

**“Diferenciación de las poblaciones microbianas
dominantes durante la maduración de queso tradicional
danés mediante la técnica de PCR-DGGE”**

Tesis de Master de

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INDEX

1.- Introduction – Objectives

2.- Literature Review

3.- Material and Methods

4.- Results and discussion

5.- Conclusions

6.- References

TITLE: PCR-DGGE as a tool for detecting dominant microbial populations in traditional Danish cheese during the ripening

Keywords: Cheese ripening; Microbial diversity; Molecular methods; PCR-DGGE

1. INTRODUCTION-OBJETIVES

Cheese, as a consequence of its nutritional and economic interest, is probably the most studied traditional food. The cheeses are usually classified on basis of the raw materials (milk and starters), the industrial technology, the texture and the chemical, biochemical and sensory properties of the cheeses. These aspects determine the bacterial growth that can influence in the safety and security of the cheeses. On the other hand, Polimerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) fingerprinting was recently introduce into food microbiology (Ercolini, 2004). This molecular biology method based on direct analysis of DNA have allowed more precise descriptions of microbial dynamics in complex ecosystems that classical microbiological methods, which are generally long and tedious, and allow only a partial inventory of the bacterial microflora. In this context, the objectives of the present project were:

- To study by the PCR-DGGE culture-independent technique the bacterial diversity in raw milk samples upon different storage conditions.
- To detect bacterial population shifts in samples of Danish cheese with different stages of ripening by PCR-DGGE.

2. LITERATURE REVIEW

2.1. Raw Cow Milk Bacterial Populations

The diversity in the microbial flora of raw milks contributes to the great differences in organoleptic characteristics among raw milk cheeses. To date, identification has been limited to the enumeration of the most represented microbiological groups, with partial identification. In brief (Desmaures *et al.*, 1997; Michel *et al.*, 2001), the dominant microflora of raw milk generally include (i) species of lactic acid bacteria (LAB; *Lactococcus* and/or *Lactobacillus* spp.), (ii) *Pseudomonas* spp., (iii) the group

Micrococcaceae (*Micrococcus* and *Staphylococcus* spp.), and (iv) yeasts. Other microbial groups present in raw milks belong to the LAB (including *Leuconostoc*, *Enterococcus*, and *Streptococcus* spp.), *Bacillus*, *Clostridium*, and *Listeria* spp. and *Enterobacteriaceae*; there are also many gram-negative (*Acinetobacter*, *Alcaligenes*, *Flavobacterium*, and *Aeromonas*) and gram-positive (*Arthrobacter*, *Corynebacterium*, *Brevibacterium*, and *Propionibacterium*).

Many factors influence milk composition and hence the nature and abundance of the microbial load. The conditions of raw milk production, in particular the hygienic practices of farmers (e.g., washing of milking equipment and pre- and post-milking udder preparation), determine the contents in useful cheese-making and spoilage microorganisms (Michel *et al.*, 2001). Intensive washing of milking equipment and udder preparation (individual washings) result in raw milks containing a majority of spoilage microorganisms (such as coliforms and *Pseudomonas* spp.). In contrast, minimal hygiene around the udder preserves microorganisms, including salt-tolerant flora (such as *Micrococcus*, *Arthrobacter*, *Microbacterium*, *Brevibacterium*, and *Staphylococcus* spp.) and also the lactic acid bacteria (LAB) (Desmaures *et al.*, 1997), yielding raw milks in which useful cheese-making microorganisms are dominant. The health status of animals, the nature of their feed (forage, ensilage, etc.), and the storage conditions of raw milk are also important factors that determine the composition of their microbial flora. Intensive washing of milking equipment associated with storage of the raw milk at low temperatures gives higher levels of contamination by *Pseudomonas* spp. Fifty percent of the psychrotrophs in refrigerated raw milk (the first day) belong to the genus *Pseudomonas*, with *Pseudomonas fluorescens* being the predominant species. Other psychrotrophs present in refrigerated raw milk belong to the genera *Acinetobacter*, *Alcaligenes*, *Flavobacterium*, *Aeromonas*, *Bacillus*, *Listeria*, and *Arthrobacter*; *Enterobacteriaceae* such as *Hafnia alvei*, *Citrobacter freundii*, or *Serratia liquefaciens* are also found (Desmaures, 1995). The dynamic change in the bacterial population in milk associated with refrigeration was also studied by Lafarge *et al.* (2004).

2.2. Importance of raw milk in cheese making

Pasteurization, since its adoption in the early 1900s, has been credited with dramatically reducing illness and death caused by contaminated milk. The pasteurization process uses heat to destroy harmful bacteria without significantly changing milk's

nutritional value or flavour. In addition to killing disease-causing bacteria, pasteurization destroys bacteria that cause spoilages. For that raw milk may harbour a host of disease-causing organisms (*pathogens*) where they finally appear in the cheese as well, such as the bacteria *Campylobacter*, *Escherichia*, *Listeria*, *Salmonella*, *Yersinia*, and *Brucella*. Common symptoms of food-borne illness from many of these types of bacteria include diarrhoea, stomach cramps, fever, headache, vomiting, and exhaustion. Most healthy people recover from food-borne illness within a short period of time, but others may have symptoms that are chronic, severe, or life-threatening. People with weakened immune systems, such as elderly people, children, and those with certain diseases or conditions, are most at risk for severe infections from pathogens that may be present in raw milk. *Listeria monocytogenes* can cause meningitis and septicemia with up to 30% mortality, and in pregnant women can result in miscarriage, fetal death, or illness or death of a newborn infant. *Escherichia coli* infection has been linked to hemolytic uremic syndrome, a condition that can cause kidney failure and death. Gastroenteritis due to *Salmonella* spp. can lead to long-term illness such as reactive arthritis. Infection with *Salmonella paratyphi* B may lead to septicemia (enteric fever). *Brucella melitensis* causes undulant fever, a severe disease that can be long-lasting and incapacitating. Other diseases that pasteurization can prevent are tuberculosis, diphtheria, polio, salmonellosis, strep throat, scarlet fever, and typhoid fever (Bren, 2004).

In spite of this health risks, there are several reasons to use raw milk to make cheese:

- i) The beneficial bacterial community who grows up naturally (no pathogenic or spoilage microorganisms) they should be inoculated as starters if the milk would be pasteurized and this increases the production costs and decreases some quality aspects.
- ii) Most of the natural milk enzymes are breakdown affected by heat during the pasteurization and this could have an important influence in the enzymatic process during the ripening stages because they are involved in the development of the flavour, taste and texture of the cheese.
- iii) Some whey proteins can precipitate by excessive heat treatment. Therefore, they are no usable for curd and as result the performance of the process is lower.
- iv) Calcium solution is also affected for the heat treatment during pasteurization process.

- v) Finally, after a ripening time of 60 days the raw milk cheeses are considered risk-free because of the most pathogenic bacteria (*Campylobacter*, *Escherichia*, *Listeria*, *Salmonella*, *Yersinia*, and *Brucella*) are supposed not to be able to grow up after this time.

2.3. Denaturing Gradient Gel Electrophoresis (DGGE)

The bacterial community of raw milk has been described by classical microbiological methods, which are generally long and tedious, and allow only a partial inventory of the bacterial microflora. New molecular approaches based on direct analyses of DNA (or RNA) in its environment without microbial enrichment have allowed more precise descriptions of microbial dynamics in complex ecosystems. One of the most-developed methods is denaturing gradient gel electrophoresis DGGE (Randazzo *et al.*, 2002; Van Beek *et al.*, 2002; Ercolini *et al.*, 2003)

DGGE is an electrophoretic method capable of detecting differences between DNA fragments (200-700 bp) of the same size but with different sequence. This is because these fragments can be separated in a denaturing gradient gel based on their differential denaturation (melting) profile. When a DNA fragment is subjected to an increasingly denaturing physical environment, it partially melts. As the denaturing conditions become more extreme, the partially melted fragment completely dissociates into single strands. Rather than partially melting in a continuous zipper-like manner, most fragments melt in a step-wise process. Discrete portions or domains of the fragment suddenly become single-stranded within a very narrow range of denaturing conditions. The rate of mobility of DNA fragments in acrylamide gels changes as a consequence of the physical shape of the fragment. Partially melted fragments migrate much more slowly during electrophoresis through the polyacrylamide matrix than completely double-stranded fragments. When a double-stranded fragment is electrophoresed into a gradient of increasingly denaturing conditions, it partially melts and undergoes a sharp reduction in mobility because it changes shape. In practice, the denaturants used are heat (a constant temperature of 60 °C) and a fixed ratio of formamide (ranging from 0-40%) and urea (ranging from 0-7 M) (Cocolin *et al.*, 2001). The position in the gradient where a domain of a DNA fragment melts and thus nearly stops migrating is dependent on the nucleotide sequence in the melted region. Sequence differences in otherwise identical fragments often cause them to partially melt at different positions in the gradient and therefore 'stop'

at different positions in the gel. By comparing the melting behaviour of the polymorphic DNA fragments side-by-side on denaturing gradient gels, it is possible to detect fragments that have mutations in the first melting domain.

Many fragments can be analyzed simultaneously on a single denaturing gel (32 wells can be load in each operational round with the Bio-Rad DCode™ System apparatus Hercules, USA) in which the direction of electrophoresis is perpendicular to that of the denaturing gradient. When a large number of different fragments are electrophoresed, the fragments can be identified by their molecular weight in the low denaturant side of the gel. By following the S-shaped curves, the characteristic denaturant concentration at which the first domain melts can be determined. When two nearly identical sets of fragments are mixed together and electrophoresed into a 'perpendicular' denaturing gradient gel, the melted domains that have sequence differences between each other will melt at slightly different positions and produce double bands.

Sequence differences are often easily detected in DNA fragments when nearly identical digests are electrophoresed in the same direction as that of the denaturing gradient. These 'parallel' gels permit the simultaneous comparison of as many sets of fragments as there are lanes on the gel, unlike the perpendicular gels. The procedures below refer almost entirely to parallel denaturing gradient gels. DGGE analyses are employed for the separation of double-stranded DNA fragments that are identical in length of the different bacteria of the raw milk and the raw milk cheese (Terzic-Vidojevic *et al.*, 2007; Flórez and Mayo, 2006), but differ in sequence. In practice, this refers to the separation of DNA fragments produced via PCR amplification. The technique exploits (among other factors) the difference in stability of G-C pairing (3 hydrogen bonds per pairing) as opposed to A-T pairing (2 hydrogen bonds). A mixture of DNA fragments of different sequence are electrophoresed in an acrylamide gel containing a gradient of increasing DNA denaturants. In general, DNA fragments richer in GC will be more stable and remain double-stranded until reaching higher denaturant concentrations. Double-stranded DNA fragments migrate better in the acrylamide gel, while denatured DNA molecules become effectively larger and slow down or stop in the gel. In this way DNA fragments of differing sequence can be separated in an acrylamide gel. For the low-GC-content (low-GC%) bacterial species (T_m of V3 sequence of <75°C), optimal resolution was achieved by temporal temperature gel electrophoresis (TTGE); for bacteria with medium- or high-GC% DNA (T_m of V3 sequence of >75°C), the best

separation was obtained by DGGE.

This sort of techniques are called “*Fingerprint technique*” because they give a quite define shape between two different DNA chains (Fromin *et al.*, 2002). These techniques are used to find differences between the strains of different bacteria in the same samples, because it is supposed that each bacterium has some genomic different, even though a lot of bacteria have a close phylogenetic relationship, for example between the bacteria of the same group as LAB, and many times to find the differences between each other with this kind of similarities are really difficult because the shape of the bands are close one an other appearing as only one band this is known as “coomigration” and It is resolved having a precise gradient of denaturant this could open the distance between each other because as closer gradient of denaturant as the precision opening the DNA chains increases.

3. MATERIALS AND METHODS

3.1. Raw Milk samples

The variety of cows which were milked for the production of the raw milk cheese was the Red Danish cow. The milk samples were collected from two dairy farms (Dairy farm A and Dairy farm B) in sterile conditions. All raw milk samples were cooled down, carried to the laboratory and stored at -20°C.

Samples of raw cow milk from dairy farm A (55085) and B (55086) and maintaining for 2 days at 37°C (incubated cow milk) were also studied.

The raw and incubated cow milk sample codes are:

| Raw cow milk sample's codes | |
|-----------------------------|--------------|
| Dairy farm A | 1,2,3,4,5 |
| Dairy farm B | 6,7,8,9,10 |
| Incubated Raw cow milk | 55085, 55086 |

3.2. Raw Milk Cheese samples

The cheese samples used in this research were taken away, in sterile conditions, from the normal production of a dairy company in different days and weeks during the ripening process. The samples were frozen at -14°C.

The raw cow milk cheese sample codes are:

| Raw cow milk cheese sample's codes: | |
|-------------------------------------|---|
| Fresh cheese | 77011c, 77012c, 77 013c, 77015c. (C: Core) 77011s, 77012s, 77013s, 77015s. (S: Surface) |
| One week | 77017c, 77018c, 77019c, 77021c. (C: Core) 77017s, 77018s, 77019s, 77021s. (S: Surface) |
| Four weeks old | 77076c, 77077c, 77078c, 77080c. (C: Core) 77076s, 77077s, 77078s, 77080s. (S: Surface) |
| Six weeks old | 77128c, 77129c, 77130c, 77132c. (C: Core) 77128s, 77129s, 77130s, 77132s. (S: Surface) |

3.3. DNA extraction in dairy samples

Cheese and milk samples (2 g) were dissolved in 20 ml of 2% (w/v) trisodium citrate and incubated 30 min at 45°C. The mixture was mechanically treated with 3 mm diameter glass pearls and vortexed for 5 min. After settling (10 min), the supernatant was transferred to clean tubes and centrifuged for 10 min at 8,000 rpm. The fat layer was removed with a sterile cotton tip and the cell pellets re-suspended in 1ml of TE buffer (10 mM Tris-Cl, 1 mM EDTA at pH 7.5) and centrifuged for 5 min at 8,000 rpm. The supernatant (approximately 900µl) was removed. The remaining (100 µl) and the pellet were mixed and transferred to a sterile 2 ml Screw-cap tube containing 0.3 g Zirconium beads and 150 µl TE-Phenol. This mixture was treated in a bead beater for 5 min at 5,000 rpm. Afterwards 150 µ of CI solution (Chloroform: Isoamyloalcohol 24:1) was added and the tubes were briefly vortexed and centrifuged for 5 min at 13,000 rpm in a vacuum hood. Aliquots of 0.5 ml from liquid phase were used for performing DNA purification.

Total DNA was obtained by phenol-chloroform extraction previously described by (de los Reyes-Gavilan *et al.*, 1992). The aliquot (0.5 ml) described above was mixed with 150 µl TE-Phenol and 150µl CI solution (Chloroform:Isoamyloalcohol; 24:1), under vacuum hood, and it was performed until a clear interface was obtained. The aqueous phase was taken and added 2 volumes of absolute ethanol (-20°C). After incubation at -20°C for 30 min the mixture was centrifuged for 20 minutes at 13.000 rpm, washed briefly with 70% ethanol and suspended in 500 µl of TE after that the DNA would be collected in the sample .Then, the sample was added to five units of DNase-freeRNase and was incubated for 5 min at 37°C.

Finally, DNA was precipitated with 1/10 (v/v) 3 M Sodium Acetate (pH 5.2) and 2 volumes of 96% ethanol (-20°C) and stored at -20°C. After 30 min the DNA was collected by centrifugation at 13,000 rpm for 20 min and suspended in 50 µl of TE Buffer.

3.4. PCR conditions

PCR amplification was performed with Taq DNA Polymerase Kit (Life Technologies, Inc., Rockville, MD). The 180 bp V3 region of the 16S ribosomal DNA of *E. coli* was amplified with the primer 338 fGC and 518r (5¢ CCTACGGGAGGCAGCAG 3¢). Reaction mixtures consisted in 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3mM MgCl₂, 50 mM of each deoxynucleoside triphosphates (dNTPs), 1.25 U of Taq-polymerase, 5 pmol of each primer and 1 µl of appropriately diluted template DNA in a final volume of 50 µl. Amplification was performed in a programmable heating incubator. Template DNA was denatured for 5 min at 94°C. A “touchdown” PCR was performed to increase the specificity of amplification and to reduce the formation of spurious by-products (Ercolini *et al.*, 2002). Afterward an hybridation block of 35 cycles with this three different steps: 94°C for 30 s, 66°C for 20 s and 68°C for 40 s. After a final extension at 68°C for 7 min the samples could be kept in the incubator machine for 24 h at 4°C. The amplification products were analyzed first by conventional electrophoresis in 2% (w/v) agarose gel to test the success of the extraction and the PCR.

3.5. DGGE analysis

PCR products were analyzed by DGGE using a Bio-Rad DCode™ System apparatus. Samples were electrophoresed on thick 9% polyacrylamide (PAA) gel (acrylamide:bisacrylamide; 37.5:1) polyacrylamide gels containing a denaturing gradient from 30 (or 40) to 65% urea and formamide (100% corresponded to 7 M urea and 40% (v/v) formamide) in the direction of electrophoresis.

3.5.1. Mounting plates

The glass plates and plastic spacers were cleaned with ethanol (70/96%). Silicone vacuum grease was applied on the plastic spacers to about 0.25 cm from the edge of the

spacers, avoiding that the vacuum grease touch the gel's side. When the plastic spacers were placed and aligned at the edge of the big plate, the smaller plate was firmly placed on the bigger plate over the two spacers, edge by edge, creating a cell in-between the plates afterwards mount plates in the clamps. The alignment of the clamped plate holder and then slot the clamped plates in the holders was checked. Plates were then ready for gel casting. Mix in a yellow screw cap centrifuge tube the denaturing solution (16.5 ml), 300 μ l loading dye (with the indications of the Bio-Rad manufacturer), APS- 90 μ l (ammonium persulfate solution 10% wt/wl) and 15 μ l TEMED. The centrifuge tube was gently roller a few times to mix. As soon as the components were mixed the denaturing solutions (containing 9% polyacrylamide (PAA) - acrylamide:bisacrylamide 37.5:1) started to polymerise then there left 5-7 minutes to cast the gel. The gradient solutions were sucked into the respective high and low syringes and gently released the bubbles by pushing the piston of the syringe vertically up till air space was removed. Both of the flexible outlet connectors of the needle nozzle were connected to each syringe and release the low solution to the connexion of both outlet connectors and high gradient fluids were pushed out to the edge of the needle, taking care to release remaining bubbles. The syringes were clamp (horizontally) on "the wheel" using some plastic screws which fix them to the structure. The needle nozzle was inserted on a side edge of the plate cell. The wheel was rolled down gently and gradually releasing the mixture into the cell until filling up it completely. The comb was inserted centrally to form the gel's wells, a total of 16. The plates were left undisturbed for about 2 hours to polymerise inside the fume hood.

3.5.2. Electrophoresis

Electrophoresis chamber was filled up with TAE buffer (prepared with sterile water) to the run level. It was covered and turned on power heater settled at 60°C. System was equilibrated for about 2 h until 60°C was reached. The plastic handle of glass cell was locked to the clap which was used to hold on the glass cells inside the chamber. It was left for at least one hour

Loading of samples: 40 μ l of loading dye solution (with the indications of the Bio-Rad manufacturer) were added to the PCR product (~ 40 μ l). The wells were filled up with buffer before load the samples. Then, 35 μ l of dyed sample were loaded into each well using the special loading pipette tip, taking care to clean the tip with the buffer, before to introduce it in the well, in order to don't contaminate with the external residue

the surroundings. The chamber was covered, put on heater and waited until temperature reached 60°C. Then, the chamber was connected to the power supply. The voltage was adjusted to 70 V. The migration was performed at least 16 hours.

The gel was introduced into a dark tray with a staining solution (1:10000) by adding 25 µl of stain (*SYBR Gold*) to 250 ml of 1x TAE buffer and placed on a horizontal shaker for about at least 20 minutes. Afterwards, the gel was transferred on UV table. The chamber was capped with lid, turned on UV light and taken pictures with exposures times from 1.5 to 3 seconds depending on the gel. As soon as the photo was taken the UV light was put off to avoid damaging the DNA. Saturation was unchecked and maximized window to have a proper view of picture. Picture and print copy were saved.

3.5.3. Purification of DGGE fragments/bands

In cases where new bands appeared or where assignments were ambiguous, DNA bands on DGGE gels were excised and purified (Ampe *et al.*, 2001). For this purpose, piece of the band was sucked with a 200 µl cut pipette tip (about 0.5 cm). The tip was placed into a labelled PCR tube and with a new pipette tip was sucked 40 µl of TE buffer and the gel fragment was pushed with the buffer to the PCR tube. DNA was eluted for a couple of hours at 5°C in a refrigerator and transferred to a deep-freezer (-20°C) until to be used. In this work, the original bands were purified by subsequent steps running gel with the same denaturing concentration (Denaturing gradient from 40 to 65%) until a clear and recognised bands were obtained in DGGE gels (examples in Figure I and II.)

Final results in red

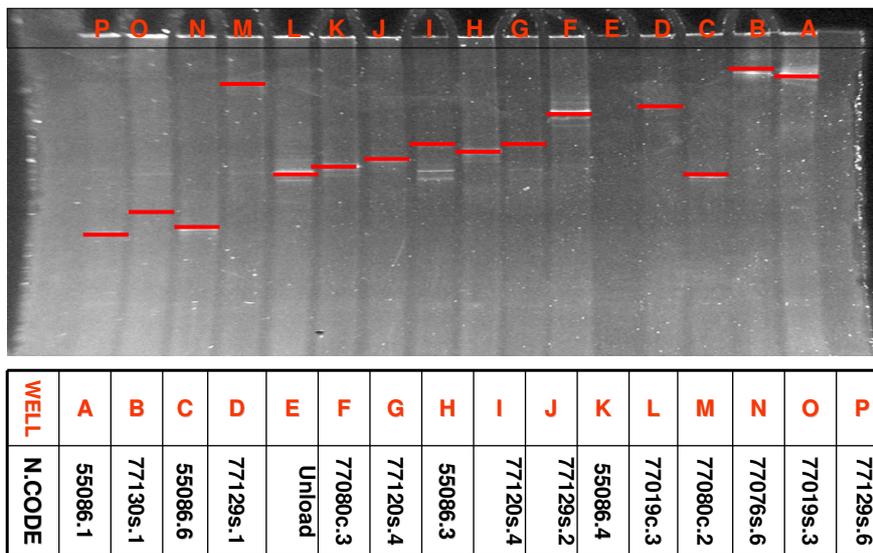


Fig. I DGGE profiles of PCR amplifications of the V3-region of the 16S rRNA gene of total DNA isolated from some samples. Denaturing gradient from 40 to 65% . Result in red are all the samples that were excised from the original gels with the denaturant concentration from 30 to 65%.

Final results in Blue

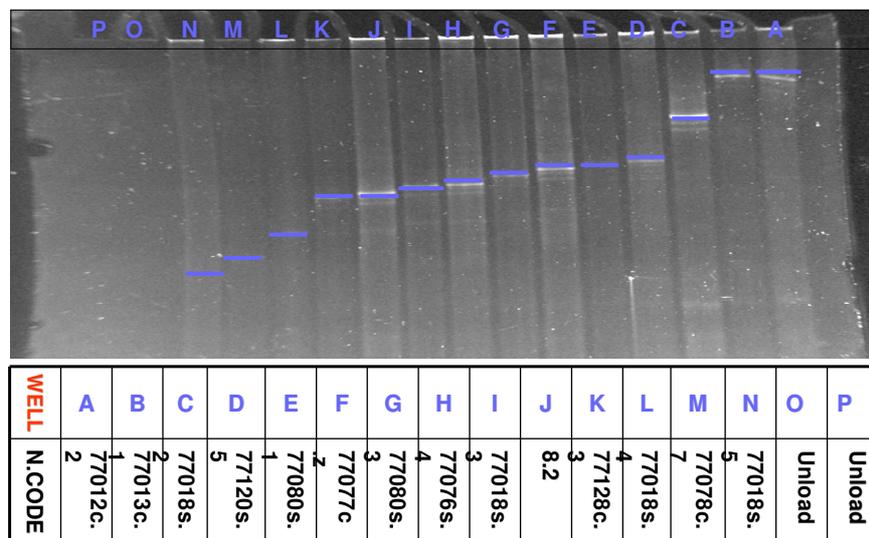


Fig. II. DGGE profiles of PCR amplifications of the V3-region of the 16S rRNA gene of total DNA isolated from some samples. Denaturing gradient from 40 to 65% . Result in blue are all the samples that were excised from the original gels with the denaturing concentration from 40 to 65%.

4. RESULTS AND DISCUSSION

4.1. DGGE profiles of raw milk

The DGGE profiles of raw milk from dairy farm A and B showed three bands and demonstrated a similar microbiological load in both milks (Fig. 1). In these two raw milks, the most intense bands corresponded to bacteria I and J. Band D was occasionally detected, probably due to contamination problems. Furthermore, the intensity of the microbiological bands was low, which indicated that the bacterial population in these samples was also low. Both bands could be part of the bacterial microflora present in the raw milk. It is interesting that the raw milk keeps these bacteria as self-primer for getting the final qualities of flavour and taste of cheese. Moreover, the results showed that these bacteria survived to refrigeration temperatures during the transport or storage.

On the other hand, incubated raw milk displayed more complex profiles, with as many as 8 bands (Fig. 1. red bands). An increase of temperature made that the bacteria had more rates of survive and development. Probably, many of these bands corresponded to spoiling bacteria and bacteria of raw milk (perhaps self-primer) which grew up with warmer temperature conditions (Spreer, 1999). Therefore, these bacteria were Mesophilic, i.e. they were able to grow up better in warm conditions and they couldn't at low temperature (Varnam *et al.*, 1995).

Direct comparison of DGGE bands (profiles) from raw and incubated milk samples was not possible because denaturing gradient of the gels were different.

4.2. DGDE profiles of fresh cheese

Fig. 2 shows the DGGE profiles of the fresh cheese manufactured with raw milk. Similar results were obtained for the different batches, because in the first ripening stage the bacterial grow was low. Three new bands appeared at the top of the patterns (A, B, C in blue). It means that their DNA fragment was opened at low denaturing dilutions, probably because these DNA fragments had a low number of GC links. These upper bands could correspond to starters (B, D in red codification) because they were detected in the raw and incubated milk samples (Fig. 1).

FINAL RESULT MILK

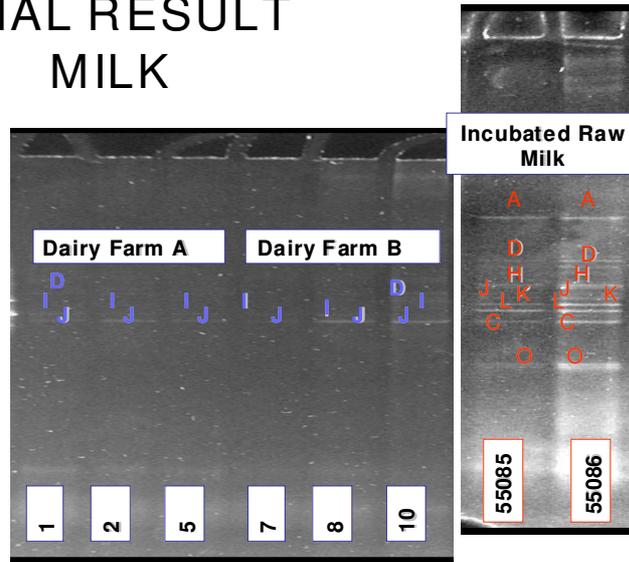


Fig. 1. DGGE profiles of PCR amplifications of the V3-region of the 16S rRNA gene of total DNA isolated from raw milk. Lanes 1, 2 and 5: milk from dairy farm A. Lanes 7, 8 and 10: milk from dairy farm B. Samples 55085 and 55086: incubated raw milk. Denaturing gradient from 40 to 65% for raw milk (blue bands) and from 30 to 65% for incubated raw milk (red bands).

A differential group of bands (F, G, I, J, and J, K, L, C) was also detected at the middle of DGGE profiles with different denaturing concentration. The most intense bands were F, I, and J, L, C which are the most representative bands, appearing on all the wells. This behaviour can represent that these bands correspond to the same microorganisms, but it would be necessary a DNA codification for confirmation. The K band, in this case appears between J, L, and with a low denaturing gradient, with a higher gradient it's not visible any band. In conclusion, on the gels with higher denaturing concentration the bands appear more separated between one another, and some bands could disappear for a coomigration effect .

The samples 77015c and 77015s were analysed with a different denaturing gradient (from 30 to 65%, red bands). In this case, the bands were closer and a new band (O) appeared at the bottom of the profile. This band wasn't detected with denaturing gradient from 40 to 65%. On the other hand, in the 77011s and 77012c samples were also observed the bands D and E. Once again, it would be necessary a DNA confirmation to know their origin. Additionally, no differences were detected between samples from core or surface.

Fresh cheese

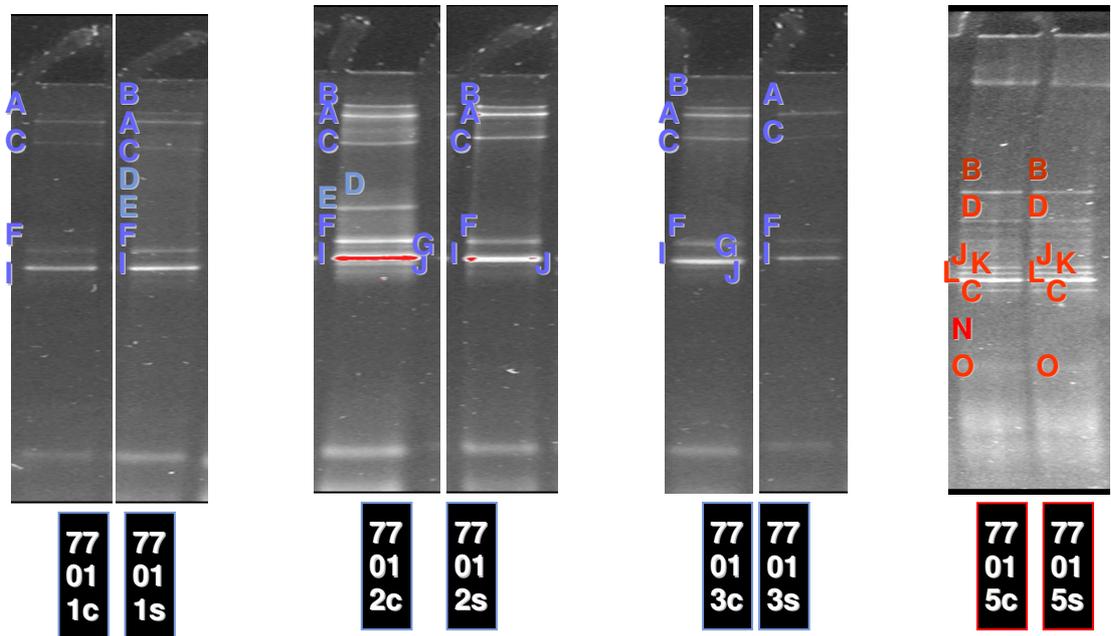


Fig. 2. DGGE profiles of PCR amplicons of the V3-region of the 16S rRNA gene of total DNA isolated from fresh cheese Denaturing gradient from 40 to 65% except for sample 77015 (30 to 65%).

4.3. DGGE profiles of cheese at one week of ripening

Fig. 3 shows some heterogeneity between samples of cheese elaborated with raw milk at one week of ripening. A comparison with the profile obtained for the fresh cheese (Fig. 2–blue codification) showed that the upper bands **A**, **B**, described above, also appeared in 77017c, 77018s. Moreover, a new band **E** was also detected. On the other hand, the band **D** was observed in all the red samples as the upper one. The central triplet **F**, **I**, **J** and **J**, **L**, **C** were observed in cheese at one week of ripening and new bands were detected (**L**, **N**, **P**) in the bottom of the patterns .

A difference was detected between samples 77017 taken from the surface or the core of the cheese, because the bottom bands were not detected in the surface sample.

One week old

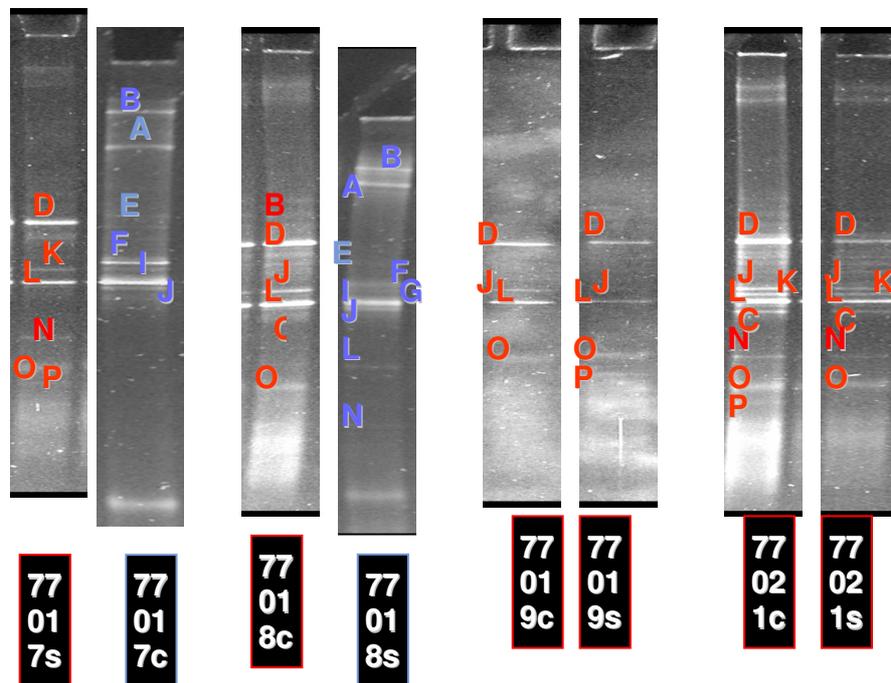


Fig. 3. DGGE profiles of PCR amplicons of the V3-region of the 16S rRNA gene of total DNA isolated from cheese at one week of ripening. Denaturing gradient from 30 to 65% except for samples (77018c, 77018s) with a concentration (from 40 to 65%)

4.4. DGGE profiles of cheese at four week of ripening

The bacterial population profiles of cheese manufactured by raw milk at four weeks of ripening examined by DGGE are shown in Fig. 4. The results obtained were more homogenous than previous ones. It suggested the bacterial community begun to stabilize itself for different ways as the competitive inhibition of the bacterial growth or the acidification of the medium by LAB bacteria that reduces the survival rates of some bacteria. Furthermore, it could be possible that some biochemical drugs like nisine or sakacine can inhibit the bacterial growth (Arques et al., 2000).

This reduction seems clearer with the bands **G** and **J** (Fig. 4), which were more frequent in the first ripening stages (Fig 3). The assumption of competitive behaviour we could explain why the number of bands (**G**, **J**) between the triplet (**F**, **I**, and a new band **K**) was reduced. This triplet is a reference itself so it is present in all the samples during

the different times of ripening process. The bands at the top (A, B, C) and at the bottom (M, N) were reduced after four weeks of ripening. They were clearly present only in three samples and in the rest were blurred. Therefore, it suggested that the number of bacteria in these samples were reduced in comparison with the samples described previously.

4.5. DGGE profiles of cheese at six week of ripening

The Fig. 5 shows that the all upper and lower down bands were blurred, and even in the sample 77128 these bands had disappeared. Therefore, the bacterial population was reduced as a consequence of the feed resources or chemical competition. In samples 77129 there were the bands D, O and P. Finally, the triplet (F, I, K) and (J, L, C) appeared in the latest ripening stages.

Four weeks old

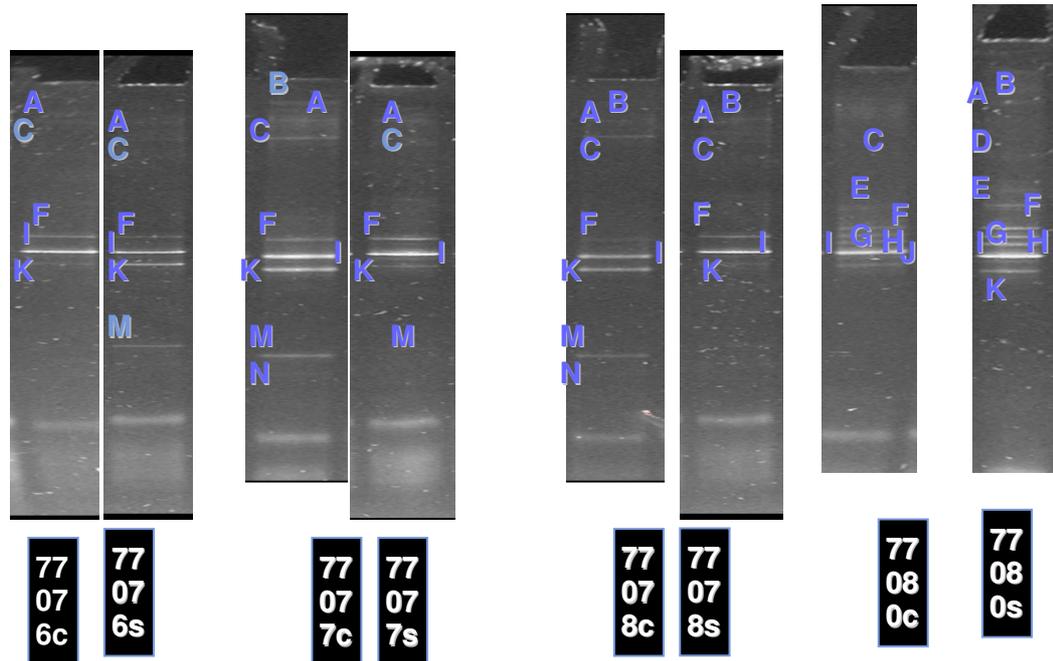


Fig. 4. DGGE profiles of PCR amplicons of the V3-region of the 16S rRNA gene of total DNA isolated from cheese at one week of ripening. Denaturing gradient from 40 to 65%.

Six weeks old

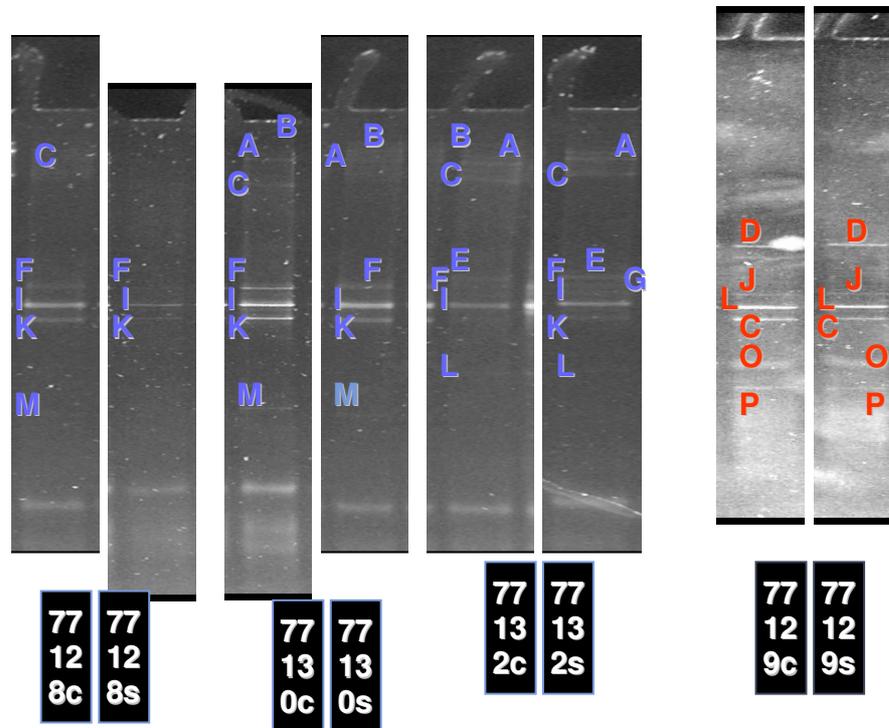


Fig. 5. DGGE profiles of PCR amplicons of the V3-region of the 16S rRNA gene of total DNA isolated from cheese at one week of ripening. Denaturing gradient from 40 to 65% except for sample 77129c, 77129s (30 to 65%).

5. CONCLUSIONS

Despite of the necessary DNA fragment confirmation to identify the bacterial communities detected in our samples, it's possible to conclude that there were three main groups of bacteria depends on its position in the gels: upper, medium and lower down.

Upper part: Bands **A, B, C, A, B, D, H**. These bands appeared at the top of the electrophoresis way. Their DNA fragments were opened with low denaturing dilutions because the DNA fragment had a low number of the strongest chains (GC).

Medium part: Bands **J, K, L, C, E, F, G, H, I, J, K**. All these bands were in the middle of the electrophoresis way between of the main bands groups. Their DNA fragments were opened with a more concentrated denaturing dilution. The triplet (**F, I, K**) and (**J, L, C**) appeared at long ripening times. It's possible that these three bacteria had an important

function in the cheese making. Between this triplet there was another bands E, G, H, J, K that could be result of coomigration effect.

Lower part: L, M, N and O, P, N. All these bands were at the bottom of the electrophoresis way. Their DNA fragments were opened at the most concentrated denaturing dilutions and therefore these DNA fragments had a high number of the strongest chains (GC).

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