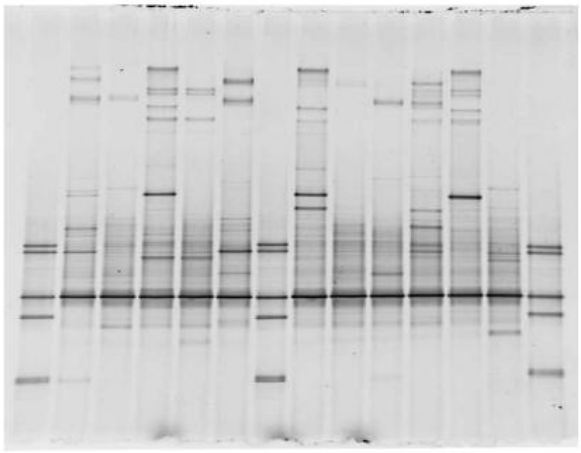
The second important step is the **PCR,** amplification of a variable region of ribosomal DNA., in fact there are a lot of parameters and conditions that have to be fixed before to run the PCR.

First of all, the region detected, usually for dairy products are "V1 and V3" (Ercolini, 2004) of 16S. The experiment could be conducted for the detection of the total population, or show the presence of particular microorganisms, in the last case there is the need of specific primers. Following the choice of PCR protocol conditions, influenced by the primer. Noteworthy, the fragment to be read by DGGE cannot be longer than 500 bp.

The product is the mixture of DNA amplicons from different species (same size but different sequence). In figure 1.7 is showed the theory of base principle of PCR-DGGE, and one real example of gel.



Theory of base principle (Temmerman et al., 2004)



A real example of gel (http://en.engormix.com/)

Figure 1.7 The theory of base principle of PCR-DGGE, and one real example of gel

The third step, **DGGE analysis**. Such as previously aid the DGGE permit to detecte differences between DNA fragments of the same size but with different sequences. The denaturing gradient gel permit the separation of fragments based on their differential denaturation (melting) profile (Ercolini, 2004).

The gel is made of polyacrylamide, the denaturing conditions are provide by urea and formamide.

"The electrophoresis is carried out at a constant temperature between 55 and 65°C, mostly 60°C. Thanks to this temperature the double-stranded DNA are denaturing and encounter increasing concentrations of the denaturing agents and partially melt in discrete regions called "melting domains". "The part of fragment becomes partially melted, creating branched "breaking" molecules, which run in different way for their sequence" (Ercolini, 2004).

Bands in the DGGE gels can be revealed by ethidium bromide staining, or by silver (Felske et al., 1996). The product of DGGE is the sample-specific fingerprint.

The following step is divided in two procedure with the same aim: identification of the microorganism. One of the method is sequencing. The problems of this choice are the need to prepare the sample of DNA, the purification of DNA, and the price of sequencing.
The other method is the comparison between the different migration distances of the markers microorganism and samples. The critical point is the choice of the microorganism marker. Although this procedure is much easier than the sequencing of the band, it does not give the absolute certain identification. In fact one of the problem of the DGGE is the possible co-migration of amplicons. Many species of bacteria are closely related and there is not particular differences in the 16S rDNA fragments (Ercolini, 2004), moreover PCR-DGGE is not a quantitative method, band intensity is not always in agreement with plate counts (Ndoye et al., 2011). Other limits is the sensitivity of DGGE for the analysis of cheese, in fact to estimate to it the number should be 10^4 cfu $g^{-1}$and not detectable of death cells.

Other important disadvantages of the PCR-DGGE are connected with the working part. First the use of formamide, which is a cancerogenic in liquid state, but when it becomes solid it does not present particular problem, from this aspect it is necessary to work under particular conditions, with dedicated equipment and over chemical hood, other cancerogenic element is ethidium bromide.

1.10.2. Future prospective of PCR-DGGE

Ercolini (2004) wonders if the technique may be considered a new tool in food microbiology. Actually is not used to monitor food fermentations and microbiological food quality in real production chain, but only for the research purposes due to its characteristic.

However the technique could be employed to defined microbial compositions, to reveal food adulteration; (Ercolini, 2004).

Moreover these techniques can be applied in the dairy. In order to improve the performance of microbial starter cultures; like help to standardize the technology and food quality.

1.11. The aim of thesis

The aim of this work was to study the Microbial dynamic in the in a Gouda type cheese applying a comparison between culture-dependent technique, plate count, and culture-independent technique as PCR-DGGE. The study was also focus on the analyses of the efficiency of the pasteurizer, and its cleaning steps.

2. Materials and Method

2.1. Pure strain

The pure strains used in this study were: *Lactobacillus (Lb). paracasei* INF 448, *Lb. curvatus* NSLAB 47, *Lb. plantarum* 2060, *Lb. rhamnosus* LGG, *Leuconostoc(Leu.) pseudomesenteri ssp. mesenteroides* ENV58, *Lb. helveticus* ENV26, *Lactococcus (Lc.) lactis susp. cremoris* BF2, *Streptococcus (St.) thermophilus* LMG 2726, *Enterococcus faecium* 2086, *Pediococcus pentosaceus* LMG2722, *Lc. lactis susp. lactis* ML8. The pure strains were incubated in M17 broth (Merck, Darmstadt, Germany) at 30°C for lactococci and enterococci, *St. thermophilus* was incubated at 37°C. MRS broth was used for lactobacilli and pediococci (MRS; Difco Laboratories, Detroit MI, USA) at 30°C, overnight.

2.2..Sampling of dairy product

The samples were collected from the production of Dairy A from four vats on Monday and four vats on Friday. The daily production of cheese is 32 vats/day. Raw milk (RM), Pasteurized milk (PM), cheese milk after rennet (YML), starter (SK), cheese after pressing (OEP), cheese after brine (OEL), were collected from each vat. The samples were stored at -80°C until analysis.

2.3. Plate count

The samples for microbial analysis were collected in accordance with IDF-standard 50c (1995). The *lactococci* were enumerated by M17 agar plateing (Merck,) after 2 days at 30°C. *Lactobacilli* were counted in LBS Agar plates (Difco) after anaerobic incubation for 4 days at 30°C. Presumptive *leuconostoc* and *pediococci* were enumerated on De Man Rodgosa Sharpe agar plates (MRS; Difco Laboratories, Detroit MI, USA) supplemented with 100µg mL^{-1} vancomycin, after incubation at 30°C for 4 days (Østlie, Eliassen et al. 2004).

2.4.DNA isolation

DNA from the pure strains was extracted from 1 ml of the broth following the GelElute Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MO) instructions.

Cheese milk and cheese samples were collected for total DNA extraction at the sampling times described earlier. Cheese samples (11 g) were added to 99 ml sterile 2% w\v sodium citrate solution and homogenized for 2 min, speed 4 in an Omni mixer (Omni International, Waterbury, CT, USA), and 10 ml of supernatant was collected in a 15ml tube, after centrifugation at 8000 rpm for 10min (Thermo Fisher Scientific, Soeborg, Denmark). The fat layer was removed with sterile cottons. For the milk samples, 10 ml were collected in a 15 ml tube. The supernatant was discarded, and the cell pellet collected and washed with sodium citrate water. The DNA isolation from cheese and milk samples was performed by GelElut Bacterial Genomic DNA kit (Sigma-Aldrich), according to the manufacturer's instructions with minor changes; lysozyme and proteinase K treatment were prolonged to 60 and 40 min, respectively (Porcellato, Grønnevik et al. 2012).

2.5.PCR conditions

For amplification of the V1 region of the 16S rDNA gene, universal primer V1allF (5'-GGCGTGCCTAATACATGC-3') and V1all340R (5'-CGTGTCTCAGTCCCAATGTG-3') were used. For V3 region primer Lac1all F (5'- CTCCTACGGGAGGCAGCA-3') and Lac2all R (5'-ATTTCACCGCTACACATGGA-3'). For each sample the reaction mixture consist of: 1x dream taq buffer, 10 Mm dNTP, 5 mM $MgCL_2$, primer F 20 µM, primer 20 µM, 1.25 U, DNA polymerase, 1 µl DNA, total volume of 50 µl. The PCR was performed in a C1000 thermal Cycler, (Biorad, Fostercity, Califonia). The mixture was subjected both to amplify the V1 region of V3 region. To amplify the V1 region the protocol was: initial denaturing 30 min 95°C, denaturing 30s 95°C, annealing 30s 55°C, extension 1 min 72°C, 34 cycle of denaturing, final extension 30 s 72°C. To amplify the V3 region the protocol was: initial denaturing 30min 95°C, denaturing 30s 95°C, annealing 30s 55°C extension 1min 72°C, 34 cycle of denaturing, final extension 30min 95°C.

2.6.Amplification with specific primer for Lactobacillus, Leuconostoc and Pediococcus and Lactococcus, Enterococcus and Streptococcus

In addition to the amplification of V1 and V3 specific primers for Lactobacillus, Leuconostoc and Pediococcus ssp. were used: LAC1 (5'-AGCAGTAGGGAATCTTCCA-3') and LAC2 (5'-ATTTCACCGCTACACATG-3') (Walter, Hertel et al. 2001; Endo and Okada 2005).

For amplification of Lactococcus, Enterococcus and Streptococcus ssp., LAC 2 and LAC 3 (5'-AGCAGTAGGGAATCTTCGG-3') were used. The PCR was conducted in a 20 µl and included 1x LightCycler© 480HRM MasterMix (Roche, Mannhein, Germany), 2 mM of MgCl2, 0.4 µM of each primer and 1µl of extracted DNA from dairy samples. The protocol was: initial denaturation 5min at 95°C, denaturing 30s at 95°C for 30 cycle, annealing 30s at 61°C and final extension 1min at 72°C. The PCR was run in a 96 multiwell LightCycler© 480 Real-Time instrument (Roche) (Porcellato, Grønnevik et al. 2012).

2.6.Agarose gel

The 1% agarose gel was used to check PCR product. Agarose gel was stained with 3 mg L^{-1} of ethidium bromide. Three µl of samples were loaded and run for 30 min at 80 V. Agarose gel was visualized under UV light (Biorad, Fostercity, Califonia).

2.7.DGGE gel

The INGENYphorU system (Ingeny International BV, Goes, the Netherlands) was used for DGGE analysis. The PCR product (20µl) was load into the gel. The gel was characterized by a 35-55% urea-formamide denaturing gradient (100% denaturant corresponding to 7M urea and 40% [v/v] formamide), in 1X TAE buffer (40Mm Tris base, 20Mm acetic acid, 1mM EDTA [pH 8]). Gels were run at a constant voltage of 75 V for 16h at 60°C. After the run the gel was stained in 1X TAE buffer containing 0.5 mg L-1 ethidium and analysed under UV light.

2.8.Band identification and sequencing

The selected band was cut with a sterile scalpel and DNA diffusion was performed in 50µl of 1X TE buffer at 37°C for 4 hours. The band was applied in a new PCR. PCR protocol has to be the same of the previously described (2.5; 2.6). Followed a agarose gel to confirm the presence of DNA was made. The bands were identified also comparing the migration distance with pure strain and other bands sequenced before, and other gels with the same characteristic.

The DNA was purified by QIAquick© PCR Purification Kit(250) (Qiagen, Sverige, Norway). The sample of DNA purified was quantified in the nanodrop (Thermo Fisher Scientific, Soeborg, Denmark). The samples of DNA prepared following the protocol of GATC (http://www.gatc-biotech.com/en/sequencing/single-reads.html) was send to GATC (GATC biotech AG, Konstanz, Germany) to be sequence.

3. Results

3.1.Plate count

Both production day, Monday and Friday, showed the same plate count trend on M17 media, indicating similar growth of *Lactococci* for all samples. The raw milk numbers showed the same trend for each batch from log 3 cfu ml^{-1} to log 4 cfu ml^{-1}(table 3.1). Between batch 16 and 17 there was a quick cleaning both on Monday and Friday. On Friday after the quick cleaning, between batch 16 and batch 17, there was a reduction from log 4 cfu ml^{-1} to log 2 cfu ml^{-1} in the pasteurized milk, but in batch 32 there was again an increment to log 4 cfu ml^{-1}. Between batch 32 of the previous day and batch 1 the next day there was a long cleaning. This cleaning decreased the bacteria to < log 1 cfu ml $^{-1}$ in the first vat. Cheese milk before rennet showed the same trend for both days but at this stage the starter was added to the cheese milk. The starter count on Monday showed a reduction of bacteria from batch 17 to batch 32. However, the trend was different during the Friday production with log 7 cfu ml^{-1} in the starter of vat 1 and log 8 cfu ml^{-1} in batch 16, but then reduction in batch 17 and 32. In cheese after pressing the count was similar for both production days, around 8 cfu ml^{-1}. In cheese after brining the plate count were log 8 cfu ml^{-1}, and showed a larger variation compared to the cheese before brining.

Table 3.1 Plate count in M17, incubated at 30°C for 24h for the samples of Monday and Friday

day	**MONDAY**				**FRIDAY**			
batch	**1**	**16**	**17**	**32**	**1**	**16**	**17**	**32**
Raw milk	3.17	3.053	3.015	3.03	3.34	3.38	3.01	3.019
Pasteurised milk	<1	3.3	3.23	3.04	<1	4.26	2.31	4.12
Cheese before rennet	6.53	6.71	6.68	6.51	6.57	6.7	6.68	6.61
Starter	8.31	8.8	8.3	7.4	7.61	8,2	7,91	7.55
Cheese after pressing	8.077	8.12	8.09	8.12	8.08	8.12	8.09	8.2
Cheese after brine	8.055	7.25	8.17	8.14	8.46	8.19	8.17	7.79

The plate count in MRSV, selective media for *Leuconostoc (Leu.)*, evidenced the presence of presumptive *Leuconostoc* in raw milk (table 3.2). No colonies were counted in the pasteurized milk, while in the cheese before rennet at batch 1 a low number of colonies were counted. In the starter the presence of *Leuconostoc* was showed in batch 1, but not in the other batches on the Monday production. On the Friday production the presence of *Leuconostoc* showed the same trend as the Monday production from raw milk to cheese after brine, with counts of log 3 cfu ml^{-1}. *Leuconostoc* was found in cheese after pressing in the Friday production with the same trend as on Monday, and in cheese after brine. On Monday *Leuconostoc* was not found probably due to too high dilutions.

Table 3.2 Plate count in MRSV agar plate presumptive *Leuconostoc* for the samples of Monday and Friday

day	**MONDAY**				**FRIDAY**			
batch	**1**	**16**	**17**	**32**	**1**	**16**	**17**	**32**
Raw milk	2.47	2.14	2.09	2.19	2.23	2.214	2.2	2.33
Pasteurised milk	<1	<1	<1	<1	<1	<1	<1	<1
Cheese before rennet	<4*	<4*	<4*	<1*	1.65	<1*	<1*	<1*
Starter	5.02	<4*	<4*	<2*	3.23	3	3.2	3
Cheese after pressing	<3*	<3*	<3*	3.1	<3*	2.61	2.54	3.1
Cheese after brine	<6*	<6*	<6*	<6*	<5*	3.21	3.18	3.053

* There was not of colonies present above this dilution. This was the lowest dilution plated.

The LBS plate count underlined the presence of *Lactobacilli (Lb.)* in the raw milk (table 3.3). *Lactobacillus* were not found in the pasteurized milk, in the cheese milk before rennet and in the starter. In the cheese before pressing the *Lactobacilli* count was log 3 cfu ml^{-1} in vat 16, while count were < log 5 cfu ml^{-1} for the other batches. On the Friday production *Lactobacilli* were detected between log 2 cfu ml^{-1} and log 3 cfu ml^{-1} in cheese after pressing and cheese after brining of each batch, except for batch 1.

In the pasteurized milk *Lactobacilli* spp. was not detected in batch 1 on Monday while in batch 1 on Monday around log 5 cfu ml^{-1} were enumerated in the cheese after rennet.

For batch 16 and 17 between log 4 cfu ml^{-1} and log 1 cfu ml^{-1} were enumerate, respectively. In batch 1 of Friday *Lactobacilli* were enumerated log 2 cfu ml^{-1} and no presence of *Lactobacilli* were detected in the other batches. In the starter *Lactobacilli* were enumerated

less than 5 cfu ml $^{-1}$ on Monday and less than log 2 cfu ml $^{-1}$ on Friday. On Friday *Lactobacilli* are present in batch 16, 17 and 32 after pressing and also for all the samples of cheese after brine.

Table 3.3 Plate count in LBS, dived in Monday and Friday and for each batch.

day	**MONDAY**				**FRIDAY**			
batch	**1**	**16**	**17**	**32**	**1**	**16**	**17**	**32**
Raw milk	2.69	2,2	2,04	2,14	2,23	2,2	2,2	2.45
Pasteurised milk	<1	<1	< 1	<1	<1	<1	<1	<1
Cheese before rennet	<5	< 4	< 4	<1	2	<1	<1	<1
Starter	<5	< 5	< 5	<2	<2	<2	<2	<2
Cheese after pressing	<5	<5	<5	<5	<5	2,68	2,32	3.07
Cheese after brine	< 5	< 5	< 5	<5	<5	3,2	3,13	3.3

3.2. DGGE gel

The DGGE analysis with universal primer specific for the V1 and V3 regions of the 16S were used to compare the two production days.

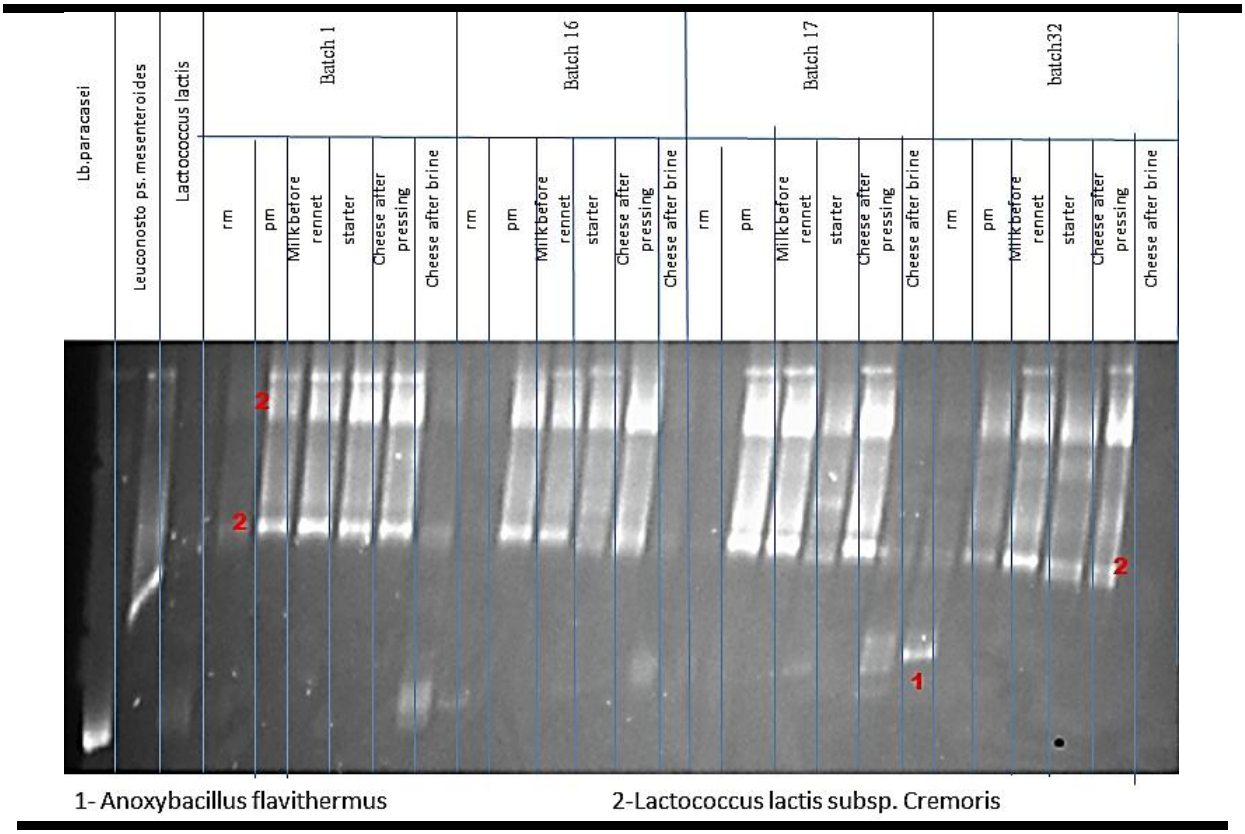
In the raw milk (rm.) of 1, the presence of *Lactococcus (Lc.) lactis* subsp. *cremoris* as the major species was shown (figure 3.1). Pasteurized milk showed a particular DGGE fingerprint, which was not in accordance with the plate count, meaning that also died microorganisms were detected. In the milk before rennet *Lc. lactis* subsp. *cremoris* was the predominant species. The DGGE analysis showed a more pronounced presence of *Lc. lactis* subsp. *cremoris* than *Leuconostoc* in the starter. The same fingerprint as the starter was shown in the cheese after pressing, while in the cheese after brine *Lc. lactis* subsp. *cremoris* and *Leuconostoc* was less present. With the amplification of the V3 region for the same batch it was showed *Halomonas* (93% identity, GenBank accession no.: JQ228579.1) was present in the pasteurized milk (fig. 3.2). This microorganism was also found in the following sample. From the plate count it was not possible to detect this microorganism.

In **batch 16** from Monday, amplification of V1 showed the presence of *Lc. lactis* subsp. *cremoris* in particular in the pasteurised milk and in milk before rennet. However in the starter the presence of *Lc. lactis* subsp. *cremoris* was lower. In the cheese after pressing *Lc. lactis* subsp. *cremoris* was found. In the batch of the Friday production raw milk had the presence of *Lb. casei*, band 3 figure 3.1, (97% identity GenBank accession no. HM070024.1), but this specie was not found in the other samples from the same vat. For the pasteurized milk the population microorganism were the same as for the other samples. In the same batch with the amplification of the V3 region of the 16S gene it was detected *Geobacillus*, band 2 figure 3.2 (92% identity, GenBank accession no.: JN867331.1), in the raw milk. The Friday production *Lc. lactis* subsp. *cremoris* was the major microorganism in all samples.

In **batch 17** of Monday, by amplification of the V1 region of the 16S rRNA gene, *Lc. lactis* subsp. *cremoris* (100% identity, GenBank accession no.: CP003132.1) was the dominant specie. In batch 17 of Friday, *Lb. casei* (97% identity GenBank accession no. HM070024.1) was detected in the raw milk, while the DGGE analysis of the other raw milk samples were the same as the Monday production, with the dominance of *Lc. lactis* subsp. *cremoris.* By amplification of V3, *Halomonas* was found in all Monday productions in milk after rennet, cheese before pressing, cheese after brine. *Geobacillus* was also found following the same trend in the same samples, while in the Friday production *Lc. lactis* subsp. *cremoris* was the dominating microorganism.

In the **batch 32** of the Monday production, amplification of V1 showed that *Anoxibacillus flavithermus* (98% identity, GenBank accession no.: CP000922.1) band 1 figure 3.1 was present in the raw milk. In the other samples *Lc. lactis* subsp. *cremoris* dominated the microflora population. In the DGGE gel of V3 region of the raw milk (fig. 3.2) presence of *Halomonas* and *Geobacillus* was seen, while no presence of *Lc. lactis* subsp. *cremoris* was detected. In the Friday production, the V1 amplification detected *Lb. casei* and in the samples of Friday the same trend was shown with the presence of *Lc. lactis* subsp. C*remoris* (fig. 3.2).

MONDAY PRODUCTION



FRIDAY PRODUCTION

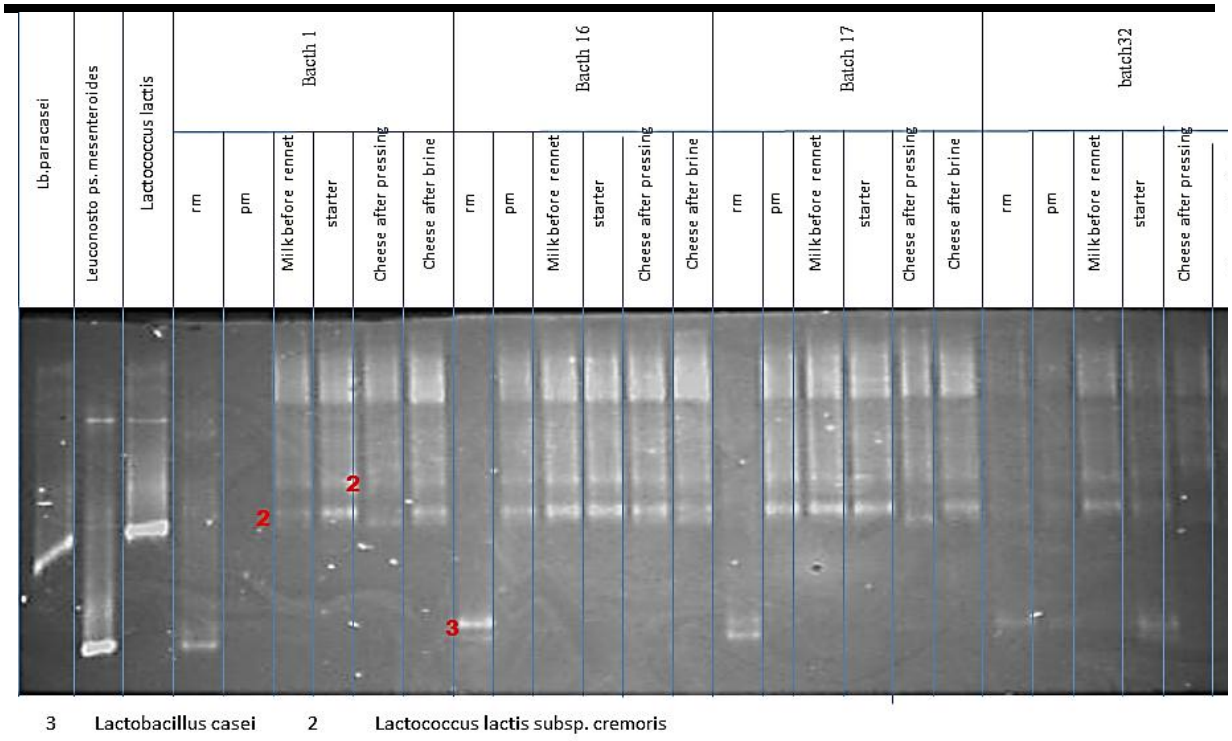
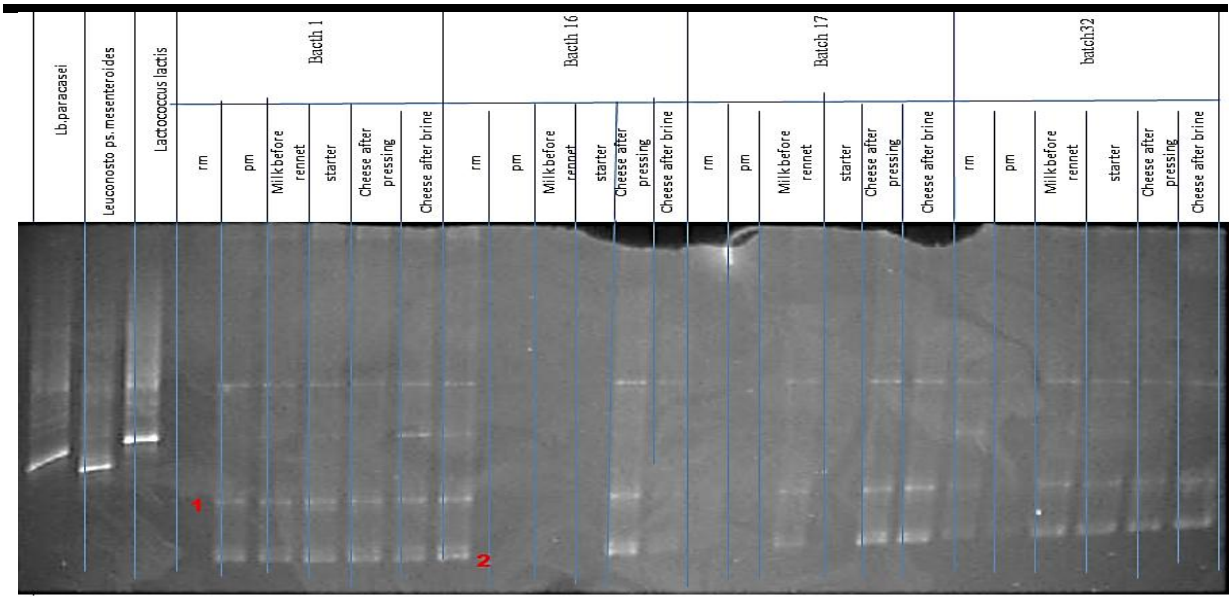


Figure 3.1 DGGE of PCR products of the V1 for the Monday and Friday production.(rm: raw milk; pm: pasteurized milk)

Comparing the result of plate count and PCR-DGGE (figure 3.2) there was a predominant presence of *Lactococci* and minority of *Lactobacilli.* In the raw milk amplification of the V3 region detected the presence of *Lb. casei.*

MONDAY PRODUCTION



1 Halomonas 2 Geobacillus

FRIDAY PRODUCTION

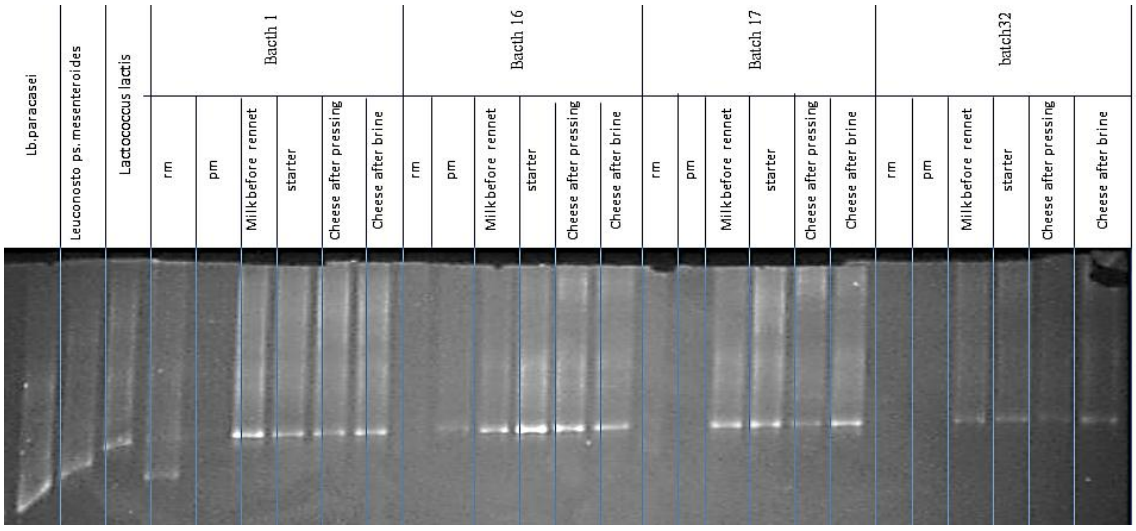


Figure 3.2 DGGE of PCR products of the V3 for the Monday and Friday production. .(rm: raw milk; pm: pasteurized milk)

In figure 3.3 the gel of the PCR-DGGE product with specific primers for V1 and V3 for the

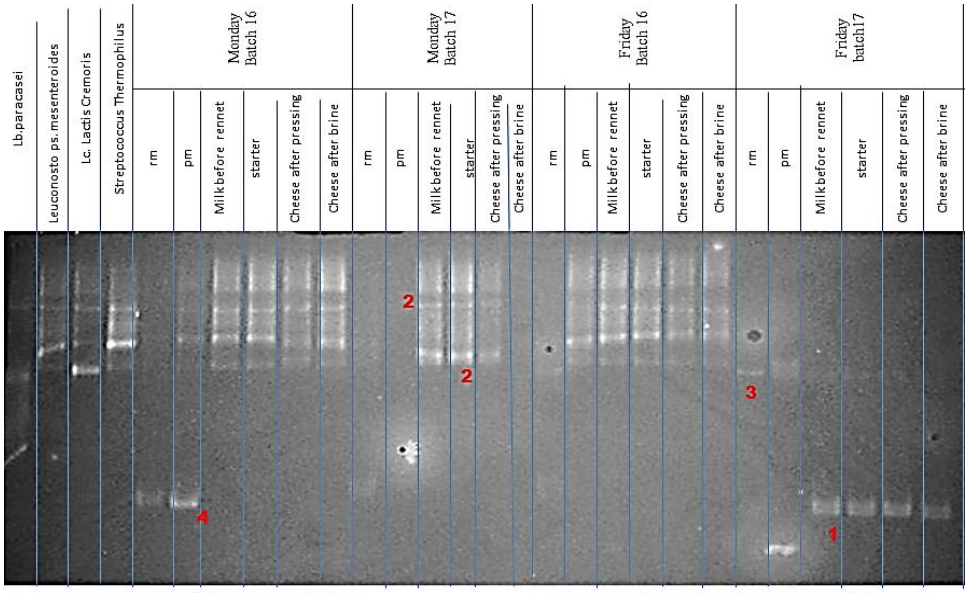
sample before and after the short cleaning of the pasteurizer for the Monday and Friday production is shown (batch 16 and 17).

Amplification of V1 of the Monday **batch 16** showed the presence of *Anoxibacillus flavithermus* band 4 figure 3.3 (98% identity, GenBank accession no.: CP000922.1) in the pasteurized milk. In the cheese making process it was not found in batch 16 on Monday and Friday. Both V1 and V3 amplification showed the presence of *Streptococcus dysgalactiae* subsp. *dysgalactiae* band 3 figure 3.3 (99% identity, GenBank accession no.: JF789447.1) in the raw milk of batch 16 on Friday. Amplification of V3 showed the same trend for both days of production. Anyway, in the Monday production the presence of *Lactococcus raffinolactis,* band 7 figure 3.3 (97% identity, GenBank accession no.: HM218712.1) in the cheese after pressing was shown. In the same sample *Lc. lactis* subsp. *lactis* (99% identity, GenBank accession no.: JQ724544.1), band 6 figure 3.3 was present. Comparing the result of plate count and PCR-DGGE predominant presence of *Lactococci* in all samples of batch 16 was shown.

In **batch 17**, the V1 amplification, showed that *Streptococcus thermophilus* was not present. In the raw milk of Friday *Streptococcus dysgalactiae* subsp. *dysgalactiae* (99% identity, GenBank accession no.: JF789447.1), band 5 figure 3.3, was present. From the milk after rennet until cheese after brine *Aeribacillus pallidus* (93% identity, GenBank accession no.: JN701188.1), band 1 figure 3.3 and *Geobacillus pallidus* (93% identity, GenBank accession no.: HM030740.1), band 1 figure 3.3 were present, underling the environment contamination. With the amplification of V3, the trend is the same for both production days, with the relevant presence of *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris.*

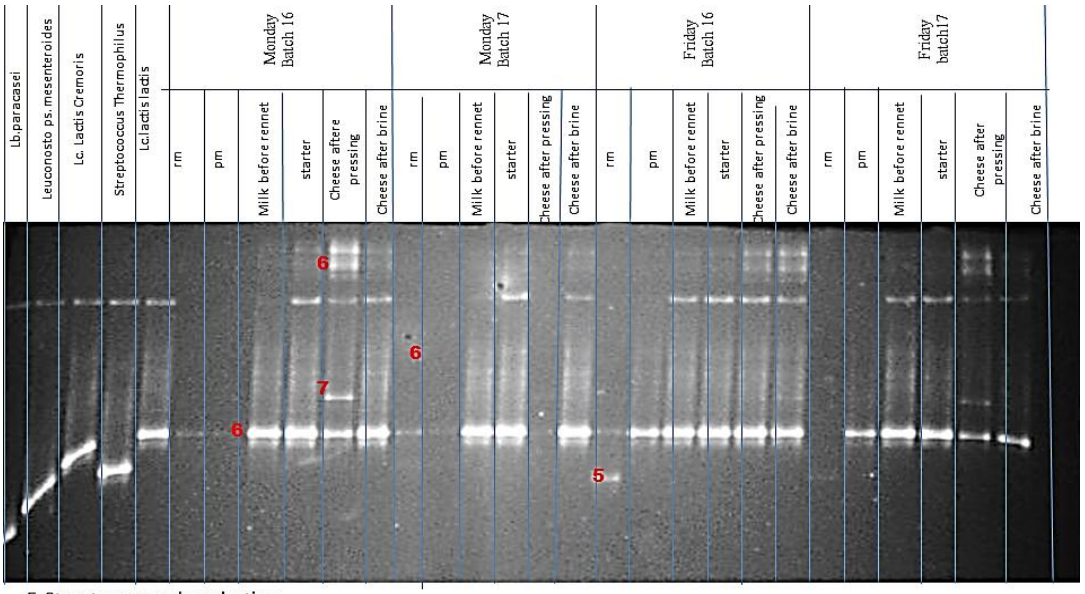
Batch of 16 and 17 were analysed with two set of primers (Lac1-Lac2 and Lac3-Lac2) specific for the *Lactobacillus/Leuconostoc/Pediococcus* (*Lb./Leu./Ped.*) and the *Lactococcus/Streptococcus/Enterococcus* (*Lc./St./En.*) genus, respectively. These primers amplified the V3 region of the 16S rRNA gene.

V1



1-Aeribacillus pallidus/Geobacillus pallidus
2- Lactococcus lactis subsp. cremoris
3-Streptococcus dysgalactiae subsp. dysgalactiae
4-Anoxybacillus flavithermus

V3



5-Streptococcus dysgalactiae
6-Lactococcus lactis subsp. lactis
7-Lactococcus raffinolactis

Figure 3.3 DGGE of PCR products of the V1 and V3 for the sample before and after cleaning of pasteurized for the Monday and Friday production .(rm.: raw milk; pm: pasteurized milk)

Lactococci for batch 16 and 17 before and after cleaning of the pasteurized for Monday and Friday production are showed.

In the PCR-DGGE gel from batch 16, the dynamic of the microorganisms was similar from milk before rennet to cheese after brine. However in the Friday production had higher intensity of the bands, meaning a major presence of this microorganism. The main difference was in the pasteurized milk. At the Friday production *Leuconostoc* was detected contrary to the Monday batches. Moreover the Friday raw milk showed presence of more microorganism, in particular *Lactobacilli.*

Batch 17 of Monday, had a similar fingerprint as batch 16 of the same production day. The samples from starter to cheese after brine on Friday production did not present any bands, possible due to technical bias of DGGE such as the amount of DNA or incomplete PCR.

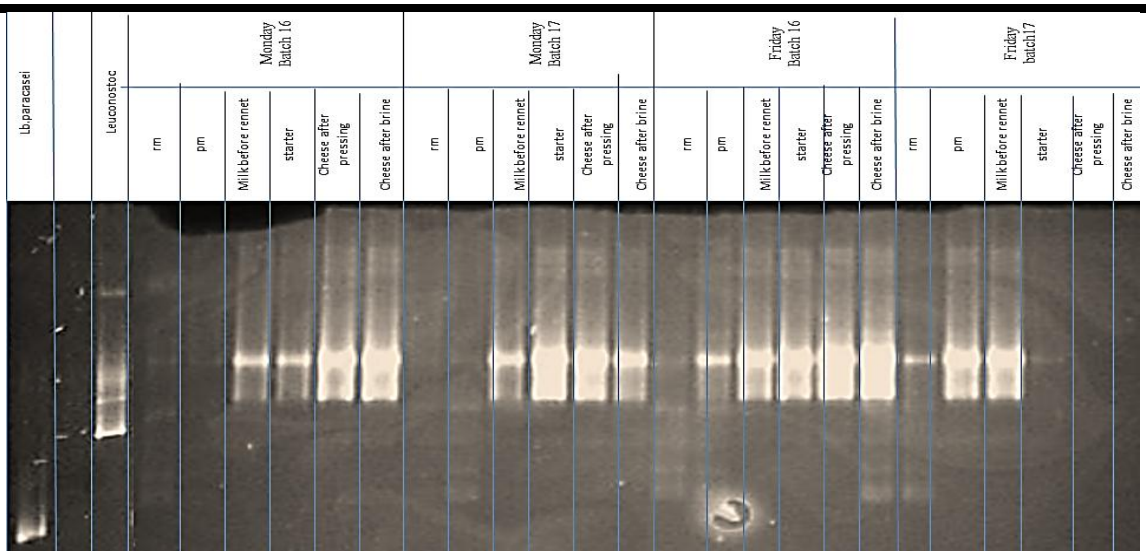
The gel for *Lactococci* (fig 3.4) underlined the presence of *Streptococcus dysgalactiae* subsp. *dysgalactiae* in the raw milk of the Friday production. In the cheese after pressing of both batches of Monday *Lc. raffinolactis* was present, *Lc. raffinolactis* was also detected in the V3 gel band 7 in figure 3.3. In all productions in both raw and pasteurized milk there was a presence of *Lc. lactis* subsp. *lactis.*

In figure 3.5 the gel of the PCR-DGGE product with the specific primers for *Lactobacilli* and for *Lactococci* for the raw milk and pasteurized milk for each batch of the two days investigated are shown.

PCR-DGGE for *Lactobacilli*/*Leuconostoc*./*Pediococci* of the **Monday** production for each batch showed the presence of *Leu. pseudomesenteroides* (99% identity, GenBank accession no.: JN167933.1), band 3 figure 3.5, however with less intensity in batch 32. In the raw milk of batch 1 there were in addition to *Leu. pseudomesenteroides, Leuconostoc* ssp. *mesenteroides* and *Lb. paracasei.* All of them persisted in the following samples, with the highest intensity in batch 16. In the **Friday production** the intensity of the bands was changed for all raw milk samples with more intensity of the bands of the *Leu. mesenteroides* (94% identity, GenBank accession no.: FR670534.1), band 2 figure 3.5 present in batch 1.

The analysed PCR-DGGE for Lactococci/ Streptococci/ Enterococci showed for the **Monday** production underlined the trend between after and before cleaning of the pasteurized milk , with more band intensity in the pasteurized milk of batch 16 and 32. On **Friday** the same trend was shown, but in particular in the raw milk the presence of *Streptococcus dysgalactiae* subsp. *dysgalactiae* (99% identity, GenBank accession no.: JN639391.1), band 5 figure 3.5 and *Lc. lactis* subsp. *lactis* (99% identity, GenBank accession no.: JQ411245.1) were shown.

Lactobacilli



Lactococci

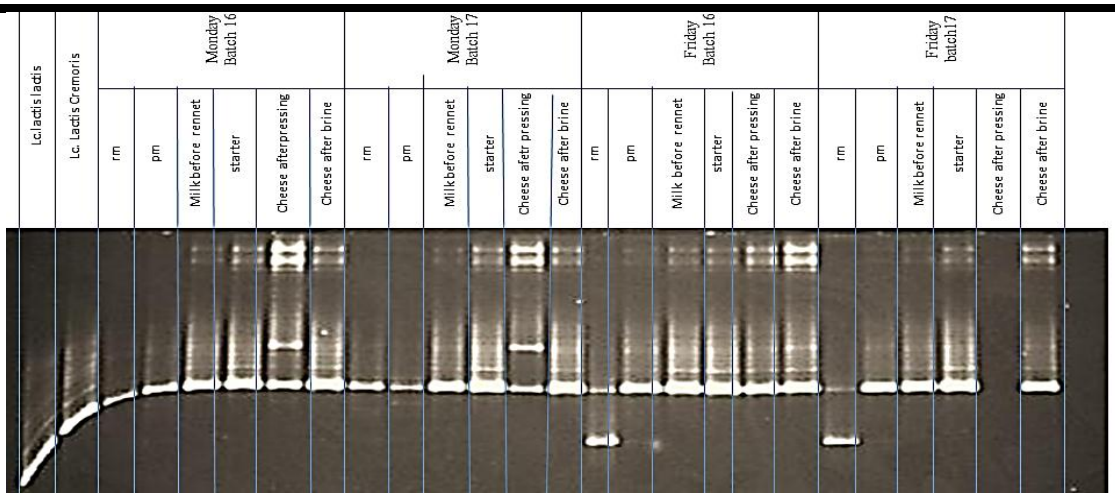
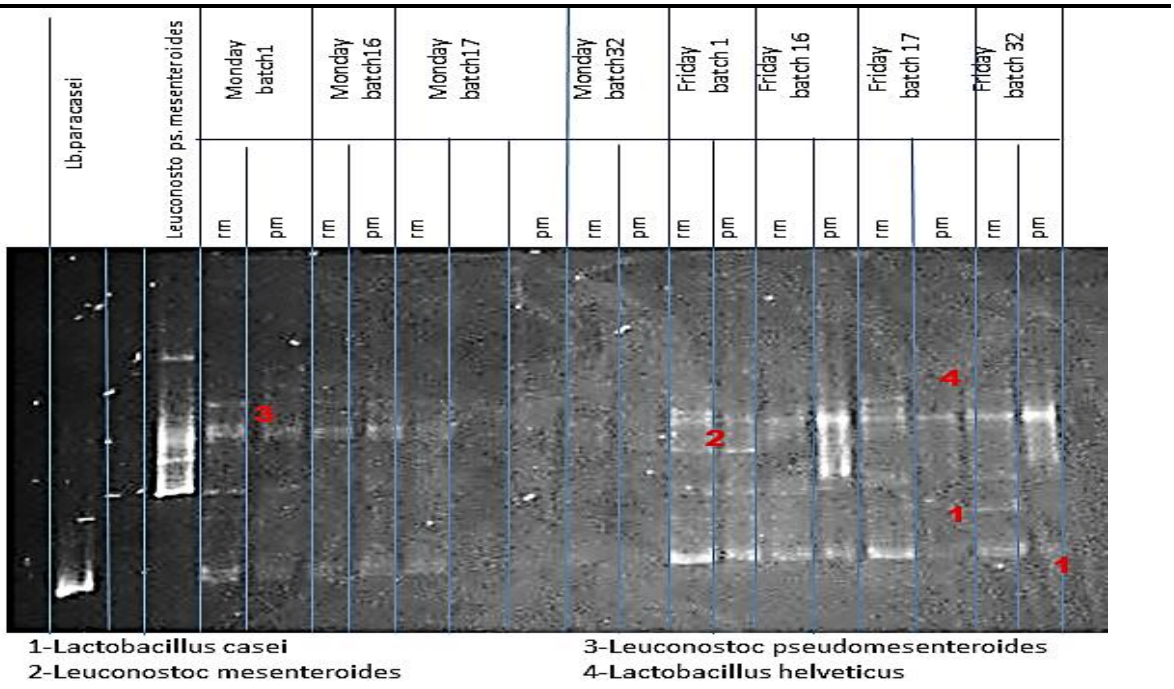


Figure 3.4 DGGE of PCR products of the specific primer *Lactobacilli* and for *Lactococci* for the sample before and after cleaning of pasteurized for the Monday and Friday production. (rm.: raw milk; pm: pasteurized milk)

Lactobacilli



Lactococci

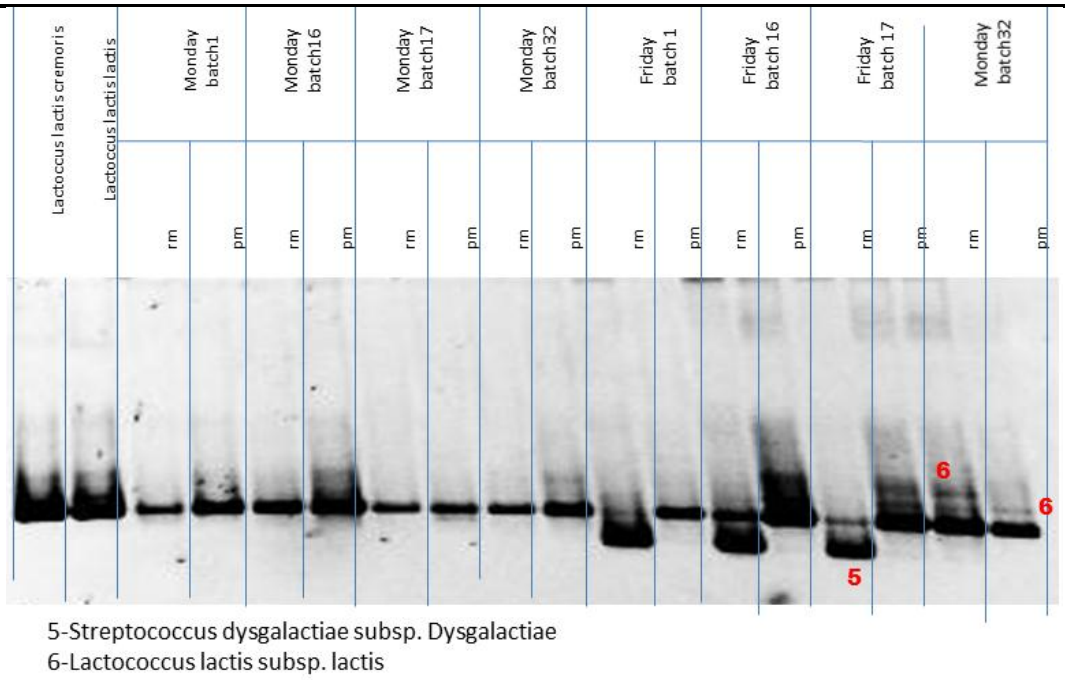


Figure 3.5 DGGE of PCR products of the *Lactobacilli* and for *Lactococci* for the sample of raw milk and pasteurized milk for each batch of two days.(rm: raw milk; pm: pasteurized milk).

4. Discussion

In this work the study of the dynamic population in early step of the cheese making was investigated by culture-dependent and culture-independent techniques from raw milk to cheese after brining. The samples were analysed during two day of productions, Monday and Friday, and for each days 4 batches were followed. During the day of production 32 batches are normally produced. The batches studied were the first the batch number 16, before the quick cleaning of the pasteurizer, batch 17, after the quick cleaning of the pasteurizer, and the last batch of the day.

The raw milk showed a trend in the plate counts according to a previous study (Jarvis et al., 2012) with a count of 3 cfu ml^{-1} in the M17 media. The indigenous microbiota in the raw milk may include *Lactococcus (Lc.)* ssp., *Lactobacillus(Lc.)* ssp., *Leuconostoc(Leu.)* ssp., *Streptococcus* ssp., *Micrococcus* ssp., *Staphylococcus* spp., *Arthrobacter* spp., *Corynebacterium* spp., *Brevibacterium* spp., *Enterobacter* spp., *Citrobacter* spp. and *Acinetobacter* spp. (Masoud et al., 2011). By molecular techniques, as PCR-DGGE, it was possible to detect the microorganisms present in the raw milk, and to show the difference between the raw milk at different production days. Sequencing of the band showed presence of *Streptococcus dysgalactiae* susb. *dysgalactiae*, which may indicate a problem of mastitis in the raw milk (Zadoks et al., 2011). *Streptococcus dysgalactiae* subsp. *dysgalactiae* was detected in all raw milk of Friday production. *Anoxybacillus flavithermus* was present in the raw milk of batch 16 and 32 during the Monday production. *Streptococcus dysgalactiae* subsp. *dysgalactiae*, and *Anoxybacillus flavithermus* may create biofilm, and for this it may be present in the pasteurized milk. *Anoxybacillus flavithermus* had previously been found at low level in raw milk. *Anoxybacillus flavithermus* is part of the typical microflora in the milk powder, and their presence can be associated with the selling price, (Palmer et al., 2010). Furthermore, another important aspect is the optimum temperature (50-70 °C) of growth for *bacilli*. *Bacilli* produces spores which are heat resistant (Somerton et al., 2012). In this case, *Anoxybacillus flavithermus* was considered as an autochthonous microorganism in the raw milk, while in the pasteurized milk, it may be considered as one of the causes of biofilm. The comparison of the plate count and PCR-DGGE analysis highlighted how PCR-DGGE was not able to detect dead microorganisms in some of the sample after complete cleaning. *Anoxybacillus flavithermus* was not detected with the use of specific primers for *Lactobacilli*, due to the high presence of *Lb. casei* and to the possible low number of the microorganism. Some of the pitfall of PCR-DGGE may be the co-migration of bands belonging to different species, and the limit detection which may be around log 3 cfu ml/g^{-1}. Another important bias

is that PCR-DGGE is not able to distinguee the live cell from dead cell. This difference is important in the samples of pasteurized milk, because the plate count with M17 (Table 3.1), MRSV (Table 3.2) and LBS (Table 3.3) were below 10 CFU ml/g^{-1}, however DGGE detected these species present in the samples. In previous work on raw milks, presence of *Lb. casei*, *Leu.* spp. *mesenteroides, Lc. lactis* subsp. *lactis, Lc. lactis* subsp. *cremoris* were shown (Ercolini, 2004).

The plate results indicated that the critical control point was the pasteurization step and the cleaning between batches 16 and 17. In pasteurized milk *Halomonas* was present, but it was a possible bias of PCR-DGGE, because *Halomonas* was only found in this gels. *Lc. lactis* subsp. *cremoris* and *Lc. lactis* subsp. *lactis* were the dominant microflora in all samples.

Moreover the presence of *Lb. casei* and *Lb. paracasei* in the pasteurized milk was showed, with higher intensity of the band in batch 16, before cleaning. From these results the problem of pasteurization was not only connected with the streptococci biofilm, but also with the presence of lactobacilli. The presence of *Lc. casei* and *Leuconostoc* ssp. in the pasteurized milk was impossible to detect with the amplification of V3 region of 16S and V1 region of 16S with universal primer because of the *Lactococci* amount in the sample which may hide *Lactobacilli* and *Leuconostoc* bands.

The Norwegian law set the limits for the presence of the pathogens, *Salmonella* and *Staphylococci*. Moreover the law forbids the distribution and trade of raw milk for consumption. Its regulation is different from other countries, where raw milk cheeses are made and sold, for example Italy and France.

In the milk before addition of rennet at the Monday production, there was the predominant presence of *Lc. lactis* subsp. *cremoris* and *Lc. lactis* subsp. *lactis*. At the Friday production, in particular in batch 16, there was the presence of *Aeribacillus pallidus/Geobacillus pallidus*, which may evidence an environment contamination. *Aeribacillus Pallidus/Geobacillus Pallidus* may create spores which are heat-treatment resistant (Burgess et al., 2010).

The starter used every day was a bulk starter and after pasteurization of the milk, it was added to the milk. The plate count trend for each batch of starter of both production days was in according with result ground literature (Fox et al., 2004), between log 7 cfu ml^{-1} and log 8 cfu ml^{-1} for *lactococci*. According to other works and with the trend of plate count *Lc. lactis* subsp. *cremoris* and *Lc. lactis* subsp. *lactis* were the dominant microorganisms. *Leuconostoc*,

Lb. casei and *Lb. paracasei*, was detected as a minor population (Fox et al.,2004). Even if the composition of the starter was the same, there was a little difference between the days. The difference consisted in the balance between the starter strains and *Lb. casei* and *Lb. paracasei*, *lactobacilli* may be part of the NSLAB flora.

In the cheese after pressing there was a predominant presence of *Lc. lactis* subsp. *cremoris*, *Lc. lactis* subsp. *lactis*, without the presence of *Leuconostoc.* In the starter there was only *Lactococci*. This data can underlined also the comparison between the result of PCR-DGGE with the amplification of V1 and V3 regions with specific primer for *Lactobacilli* and *Leuconostoc*. In both *Leuconostoc* was not present, while in the cheese after pressing of Monday, *Lactococcus raffinolactis* was detected.

Lactococcus raffinolactis may be considered one non-starter lactic acid bacteria (NSLAB), able to ferment α-galactose, such as melibiose and raffinose. *Lactococcus raffinolactis* is normally found in raw milk, it is able to grow in the presence of 4% NaCl, in M17 but it is not able to growth at 40°C (Boucher et al., 2003). The presence of *Lactococcus raffinolactis* may underlined the absence of yeast and glucose (Kimoto-Nira et al., 2012). The presence of *Lactococcus raffinolactis* can be derived from the starter (Kimoto-Nira et al., 2012).

In the cheese after brine *Lc. lactis* subsp. *cremoris*, *Lc. lactis* subsp. *lactis* were the dominant microorganisms. From the PCR-DGGE with specific primer for Lactobacilli *Leuconostoc* ssp. *mesenteroides* was detected. The presence of *Leuconostoc* was show both from the plate count and from the intensity from the band. The plate count trend lead to study with particular attention the pasteurization step, before and after cleaning. Before the experiment, it suggests that this dairy may have problem with the presence of *Streptococcus thermophilus* (Skeie, Porcellato personal communication), which was suggest to be a part of the biofilm on the surface of pasteurizer and may be present, during the cheese making process in the pasteurized milk (Fox et al., 2004). However with the molecular technique other microorganisms were identified which may create biofilm. *Aereobacillus pallidus* and *Geobacillus* were found in the milk after pasteurization and in the cheese before rennet.

The problem of thermophilic bacillus

The presence of *Anoxybacillus flavithermus, Aeribacillus pallidus* and *Geobacillus* underlined the presence of different species of bacillus in cheese before rennet and in the raw milk.

Anoxybacillus flavithermus and *Geobacillus spp*. are one of the main contaminants in the dairy products, not pathogenic, but they are shown a poor hygiene (Burgess et al., 2010). *Bacilli* are able to produce spores, resistant to heat treatment, ultra-high-temperature (UHT) treatment (134– 145 °C, 1–10 s). (Burgess et al., 2010)

Anoxybacillus flavithermus is a facultative anaerobic thermophile and motile bacillus. "The growth temperature is 30-70 °C with optimum around 60°C and pH 6-9" (Burgess et al., 2010, Heinen et al., 1982).

Geobacillus ssp. optimum temperature is above 50°C, (Burgess et al., 2010; Holt et al.,1985). Moreover, the presence of salts can contribute to creation of spores (Burgess et al., 2010).

"Biofilms are generally resistant to cleaning-in-place (CIP) chemicals and sanitizers. There is evidence that biofilms may protect bacterial cells against CIP chemicals and that viable bacterial cells can remain attached to dairy manufacturing surfaces following a CIP cleaning" (Burgess et al., 2010).

Sometimes, in the cheese making process there are some critical points as the addition of $CaCl_2$ after pasteurization. After rennet the pH was 6.50-6.55, optimum for the bacilli growth. The brining step can be considered as another critical point for the ability of salt to promote the production of spores. Other problems may be connected to the quality of raw milk. If the raw milk contain bacillus, such as *Aeribacillus Pallidus*, *Geobacillus* and *Anoxybacillus flavithermus* it may be difficult to remove these, while the UHT treatment, pasteurized condition, may promote to the formation of spores. Another problems might be the biofilm formation, which is resistant to chemical compound and to physic stress, using to clean the pasteurized. In order to reduce this hazard the attention should focus on the cleaning operation and raw milk quality (Burgess et al., 2010).

5. Conclusion

The present study about the dynamic of microorganisms in a Gouda type cheese underlined the major presence of *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis*, only in one case *Lactococcus raffinolactis* was also identified, while *Leuconostoc* seems is minority. The NSLAB was represented by *Lactobacillus casei*, *Lactobacillus paracasei* and species of bacillus. Bacillus may create heat resistance spores, which may create biofilm on the pasteurized surface. Since the conservation of bacillus species were higher in the pasteurized milk than in the raw milk, it is likely that these participate in biofilm creation during the pasteurizer process.

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