CHANGES IN BACTERIAL POPULATION AND ANTIMICROBIAL RESISTANCE DURING COLD STORAGE OF CONVENTIONAL AND ORGANIC RAW MILK

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Tiivistelmä — Referat — Abstract

Psychrotrophs, which are mostly gram negative bacteria, can produce heat stable proteases and lipases. Even though these bacteria cannot survive pasteurization of milk, the enzymes can. This can cause problems for the dairy industry. Psychrotrophic milk isolates also have multidrug-resistant traits for antimicrobials and may act as reservoir for resistance genes.

The aims of the study were to test denaturing gradient gel electrophoresis (DGGE) for PCR products obtained from organic and conventional raw milk bacteria, to optimize DNA-extraction protocols and PCR-conditions for the raw milk samples and study the bacterial population changes during the cold storage. The aim was also to study the antimicrobial susceptibility of the bacterial isolates.

The DNA was extracted from the raw milk samples, when received and after 4 days cold storage, using commercial kits. Nested-PCR was performed and samples were analysed using DGGE. Susceptibility to antimicrobials was determined by growing bacteria on plates that contained two different concentrations of five antibiotics. From the five antibiotics two were used as a combination.

The composition of the bacterial population changed during the cold (4ºC) storage: the difference in DGGE profiles was clear between 0 and 4 days cold storage. Fingerprint profile analysis showed that irrespective of the origin of the raw milk, the sample profiles were clustered according to the sampling date (day 0 or day 4). There was no clear difference between DGGE-profiles from conventional and organic milk. Proportion of psychrotrophic bacteria increased and antimicrobial resistance seemed to be more prevailing in conventional than in organic raw milk. Antimicrobial resistance decreased after four days storage at 4ºC, in most of the cases.

We showed that the PCR-DGGE-method is an efficient tool to analyse the changes in bacterial populations in raw milk and that cold storage has an evident effect in population composition.

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1 Introduction

The microbiology of raw milk has been extensively studied. The current practises of storage of raw milk and processed milk products have also intensified the study of the bacterial community that is able to thrive in cold conditions, i.e. psychrotrophs.

Psychrotrophic bacteria, mainly *Pseudomonas* spp., produce enzymes that can survive pasteurization and even ultra-high temperature (UHT) processing and cause e.g. flavour defects and texture problems in milk products. Therefore these bacteria and the proteases and lipases they produce can cause major economical losses to dairy industry. The population structure of psychrotrophic community is mainly characterized, but a recent study by Hantsis-Zacharov & Halpern (2007) suggests that there are still many unidentified species or even genera in the population. Need for molecular tools, in combination with traditional identification methods, is therefore needed to get the full picture of raw milk bacterial community in its initial state and after cold storage.

The antimicrobial treatment of dairy cattle has also raised a question about the resistance and susceptibility of raw milk bacteria to the antibiotic agents. Susceptibility of clinical isolates to antibiotics has lowered since the introduction of antimicrobials and many strains have developed multidrug-resistant features. This has been also noticed in raw milk isolates by Munsch-Alatossava and Alatossava (2007). The differences in the medical treatment of organic and conventional dairy cattle might also lead to a difference in the resistance patterns of raw milk bacteria from these sources.

2 Literature review

2.1 Microbiology of raw milk

Milk is an excellent growth medium for many microbes because it has neutral pH, the water content is high and it has a complex biochemical composition (Frank & Hassan 2003). Although milk in alveolar tissue of a healthy cow's udder does not carry any bacteria (Mantere-Alhonen 1995), milk is exposed to many sources of contamination during processing practices. The basic sources of microbiological contamination of milk come from the teat canal epithelium within the udder, from the surface of the teats and from the handling and storage of the milk (Chambers 2002).

Most of the microbes in milk are bacteria, even though some yeasts and moulds are also able to grow in milk (Walstra et al. 2006). When total counts are still low, raw milk is dominated by micrococci and streptococci (Chambers 2002). These groups are part of normal udder and teat skin flora. As the number of bacteria starts to rise, dynamics in the population also shift. Gram negative rods (GNR), e.g. *Pseudomonas*, *Acinetobacter*, *Flavobacterium* and *Enterobacter*, begin to dominate at the expense of micrococci (Chambers 2002). Many of the pathogenic bacteria, for example *Mycobacterium tuberculosis* and *Brucella* spp., grow very slowly in milk. Growth is limited by cold storage and competing non-pathogenic bacteria. The risk posed by pathogens depends on the initial amount of contamination (Frank & Hassan 2003).

2.2 Effect of cold storage on the bacterial population

The storage temperature and the time on the farm affect the initial bacterial microflora in milk. If milk is stored under 4ºC, the duplication of bacteria takes more than 24 hours. On the other hand, cold storage can mask the contamination caused by unhygienic conditions on a farm (Chambers 2002). In Finland milk is collected from farms in alternate days, thus the storage time at the farm can be up to two days. The quality of milk stored in cold does not suffer during this time but the growth potential of bacteria has increased significantly. When milk arrives to a dairy, part of the bacterial flora is in the brink of exponential growth phase and the storage time in a dairy becomes substantially shorter.

Griffiths et al. (1987) investigated the effect of low temperature storage on raw milk and found out that the initial count and storage temperature affected the time until the bacterial count in the raw milk samples reached 10^7 cfu/ml. Farm bulk tank raw milk

had a lower initial count and it also showed a longer lag-phase when compared to creamery silo raw milk. This resulted in a longer storage life for farm bulk tank raw milk, 5,1 days and 2,9 days at 2ºC and 6ºC, respectively (an average of 7 samples). The corresponding numbers for the creamery silo raw milk were 2,3 days and 1,3 days.

In the same study Griffiths et al. (1987) also investigated the bacterial isolates from the raw milk samples after the count exceeded 10^7 cfu/ml. In farm bulk milk the genus *Pseudomonas* was predominant. As the storage temperature was lowered the proportion of this genus from the total bacterial load increased from 82,6% at 6ºC to 96,4% at 2ºC. Lafarge et al. (2004) stored raw milk samples for 24 hours at 4ºC and noticed a marked difference in the population composition during the storage period. Some species that were not detectable in the beginning appeared and some species were not anymore present in the population after the cold storage. They also demonstrated that the psychrotrophic bacterial community increases significantly in 24 hours of cold storage.

The present-day practices for collection and cold storage have improved the quality of milk and milk products, but they have also led to a selection of psychrotrophic bacteria (McPhee & Griffiths 2003).

2.2.1 Spoilage bacteria and psychrotrophs in raw milk

The most important spoilage bacteria of raw milk are GNRs (e.g. *Pseudomonas* and coliforms), gram positive sporeforming bacteria (*Bacillus* and *Clostridium*), lactic acid bacteria and coryneform group (Frank & Hassan 2003).

Psychrotrophic bacteria can grow below 7ºC although their temperature optimum may lie between 20ºC and 30ºC (McPhee & Griffiths 2003). Most of psychrotrophs belong to GNRs and *Pseudomonas* spp. account for more than 50% of these (Chambers 2002). The most common species isolated from milk is *Pseudomonas fluorescens*, who dominates the bacterial flora at the time of spoilage (McPhee & Griffiths 2003). Other common GNRs in milk are *Pseudomonas putida*, *P. fragi*, and *P. aeruginosa* as well as *Flavobacterium, Acinetobacter, Moraxella, Achromobacter, Alcaligenes, Chromobacterium, Aeromonas, Klebsiella* and coliform group. There are also few species of gram positive psychrotrophs in milk, including *Bacillus cereus, Arthrobacter* and some streptococci (Chambers 2002).

Hantsis-Zacharov & Halpern (2007) studied the diversity of culturable psychrotrophs in raw milk from four Israeli farms with molecular tools. They concluded that dominance of different bacterial classes varied according to the season that the

sample was collected and the geographical location of the farm. *Gammaproteobacteria* (the dominant genera *Pseudomonas* and *Acinetobacter*) dominated during spring and winter, *Bacilli* (*Leuconostoc* and *Lactococcus*) in the summer and *Actinobacter* (*Microbacterium*) in autumn. The results of the same study showed that 20% of the psychrotrophic isolates were novel species, i.e. the similarity to the closest known relative in GenBank was < 97,5%. Six isolates might have also represented new genera since the similarity was < 93,8%. These results suggest that there is still a lot to learn about raw milk psychrotrophic community.

2.3 Pseudomonads

Pseudomonads are gram negative rods and they move with polar flagella. They are aerobic, catalase-positive and most of them are also oxidase-positive. Their metabolism is never fermentative (McPhee & Griffiths 2003). Even though pseudomonads are regarded as aerobic organisms, some of them can also grow in the absence of oxygen (Tortora et al. 2004).

Pseudomonads have a high genetic capacity, almost that of eukaryotic yeasts. Therefore they can produce large amounts of different enzymes and utilise many different substrates as energy source. Bacteria from this group are also resistant to many antibiotics. Their resistance is probably due to cell wall porines that regulate the movement of molecules through the cell wall. In addition, the genome of pseudomonads codes for several efficient efflux pumps (Tortora et al. 2004).

In fresh raw milk, pseudomonads comprise less than 10% of the microflora, even though they are one of the most important spoilage psychrotrophs in milk. Pseudomonads dominate the microflora that adheres to stainless-steel transfer pipelines and the contamination is mainly a result of inadequately sanitized milking and storage equipment (McPhee & Griffiths 2003). Pseudomonads cannot survive pasteurization, but they can cause post-pasteurization contamination. Pasteurized milk products with short storage time (< 5 days in 4 - 6ºC) carry almost solely *Pseudomonas* species (90,7%). Products with longer storage time (> 10 days) have also other types of micro-organisms and pseudomonads comprise 68% of the population (McPhee & Griffiths 2003).

2.3.1 Enzymes produced by pseudomonads

Different *Pseudomonas* strains produce different proteinases and one strain can produce several types of enzymes. Most of the proteinases cause coagulation of milk and they degrade κ-, α_{s1}- and β-casein. Proteinases can be divided in two classes according to their pH optimum: neutral proteinases with optima at ~pH 7 and alkaline proteinases with optima at pH 8 - 9. Temperature optimum varies between 30ºC and 50ºC. Activity of the proteinases decreases strongly when the temperature drops below optimum, but all the proteinases produced by pseudomonads studied so far maintain some activity also at 4ºC (McPhee & Griffiths 2003).

Skean & Overcast (1960) demonstrated first that the inoculation of pasteurized milk with *Pseudomonas* spp. alters the total content of casein and whey proteins and the relative amounts of protein fractions during an extended refrigerated storage. *P. putrefaciens* was the most effective in the degradation of casein of the three species that were studied. The electrophoretic profile of casein was similar after 14 days and after 42 days of cold storage. In the study by Adams et al. (1976) *Pseudomonas* spp. isolate MC60 attacked readily κ-casein and the protein could not be detected in the raw milk sample after 6 days of refrigerated storage. Also other *Pseudomonas* spp. isolates were effective on the degradation of caseins. In this study the proteolysis caused by *Pseudomonas* spp. was not so pronounced as in the study by Skean & Overcast (1960). Adams et al. (1976) also showed that a relatively low amount of isolate MC60 (10^4 cfu/ml) can lead to a statistically significant decrease in the κ-casein content.

The influence of lipases in milk is not as pronounced as that of proteinases (McPhee & Griffiths 2003). The defects caused by lipases are not apparent until the total population exceeds 10^7 cfu/ml (Shelley et al. 1987). On the other hand, Hantsis-Zacharov & Halpern (2007) showed that most of the bacterial isolates in their study from raw milk exhibit lipolytic activity alone or both lipolytic and proteolytic activities. Only few isolates showed only proteolytic activity. Lipases produced by *Pseudomonas* spp. can hydrolyze large variety of natural oils and fats. One of the most important lipases found in milk is lecithinase (a phospholipase) that hydrolyzes the thin membrane of the milk fat globule. The membrane that is mainly formed by lecithin keeps up the globular structure of the milk lipid. The hydrolysis of the membrane exposes lipids to native and microbial lipases in milk. The lipases produced by *Pseudomonas fluorescens* form aggregates with milk lipids or lipase-polysaccharide complexes. These lipases

have a pH optimum of 7 - 8 but they can function in a pH range of 5 - 11 (McPhee & Griffiths 2003). The lipases have been shown to function at temperatures below 0ºC even though the temperature optimum is between 22ºC and 55ºC (Andersson 1980).

Shelley et al. (1987) isolated lipolytic psychrotrophs for taxonomic characterization from 36 raw milk samples and found out that all of the samples were dominated by pseudomonads. The most commonly found isolates were *Pseudomonas fluorescens* and *P. fragi*, 63,9% and 31,2% of the total lipolytic isolates, respectively. In the study by Hantsis-Zacharov & Halpern (2007) *Pseudomonas* and *Acinetobacter* isolates were the dominant genera in raw milk and showed high lipolytic activity.

All pseudomonads found in milk cannot produce proteinases and lipases. In those strains that can produce these enzymes, the production levels are affected by many factors including growth phase, availability of nutrients and different environmental factors like oxygen tension and temperature (McPhee & Griffiths 2003).

2.3.2 Heat-stability of proteases and lipases

Both proteinases and lipases can show high heat-stability, especially those produced by *P. fluorescens*. This feature causes problems for dairy industry since the presence of proteinases and lipases even after ultra high temperature (UHT) processing can lead to quality defects in milk products (McPhee & Griffiths 2003). *P. fluorescens* proteases show low temperature inactivation and the structure is more stable at higher temperatures. Patel & Bartlett (1988) studied purified proteases and showed that the unfolding was increased from 25ºC upwards and the temperature inactivation of the protein was highest at 45ºC. If the temperature was raised even further (60 - 95ºC), the molecule was stabilized by metal ions such as calcium. Andersson et al. (1981) added a lipase obtained from *P. fluorescens* to pasteurized cow milk. The lipase survived the UHT-treatment and caused an off flavour in sterile, cold stored milk. Earlier Andersson et al. (1979) had shown that lipases produced by *P. fluorescens* can withstand extremely high temperature for a long time. To reach 90% inactivation of the enzyme in skim milk, a heat treatment of 2,0 minutes at 140ºC was needed.

Bucky et al. (1986) suggested that the storage of the milk in the higher temperatures than is normally used in commerce might reduce the production of lipases. They tested the lipase production of *P. fluorescens* in UHT-treated whole milk and sterile skimmed milk at 25ºC, 10ºC and 4ºC. The lipase activity was lowest at 25ºC incubation of both whole and skimmed milk.

2.4 Production and quality of conventional and organic raw milk in Finland

There were 12 143 farms in Finland producing conventional milk in 2008 and the number dropped by 7,6% from 2007. The number of organic milk producers increased by 2,9% from 2007 being 124 farms in 2008. About 2 200 million litres of milk were delivered to dairies, from which 28 million litres were organically produced (Information Centre of the Ministry of Agriculture and Forestry 2009).

According to the statute issued by the Finnish Ministry of Agriculture and Forestry (2001) about hygienic requirements of milk and milk-based products (31/EEO/2001) raw milk has to be sampled on farms at least twice a month for bacteriological studies. The geometric average in two months must not exceed 100 000 colony forming units per millilitre of milk (cfu/ml). It is also stated that if the milk from a farm does not meet the standards in three consecutive investigations, its milk cannot be used for food processing before the standards have been met. The number of somatic cells is monitored the same way as the number of bacteria and the geometric average must not exceed 400 000 cells/ml. Somatic cell counts give information of the health status of a cow, since the counts increase as a response to the presence of pathogenic bacteria.

The Finnish Association for Milk Hygiene produces statistical information about the bacteriological quality of raw milk. In 2008 the geometric mean (national mean calculated from means of all single farms) of bacterial counts in raw milk was 5 200 cfu/ml. Only 0,1% of farms exceeded the limit of 100 000 cfu/ml. The geometric mean for somatic cells was 129 000 cells/ml. Organic milk is monitored separately from conventionally produced milk. In 2008 for organic farms, the geographic mean of bacterial counts was 5 000 cfu/ml and of somatic cells 144 000 cells/ml. The quality of Finnish raw milk is excellent when compared internationally and even within the Nordic countries (Finnish Association for Milk Hygiene 2009).

2.5 Bovine mastitis in Finland

Mastitis is a common inflammatory disease in dairy cattle. The herd prevalence of mastitis (prevalence of mastitis in herds as mean of all herds) in Finland has been decreasing since 1988 and was 30,6% in 2001 (Pitkälä et al. 2004). The most common causes of mastitis are coagulase negative staphylococci (CNS), *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis*,

Enterococcus spp., *Lactococcus* spp., *Aerococcus viridans*, *Escherichia coli*, *Klebsiella* spp., *Pseudomonas aeruginosa*, *Arcanobacterium pyogenes* and *Corynebacterium bovis*. More infrequent causes of mastitis are for example *Pasteurella multocida*, *Mannheimia haemolytica*, *Corynebacterium ulcerans* and yeasts. Very rare causes of mastitis are for example *Serratia marcescens*, *Listeria monocytogenes*, *Bacillus cereus* and *Prototheca* spp. (Finnish Food Safety Authority 2006).

Earlier the main cause of mastitis in dairy cattle was *Streptococcus agalactigae* and after that *Staphylococcus aureus*. Today their proportion has decreased, probably due to changes in dairy industry practices (Pitkälä et al. 2004). *C. bovis* and CNS, that earlier were uncommon causative pathogens, are becoming more common, or are already the most common cause of mastitis in many countries (Myllys et al. 1998, Pitkälä et al. 2004, Haltia et al. 2006 and Tenhagen et al. 2006).

2.5.1 Treatment of mastitis

The use of antimicrobials in Finland for mastitis treatment should be based on the recommendations of the Ministry of Agriculture and Forestry (2003). The first-line treatment depends on the causative pathogen. The use of antimicrobials should be well-grounded, because every treatment selects for resistant strains and in many cases susceptibility test are recommended to determine effective treatment. For β-lactamase negative CNS and for *S. aureus*, G-penicillin (class of β-lactams) is used as a first-line treatment. For β-lactamase positive strains, cloxacillin is used. In mastitis caused by *E. coli* no antimicrobial is recommended, though in life-treathening cases trimethoprim-sulfonamides (classes diaminopyrimidine and sulfonamide) or enrofloxacin (class of fluoroquinolone) is used. Aminoglycoside class is used sometimes in combination with β-lactams to treat mastitis cases caused by gram positive bacteria (Lehtolainen et al. 2003).

Organic dairy cattle are treated for mastitis with the same antimicrobials as the conventional dairy cows, but there are a few differences in the practices. European Union Council Regulation 834/2007 (2007) states that chemically synthesized pharmaceuticals, including antimicrobials, can be used, when necessary, with strict conditions. Precautionary-time after medicinal treatment is twice as long as normal precautionary-time indicated in a selling permit or directed by a veterinarian and the number of treatments is limited to three. If four or more treatments are done on a single

animal within a year, it can no longer be regarded as organic (Finnish Food Safety Authority 2009).

The actual usage of different antimicrobials for cattle in Finland was surveyed recently by Thomson et al. (2008). During one week period, 1308 cows were treated with 30 kg of antimicrobial substances. From this over 85% were β-lactams. In most cases the treatment was for acute mastitis. The most commonly used antimicrobial for acute mastitis was benzyl penicillin (83%). Also fluoroquinolones were used (11%) and some cows received trimethoprim-sulfonamides. In dry cow therapy, i.e. treatment of mastitis during non-lactating period, cloxacillin alone or in combination with ampicillin (50%) was used or a β-lactam combined with an aminoglycoside (43%).

2.6 Passage of antimicrobial drugs to milk

The bovine udder gets a rich supply of blood through many arteries. The ratio of blood flowing through mammary gland and volume of milk produced is estimated to be 670:1. This provides abundant opportunities for unbound antimicrobials to passively diffuse into milk (Baggot 2006).

Milk that contains antimicrobial residues is considered as a health hazard and it also affects the processing of milk on dairy plant. Adverse effects include e.g. decrease in acid and flavour production in butter manufacture, improper ripening of cheeses and delay in starter activity for cheese, yoghurt and butter (Dowling 2006). National Milk Drug Residue Data Base (2008) reported that in the USA in fiscal year 2007 over 4 million milk samples were tested for antimicrobial residues and only 0,04% were positive.

Because of tightly controlled drug use, the residues of antimicrobial drugs are rarely encountered also in Finland. The residues of antimicrobial drugs are investigated by Finnish Food Safety Authority (Evira) and by dairies according to their in-house control plan. From 708 samples investigated by Evira during 2007 not a single sample contained traces of antimicrobial drugs. At dairies, almost 21 000 samples were tested in 2008 and only 29 were positive for antimicrobial drugs (Finnish Association for Milk Hygiene 2009).

2.7 Antibiotic resistance

Boerlin & White (2006) define antimicrobial resistance as the ability by a micro-organism to withstand the action of normally effective concentration of an antimicrobial agent.

Bacteria can be inherently resistant and display the same resistance characteristics across the taxonomic group they belong to, or the resistance can be acquired. Acquired resistance can result from acquisition of foreign gene, or by mutation, or by combination of these. The mutation rate increases in stressful environments e.g. during antimicrobial treatments. Pathogens are more frequently resistant to antimicrobials than normal flora, probably because pathogens face higher selection pressure from repeated treatments (Boerlin & White 2006).

The majority of the resistance in clinical isolates comes from extra-chromosomal resistance genes (Boerlin & White 2006) that are acquired by bacteria in three different ways: transformation, transduction and conjugation. If transformation happens bacteria take up naked DNA from the environment, transduction involves gene transfer by bacteriophage and in the case of conjugation the genetic material is transferred from one bacterium to another by direct cell-to-cell contact. Ochiai et al. (1959, ref. Watanabe 1963) and Akiba et al. (1960) were the first ones to show that antibiotic resistance features of multiresistant bacteria can be transferred by conjugation to susceptible bacteria. Both demonstrated independently that resistance features were transferred from multidrug-resistant *Escherichia coli* to susceptible *Shigella*.

2.7.1 Antibiotic resistance features of psychrotrophs

Munsch-Alatossava & Alatossava (2007) showed that some psychrotrophs isolated from raw milk samples were multidrug-resistant. They had isolated spoilage bacteria from raw milk and tested the resistances of the isolates to different antibiotics with commercial susceptibility test strips. From *Pseudomonas fluorescens* isolates 4 out of 8 were considered multidrug-resistant (i.e. resistant to more than two antibiotic classes), from the group of isolates recognised as *P. fluorescens* or *P. tolaasii* 7 out of 8, from *Stenotrophomonas maltophilia* 2 out of 5 and all 5 *Burkholderia* spp. isolates. Altogether 50% of the studied raw milk isolates were resistant to at least 5 antibiotics.

Kelch and Lee (1978) tested the resistance with 11 antibiotics and found out that out of 658 *Pseudomonas* spp. isolates from environmental sources 20% or more were resistant to 7 antibiotics. The resistance was highest for nitrofurazone, penicillin and

ampicillin (86%, 85% and 79%, respectively). In *Acinetobacter* spp. isolates, the resistance was also highest for nitrofurazone, penicillin and ampicillin and 20% or more of the isolates were resistant to 3 antibiotics. Both of these bacteria genera are found in raw milk. Antibiotic resistance in *Pseudomonas* spp. isolated from farm soil after the spread of animal manure was studied by Jensen et al. (2001). *Pseudomonas* spp. isolates showed resistance for carbadox, streptomycin and tetracycline (44,7%, 6,9% and 0,3% of isolates, respectively). *Acinetobacter* spp. isolates from raw milk (Munsch-Alatossava & Alatossava 2007) did not show multiresistant features, but the review by Van Looveren et al. (2004) of over 200 articles, states that *Acinetobacter* spp. has become resistant to almost all antibiotics that are currently available. *Acinetobacter* spp. has shown significant susceptibility only to imipenem that belongs to a class of β-lactam antibiotics.

For *Stenotrophomonas maltophilia*, multidrug-resistance has been reported e.g. by Alonso & Martínez (1997), Berg et al. (1999) and Gales et al. (2001). Berg et al. (1999) tested the antimicrobial resistance of *Stenotrophomonas maltophilia* isolates of environmental and clinical origin. They concluded that the isolates were multidrug-resistant irrespective of the origin and 20% or more of the isolates were resistant to 16 out of 19 tested antibiotics. Gales et al. (2001) tested 552 isolates from hospitalized patients and found out that 20% or more of the isolates were resistant to 6 out of 15 tested antibiotics. The resistance was highest for ciprofloxacin (27,1%) and gentamicin (26,2%). The antibiotic resistance of *Stenotrophomonas maltophilia* can be attributed, at least partially, to multidrug-resistance system (MDR). Alonso & Martínez (1997) selected with tetracycline those clinical isolates that express MDR phenotype and noticed that these isolates showed notably higher resistance to quinolones and chloramphenicol than tetracycline susceptible isolates. Activation of MDR phenotypic gram-negative bacteria could have implications also for the veterinary treatment of cattle. Even though the use of tetracyclines in Finland for the treatment of mastitis has nearly ceased in the end of 1990's (Finnish Food Safety Authority 2007), tetracyclines are used for treatments of other diseases (Ministry of Agriculture and Forestry 2003).

Burkholderia cepacia, which was formerly known as *Pseudomonas cepacia*, is intrinsically resistant to the aminoglycosides because the antibiotics are unable to disrupt the outer membrane of the bacteria (Moore & Hancock 1986). In 1979 Beckman & Lessie showed that *B. cepacia* can readily use penicillin G, which is a β-lactam antibiotic, as a source for carbon and energy and withstand also other derivates of this

antibiotic. In 1996 Burns et al. suggested that the multiple resistant features of *B. cepacia* result from an active efflux pump. In this study the bacteria proved to be resistant to chloramphenicol, trimethoprim and ciprofloxin antibiotics.

The multidrug-resistance of the raw milk isolates (Munsch-Alatossava & Alatossava 2007) and of those that can be found from raw milk but originate from other sources raises some questions: can psychrotrophic bacteria in raw milk act as a reservoir of antibiotic resistance in the dairy cold chain, and is gene transfer to human microflora possible? Boerlin & White (2006) stated that environmental bacteria may play an important role as reservoirs of antimicrobial resistance and resistance genes can move even between unrelated genera. They also speculated that gene transfer from ingested bacteria to gut microflora and further to pathogenic bacteria could be possible.

2.8 Denaturing gradient gel electrophoresis (DGGE)

2.8.1 Principle of DGGE

Length-independent separation of DNA fragments by denaturing gradient gel electrophoresis (DGGE) was first introduced by Stuart G. Fischer and Leonard S. Lerman in 1979. They were able to separate large EcoRI digested fragments of λ-DNA by partially separating the double-helix of the fragments in a gradient formed by urea and formamide. Fischer and Lerman continued their studies with digested fragments and in 1989 Sheffield et al. published a study on the use of PCR-DGGE method that has been also used in this study.

DGGE is a gel system that separates DNA fragments according to the differences in their sequences and resulting different melting properties. Different melting properties and melting temperatures result from different amounts of hydrogen bonds between bases. Guanine (G) and cytosine (C) have three hydrogen bonds but thymine (T) and adenosine (A) only two between them, therefore the melting temperature depends on the GC-content of the DNA fragment. When DNA fragments are electrophoresed through an increasing gradient of denaturants, the fragments remain double stranded until they reach the denaturant concentration that is equivalent to the lowest melting temperature (T_m) of the sequence (Sheffield et al. 1989). A partial melting produces branched molecules and the mobility of the molecule decreases sharply in the polyacrylamide gel. The lower-temperature melting domains of DNA fragments, differing by as little as a single base pair, will melt at a slightly different denaturant concentration. These

differences in melting cause two DNA fragments to begin slowing down at different levels in the gel, resulting in their separation from each other. The biggest problem with the original DGGE design was that only 50% of all possible single base pair mutations could be detected (Sheffield et al. 1989). When the most stable domain melts, the strand is completely dissociated and the resolving power of the denaturing gel is lost. If the DNA has its single base change in this most stable domain, the mutation cannot be detected (Myers et al. 1985).

Attachment of short GC-clamp to 5'-end of PCR fragment alters the melting properties of the DNA fragments so that otherwise undetectable single-base mutations can be detected by the system (Sheffield et al. 1989). GC-clamp will keep DNA strands together and they will not be completely separated until the highest melting domain of the actual sequence is also reached (Figure 1).

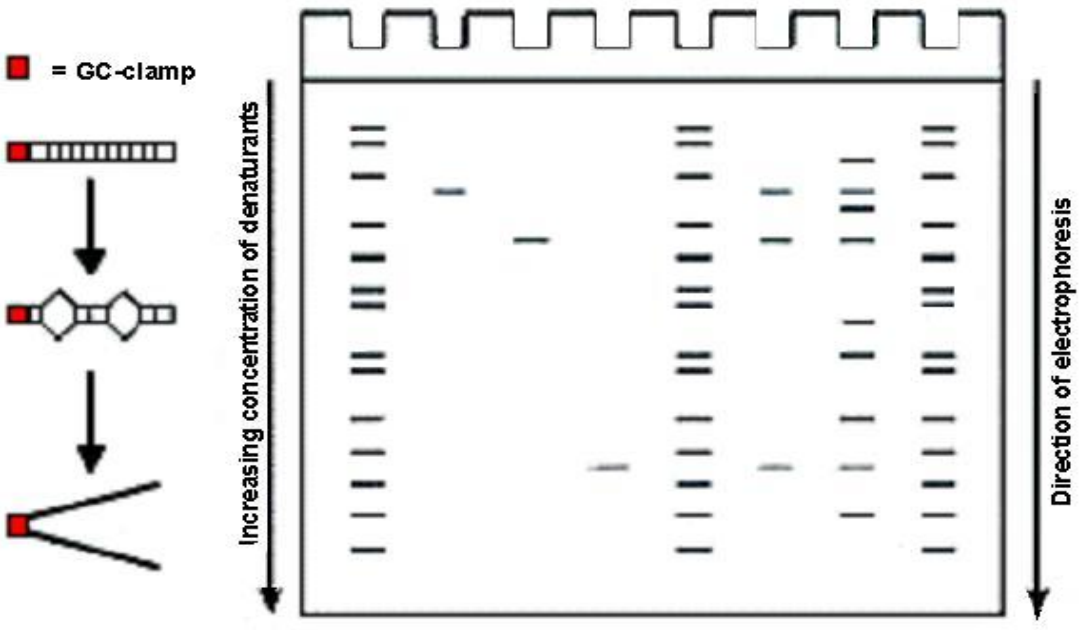

Figure 1. The principle of the denaturing gradient gel electrophoresis (DGGE). PCR amplicons are separated based on their GC-content. The increasing denaturing gradient opens double stranded DNAs starting from the most unstable melting (i.e. lowest T_m) domains. A GC-clamp prevents the complete dissociation of the amplicons. Different sequences will result in different origins of melting domains and consequently in different positions in the gel where the DNA fragments halt.
Picture adapted from Belgian Co-ordinated Collections of Micro-organisms (http://bccm.belspo.be/newsletter/17-05/bccm02.htm).

2.8.2 Limitations of DGGE

DNA-fragments longer than 500 bp cannot be resolved with DGGE. This is a limiting factor in sequence analysis and comparison with databases. Also comigration of amplicons is possible. Even if there is a difference in sequence, the melting behaviour might be identical (Ercolini 2004). Because of co-migration, underestimation of species number is possible and the retrieval of clean, independently migrating, sequences from one band is hindered.

Because DGGE is preceded by PCR, problems can also arise from that step. Preferential amplification caused by reannealing of template DNA can cause problems (Suzuki & Giovannoni 1996). Because of this, a mixture of bacterial DNA, from a complex community, may be only partially amplified by PCR, thereby leading to a product where some of the original members of the community are missing (Ercolini 2004). Formation of PCR artefacts (e.g. chimeras) can also cause problems as they suggest the existence of organisms that are not actually present in the sample (von Wintzingerode et al. 1997).

2.8.3 Community analysis with 16S rDNA

16S ribosomal RNA (rRNA) is a part of the larger 30S rRNA subunit. It is functionally constant, present in all bacteria and it has a mosaic structure of conserved and variable regions (Woese 1987). Its length (1542 bp) allows easy sequencing. 16S rRNA consists of eight highly conserved regions U1 - U8. Between these conserved regions are variable regions V1 - V9 which are thought to be functionally less important (Gray et al. 1984). All these features have promoted PCR-amplified sequences of the 16S rRNA gene (rDNA) to be widely used to infer phylogenetic relationships between micro-organisms. It is used together with PCR-DGGE to analyze complex microbial communities and directly identify the presence and relative abundance of different species. With this method it is possible to analyze microbial populations in both a qualitative and a semi-quantitative way (Muyzer et al. 1993).

There is a major drawback in 16S rDNA PCR-DGGE method. It is possible that multiple sequences of 16S rDNA exist in one bacterium (Case et al. 2007). Intragenomic copies can have sequence differences and this can lead to overestimation of ribotypes or bacterial species (Dahllöf et al. 2000) and confusion can arise about whether one 16S rDNA sequence represents one organism, or corresponds to several 16S rDNA operons from an organism (von Wintzingerode et al. 1997). Case et al. (2007)

found that 16S rDNA has regions where intragenomic sequence variation is concentrated. These are the regions that are commonly used in molecular microbiology. This information is relevant especially when relationship between closely related species is studied. These studies are better performed using single-copy protein encoding genes like the gene of the RNA polymerase beta subunit (*rpoB*) instead of 16S rDNA (e.g. Mollet et. al. 1997, Dahllöf et al. 2000).

2.8.4 16S rDNA DGGE studies done for dairy products

The use of PCR-DGGE method in population analysis is not so widely used in food as in environmental microbiological studies. A few studies have been published on PCR-DGGE with raw milk. Lafarge et al. (2004) used the V3 region of 16S rDNA to study population shifts in raw cow milk during cold storage. Both DGGE and temporal temperature gel electrophoresis (TTGE) were used and these methods were shown to be powerful tools for identifying the main bacterial species in raw milk and assessing population changes. Giannino et al. (2009) compared DGGE profiles of raw milk from different altitudes in an alpine area to collect information of inherent bacteria for authenticity assurance of a specific cheese type. PCR-DGGE has also been used to study various aspects of dairy industry including cheese ripening, distribution of bacteria in cheese and production and performance of probiotics (e.g. Ercolini et al. 2003, Minelli et al. 2004, Flórez & Mayo 2006).

3 Objectives of the study

The purpose of this master's thesis was to study the suitability of PCR-DGGE method for raw milk samples, determine whether the cold storage affects the structure of bacterial community and to investigate the level of antibiotic resistance carried by raw milk bacteria from conventional and organic raw milk. The hypotheses were that the composition of bacterial population changes notably during cold storage and organic raw milk bacteria are more susceptible to antimicrobials than bacteria from conventional raw milk. The change in bacterial population was studied using molecular fingerprinting method, and the antimicrobial resistance of milk bacteria was determined based on platings with different antimicrobial agents.

4 Materials and methods

4.1 Samples used in this study

Two different types of milk samples were used in this study: some that were produced conventionally and others that were produced according to the strict rules defined for organic milk. The earlier will from now on be referred as conventional raw milk and the latter as organic raw milk.

The conventional raw milk samples were collected from Valio Ltd. dairy in Riihimäki, Finland, on 19 January 2009. The organic samples were from Juvan Luomu Oy dairy in Juva, Finland. The dairy produces only organic milk products and the milk originates from organic farms. The production rate in organic dairy is considerably smaller than in conventional dairies and therefore gaining six independent samples took two days. Half of the organic raw milk samples were collected on 17th of February and half on 18th of February 2009. Both sets of raw milk samples contained six independent samples from tank trucks. The samples were collected aseptically from tanks to sterile bottles. All samples were registered with entries shown in Table 1.

The samples were a mix of differently aged milk, since raw milk is collected from farms on alternate days. In all the samples used in this study the age was assumed to be the maximum i.e. two days at the time of pick-up from the farm. The estimated age of all samples, when the analysis began, is given in Table 2.

Table 1. Samples' identities used in this study.

Conventional raw milk, sample numbers	Day 0	Day 4	Organic raw milk, sample numbers	Day 0	Day 4
1	C1D0	C1D4	1	O1D0	O1D4
2	C2D0	C2D4	2	O2D0	O2D4
3	C3D0	C3D4	3	O3D0	O3D4
4	C4D0	C4D4	4	O4D0	O4D4
5	C5D0	C5D4	5	O5D0	O5D4
6	C6D0	C6D4	6	O6D0	O6D4

Day 0: raw milk samples before the cold storage
Day 4: raw milk samples after the four days cold storage

Table 2. Estimated age of the raw milk samples in days when the analysis were performed.

Sample	age (d)	Sample	age (d)	Sample	age (d)	Sample	age (d)
C1D0	2	C1D4	6	O1D0	3	O1D4	7
C2D0	2	C2D4	6	O2D0	3	O2D4	7
C3D0	2	C3D4	6	O3D0	3	O3D4	7
C4D0	2	C4D4	6	O4D0	4	O4D4	8
C5D0	2	C5D4	6	O5D0	4	O5D4	8
C6D0	2	C6D4	6	O6D0	4	O6D4	8

4.2 Cold storage of the samples

The raw milk samples were processed at two different time points. The first handling was at day 0 (D0), i.e. at the time the samples arrived to the university. After this, 120 ml of the conventional raw milk samples and organic samples O1 - O3 were stored for four days in the cold room. For organic samples O4 - O6 only 40 ml of raw milk were stored in cold. The samples were placed in closed sterile glass bottles with a magnetic stirrer and the air flow was made possible through silicon tubing that had sterile filter discs at the end (Figure 2). The glass bottles were placed in a cold (4°C) water bath with on a stirring plate. The samples that had been stored in cold were labelled as the day 4 (D4) samples.

The microbiological study with testing of antimicrobial susceptibility of the bacteria and the monitoring of the changes in bacterial population with denaturing gradient gel electrophoresis (DGGE) were performed on both processing dates D0 and D4.

Figure 2. The cold storage system. Raw milk samples were stored for four days in cold (4°C) water bath with continuous stirring and air flow through sterile filters.
The original design is by Dr. O. Gursoy, Pamukkale University, Turkey and P. Munsch-Alatossava, Helsinki University, Finland.

4.3 Microbiological study and testing of antimicrobial resistance

The microbiological part of the study consisted in the determination of total counts, determination of psychrotrophic counts and evaluation of the bacterial growth in the presence of different antimicrobials.

4.3.1 Total counts

The total counts from each raw milk sample were determined on Plate Count -agar (PCA, 23,5 g/l; tryptone 5,0 g/l, yeast extract 2,5 g/l, glucose 1,0 g/l, agar no.1 15,0 g/l, lot: 091233/101, Lab M Ltd. Lancashire, UK) and Mueller-Hinton -agar (MH, 38 g/l; beef extract 2,0 g/l, hydrolysed casein 17,5 g/l, starch 1,5 g/l, agar no.1 17,0 g/l, calcium ions 50 - 100 mg/l, magnesium ions 20 - 35 mg/l, lot: 097230/130, Lab M Ltd. Lancashire, UK). On PCA plates, 20 µl of each dilution (10^{-1}, 10^{-2} and 10^{-3}) was laid

with the drop method. In the drop method, all dilutions are placed on the same plate as far from each other as possible and the drops are not spread. Three plates were prepared from each sample. MH plates were prepared by the spread method and 50 µl of either 10^{-1} or 10^{-2} dilution was laid on each plate. Three plates were prepared from each sample and dilution. The plates were incubated in normal atmosphere for 3 days at 30ºC and for 10 days at 7ºC to determine the total counts and the number of psychrotrophic bacteria respectively.

PCA was used since this media is recommended for the standard plate count (SPC) determination by the International Dairy Federation (IDF), and MH plates were used in antimicrobial resistance tests since this media is recommended for studying the effect of antibiotics (EUCAST 2000). The total counts were therefore determined on both plate types to control that there was no marked difference in PCA and MH counts.

4.4 Choice of antimicrobials

The choice of antimicrobials was influenced by previous studies done on this subject and antibiotics used in Finland to treat bovine mastitis.

The antimicrobial classes chosen for this study (Table 3) are all used in Finland to treat mastitis (Lehtolainen et al. 2003, Ministry of Agriculture and Forestry 2003). Gentamycin (G) and ceftazidime (C) are not used in Finland (Lehtolainen et al. 2003), but the classes they represent (aminoglycosides and β-lactams, respectively) are commonly used. The use of levofloxacin (L), a fluoroquinolone, is not allowed in Finland (Ministry of Agriculture and Forestry 2008) but for example enrofloxacin, a fluoroquinolone also, is used to treat mastitis (Ministry of Agriculture and Forestry 2003). Trimethoprim-sulfamethoxazole (TS) is not used in Finland, but other forms of trimethoprim-sulfonamides (eg. trimethoprim-sulfadiazine) are used in veterinary medicine (National Agency for Medicines 2009). Many spoilage bacterial isolates from organic and conventional raw milk are resistant to TS (unpublished data, Munsch-Alatossava, Xheng and Alatossava, discussion with Patricia Munsch-Alatossava 7.4.2009).

The minimum inhibitory concentrations (MIC) were derived from clinical breakpoints determined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). MIC values for *Pseudomonas* spp. were used because these are the dominant psychrotrophs in cold stored raw milk. Also *Acinetobacter*, *Burkholderia* and *Stenotrophomonas* MIC values were taken into consideration.

Table 3. Antimicrobials, their classes and concentrations used in this study.

Antimicrobial agent	Class	Concentrations used
Gentamycin (G)	Aminoglycoside	GI: 16 mg/l GII: 4 mg/l
Ceftazidime (C)	β-lactam	CI: 32 mg/l CII: 8 mg/l
Levofloxacin (L)	Fluoroquinolone	LI: 8 mg/l LII: 2 mg/l
Trimethoprim-Sulfamethoxazole 1:19 (TS)	T: Diaminopyrimidine, S: Sulfonamide	TSI: 8 mg/l (T) + 152 mg/l (S), TSII: 4 mg/l (T) + 76 mg/l (S)

Concentration I for G, C and L = 4 x MIC, for TS = 2 x MIC
Concentration II = MIC for all antimicrobials
MIC: Minimum inhibitory concentration
MIC values from European Committee on Antimicrobial Susceptibility Testing (EUCAST).

4.4.1 Preparation of the antimicrobials

The testing of the antimicrobial resistance was performed according to the instructions of EUCAST (2000). The growth medium is not specified in the instructions, but the use of Mueller-Hinton agar (MH) is recommended.

Dilutions of each antimicrobial were made from freshly prepared 5 ml stock solutions. The stock solution concentrations for gentamycin, ceftazidime and levofloxacin were 10 240 mg/l. Concentration for trimethoprim was 2 560 mg/l and for sulfamethoxazole 48 640 mg/l. All antimicrobials were dissolved with different solvents (Table 4) and all dilutions were made in autoclaved MilliQ-water.

Table 4. Antimicrobials, solvents and the final stock solution concentrations.

Antimicrobial	Solvent	Stock (mg/l)
Gentamycin	5 ml H_2O	10 240
Ceftazidime	0,1 M phosphate buffer *	10 240
Levofloxacin	0,25 ml 0,1 M NaOH + H_2O	10 240
Trimethoprim	0,8 ml 0,1 M lactic acid + H_2O	2 560
Sulfamethoxazole	3 ml 100% EtOH + 2 ml H_2O (conventional, day 0**), 5 ml 100% EtOH	48 640

* Health Canada (http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/appendix-annexe_g-h_2006-eng.php)
Other solvents EUCAST 2000
** Sulfamethoxazole was dissolved in 100% EtOH and water when the first samples were handled (conventional milk on day 0). Later sulfamethoxazole was dissolved in 5 ml 100% EtOH.

Dissolving sulfamethoxazole was difficult. When the first raw milk samples (conventional milk, day 0) were analyzed the ethanol content that was used was not enough (Table 4) and some of the sulfamethoxazole was still in powder form when it was added to warm MH agar. Subsequent dissolvings for conventional samples (day 4) and organic samples (day 0 and day 4) were made only in 100% ethanol and no water was added.

All stock solutions were sterile filtered, except for sulfamethoxazole. Stock solutions were diluted with water to gain MIC and 4 x MIC concentrations for gentamycin, ceftazidime and levofloxacin, and MIC and 2 x MIC concentrations for trimethoprim and sulfamethoxazole, which were mixed in a ratio of 1:19 (Table 3). All raw milk samples were plated from 10^{-1} dilutions and in duplicates to MH plates that contained one of the antimicrobials in MIC or 4 x MIC / 2 x MIC. Plates were incubated at 30ºC for 3 days and at 7 º C for 10 days.

4.5 Analysis of bacterial population changes

The analysis of bacterial population changes in conventional and organic raw milk samples consisted in the extraction of the total bacterial DNA, two successive rounds of PCR (nested-PCR) and denaturing gradient gel electrophoresis (DGGE) (Figure 3).

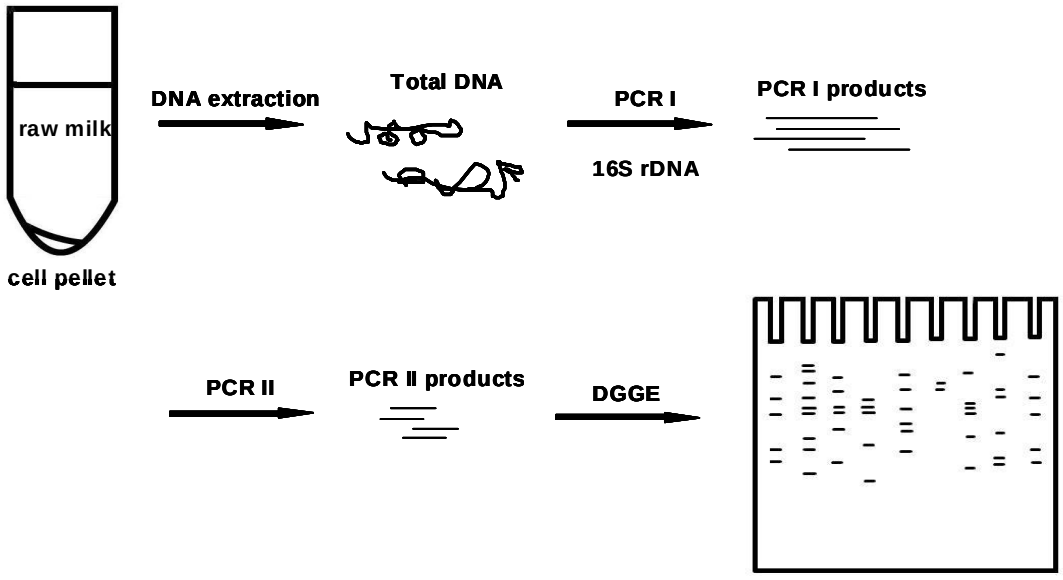

Figure 3. Flowchart of the molecular biology study and the analysis of bacterial population changes.

4.6 Extraction of bacterial DNA

Bacterial DNA from raw milk was extracted with two different commercial kits. Norgen Biotek's Milk Bacterial DNA Isolation Kit (Thorold, Ontario, Canada) was tested but only PathoProof™ Mastitis PCR Assay Kit (Finnzymes, Espoo, Finland) was used in the final analysis. The DNA extraction part of the Finnzymes' kit was used as directed in the protocol, without any modifications. With Norgen Biotek's kit some modifications were made.

DNA extraction protocol for PathoProof™ Mastitis PCR Assay Kit (Finnzymes):

1. Mix 350 µl of milk sample with 350 µl of Lysis solution 1 and 7 µl of Proteinase-K.
2. Incubate for 5 minutes at 55°C.
3. Centrifuge for 5 minutes at ~7500 rpm.
4. Remove supernatant.
5. Resuspend pellet in 100 µl of Lysis solution 2.
6. Incubate for 10 minutes at 37°C.
7. Add 20 µl of Proteinase-K and 200 µl Buffer AL.
8. Incubate for 10 minutes at 55°C.
9. Add 200 µl of 96 - 100% ethanol and mix well.
10. Apply samples in QIAamp Mini spin column and wash first with Buffer AW1 and after that with Buffer AW2.
11. Elute in 100 µl of Buffer AE.

Modified DNA extraction protocol for unknown or gram positive bacteria for Milk Bacterial DNA Isolation Kit (Norgen Biotek):

1. Centrifuge 500 µl of milk for 2 minutes at ~14 000 rpm.
2. Pour off the supernatant and add 150 µl (original protocol 100 µl) of Digestion Buffer.
3. Incubate for 1 hour (original protocol 45 minutes) at 37°C.
4. Add 350 µl Lysis Solution (original protocol 300 µl) and 10 µl Proteinase-K.
5. Incubate for 70 minutes (original protocol 45 minutes) at 55°C.
6. Add 40 µl Binding Solution and 180 µl 96 - 100% ethanol.
7. Spin down and transfer aqueous phase only to spin columns.
8. Wash first with Washing Solution I after that with Washing Solution II.
9. Elute in 100 µl (original protocol 200 µl) of Elution Buffer.

The eluted DNA was stored at 4°C until the first PCR was done and afterwards at -20°C.

4.7 PCR

16S rDNA sequences were amplified using nested-PCR technique with Perkin Elmer Cetus DNA Thermal Cycler 480. The proportions of PCR reaction components were optimized for these samples by using variable amounts of template, primers and magnesium-concentration. Different cycling parameters were also tested by raising and lowering annealing and extension temperatures and time and also by increasing the time of final extension.

The first PCR (PCR I) reaction of the nested-PCR was performed by using the primer pair W01 and W012 (Ogier et al. 2002) that amplifies about 700 base pair (bp) amplicon which includes V3 region of bacterial 16S rDNA (Table 5). The second PCR (PCR II) was performed using the product of the first PCR as a template, and primers HDA1_GC and HDA2 (Ogier et al. 2002) that produce about 200 bp amplicon. These primers flank the V3 region (*E. coli* position 436 - 499) that has variability in the base pair sequence between species. The HDA1_GC primer has a 40 bp GC-tail that is added to the 5'-end of PCR product. This GC-clamp significantly improves the separation efficiency of sequences in following denaturing gradient gel electrophoresis (DGGE). Phusion™-High Fidelity enzyme (Finnzymes, Espoo, Finland) was used because of its suitability for high GC-content amplications and its proof-reading property. PCR reaction components and programme parameters are shown in Tables 6 and 7, respectively.

Table 5. Primers used in this study, their sequence, length and their relative position in *Escherichia coli* 16S rDNA gene. GC-clamp in HDA1_GC is in bold.

Primer	Sequence (according to Ogier et al. 2002)	Length (bp)	Relative position in *E. coli*
W01	5'-AGA GTT TGA TC[AC] TGG CTC-3'	18	8 - 25
W012	5'-TAC GCA TTT CAC C[GT]C TAC A-3'	18	684 - 702
HDA1_GC	5'-**CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G**AC TCC TAC GGG AGG CAG CAG T-3'	21 (+ 40 bp GC-tail)	338 - 357
HDA2	5'-GTA TTA CCG CGG CTG CTG GCA-3'	21	516 - 536

Table 6. PCR I and PCR II reaction components used in nested-PCR for raw milk samples. PCR I reaction components were also applied for the amplification performed for the reference strains.

PCR I	Final concentration in 50 µl reaction
Autoclaved MQ-H_2O	
10 x Dynazyme II buffer (Mg concentration optimized)	Mg concentration: 1,5 mmol
Primer 1 (W01)	60 pmol
Primer 2 (W012)	60 pmol
dNTP mix	200 µM
Dynazyme II-polymerase	2 U
template (extracted DNA in elution buffer)	

PCR II	
Autoclaved MQ-H_2O	
5 x Phusion GC-buffer (Mg concentration optimized)	Mg concentration: 1,5 mmol
Primer 1 (HDA1_GC)	60 pmol
Primer 2 (HDA2)	60 pmol
dNTP mix	200 µM
100% DMSO	
Phusion-polymerase	0,6 U
template (PCR I product)	

Table 7. PCR I and PCR II cycling parameters for raw milk samples and reference strains.

	PCR I	PCR II
Initial denaturation	96ºC / 4 min.	98ºC / 45 sec.
Denaturation	96ºC / 30 sec.	98ºC / 20 sec.
Annealing	58ºC / 30 sec.	67ºC / 30 sec
Extension	72ºC / 1 min.	72ºC / 30 sec.
	30 cycles	20 cycles
Final extension	72ºC / 7 min.	72ºC / 7 min.

The quantification of DNA amounts in every PCR II sample was made by visually examining ethidium bromide (EtBr, Amresco, Solon, Ohio, USA) stained agarose (Promega, Madison, Wisconsin, USA) gels. EtBr stains DNA in a concentration-dependant manner. The more there is DNA present in a band on a gel, the more intensely it will stain. An unknown band can be compared with a standard band, whose DNA amount is known. If the intensities of two bands are similar, then they contain similar amounts of DNA (Stephenson 2003). PCR I products were not quantifiable

because of very low yield that could not be detected from EtBr-stained agarose. A band has to contain at least 140 pg of double stranded DNA (dsDNA) to be visualised with EtBr on agarose (Tuma et al. 1999).

4.8 DGGE

4.8.1 Construction of DGGE reference

For denaturing gradient gel electrophoresis (DGGE) run, a reference was constructed from PCR II products obtained from seven reference collection strains with varying GC-content (Table 8). The hypothesis was that the representatives of these seven strains could also be present in the milk samples and thus were selected as interesting reference candidates. Because DGGE separates PCR products according to their sequence and not their size, the use of common DNA ladder is not justified. With a self-prepared reference marker, the lanes on the DGGE gel could be aligned, if the gel was twisted. Also the presence or absence some species could be assessed.

Table 8. Reference strains used to construct DGGE reference, the collection they were obtained from, ATCC-number, PCR II GC-content and the GenBank access number from which the GC-content was calculated.

Strain	Collection	ATCC-number	PCR II amplicon GC-content (%)	GenBank access number
Acinetobacter johnsonii	HAMBI 1969	17909	53	Z93440
Bacillus cereus	HAMBI 250	10987	53	AJ577290
Burkholderia cepacia	HAMBI 1976	25416	54	AF097530
Escherichia coli	HAMBI 99	11775	54	NR_024570
Listeria innocua	CCUG 15531^{T}	33090	50	S55473
Pseudomonas tolaasii	LMG 2342^{T}	33618	52	AF255336
Stenotrophomonas maltophilia	HAMBI 2659	13637	54	AB008509

ATTC, American Type Culture Collection, LGC Promochem, LGC Standards, United Kingdom.
CCUG, Culture Collection University of Göteborg, Department of Clinical Bacteriology, Sweden.
HAMBI Culture Collection, Helsinki University, Faculty of Agriculture and Forestry, Department of Applied Chemistry and Microbiology, Finland.
LMG/BCCM, Belgian Co-ordinated Collections of Micro-organisms, Gent, Belgium.

Reference strains were acquired from different collections (Table 8) as lyophilized cultures. The bacteria were grown overnight in Luria Bertani broth. The extraction of the DNA was performed from 1 ml of overnight culture with Wizard® Genomic DNA

Purification Kit (Promega, Madison, Wisconsin, USA). The protocol for Isolation of Genomic DNA from Gram Positive and Gram Negative Bacteria was followed as recommended by the manufacturer with minor modifications (Appendix 1).

The reaction components of the PCR I were the same as for the raw milk samples (Table 6) and the reaction components for PCR II are depicted in Table 9. PCR I and PCR II cycling parameters for reference strains were the same as for the milk samples (Table 7). The PCR II products of reference strains were mixed so that all the species were present approximately at the same quantity. When the DGGE gel was prepared, 7 µl of reference strain mix (from now on this mix is referred as the reference) was pipetted in every fifth well on the gel.

Table 9. PCR II reaction components used in nested-PCR for the reference strains.

PCR II	Final concentration in 50 µl reaction
Autoclaved MQ-H_2O	
5 x Phusion GC-buffer (Mg concentration optimized)	Mg concentration: 1,5 mmol
Primer 1 (HDA1_GC)	60 pmol
Primer 2 (HDA2)	60 pmol
dNTP mix	200 µM
100% DMSO	
Phusion-polymerase	0,6 U
template (1:5 dilution of PCR I product)	

4.8.2 DGGE gel preparation and run

Denaturing gradient gel electrophoresis (DGGE) analysis was performed with the BioRad DCode Universal Mutation Detection System (BioRad, Hercules, California, USA). DGGE separates sequences by melting their double helix when samples are run on denaturing polyacrylamide gel. Denaturants that are used for this purpose are formamide and urea (both from Fluka Biochemika, Buchs, Switzerland). The 100% denaturant is determined as 7 M urea and 40% (v/v) deionized formamide. The denaturing gradient for this study was determined empirically. The starting point for testing the gradient was a study by Ogier et al. (2004) where the primers, same as in this study, were used to amplify the V3 region. We tested the following gradients: 20 - 60%, 30 - 70% and 35 - 70%. Last one of these was used for the raw milk samples (Table 10).

The strength of the polyacrylamide gel (acrylamide-bis 40% solution, Promega, Madison, Wisconsin, USA) gel was 10% as recommended by DGGE apparatus manufacturer (BioRad).

Table 10. The final concentrations for each component in gel solutions used to prepare the DGGE gel with 35 - 70% denaturing gradient.

	35% denaturant	70% denaturant
Acrylamide-bis 40% (37,5:1)	10%	10%
50 x TAE-buffer	1 x TAE	1 x TAE
Formamide	14%	28%
Urea	2,4 M	4,9 M
dH_2O		

The TAE-buffer used to prepare the denaturing gel solutions consisted of the following components:

50 x TAE-buffer
Tris base (Promega, Madison, Wisconsin, USA), final concentration 2 M
Acetic acid, glacial (J.T. Baker, Deventer, Holland), final concentration 1 M
0,5 M EDTA, pH 8,0 (Amresco, Solon, Ohio, USA), final concentration 50 mM
dH_2O

The gel, which was 1 mm thick and 16 x 16 cm by size, was casted with the casting wheel included in DCode system. Before casting, 10% ammonium persulfate (APS, Sigma, St. Louis, Missouri, USA) and N,N,N',N'-tetramethylethylenediamine (TEMED, Sigma, St. Louis, Missouri, USA) were added to the denaturants to the final concentration of 0,09%. The denaturants were mixed by the casting wheel in continuous manner. First the higher denaturant was solely poured to the bottom of the gel and then mixed with gradually increasing amount of lower denaturant. Finally only lower denaturant was poured to the gel. The gel was left to polymerize for 30 minutes and after that, 5 ml 10% polyacrylamide cap solution with 0% denaturant (acrylamide-bis 40% [37,5:1], 50 x TAE, dH_2O) was poured on the top of the gel and the well forming comb placed between the glass plates. The cap solution enables the entire sample to migrate into the gel. If the wells contained denaturants, part of the sample might get stuck to the wells. The gel was left to polymerize for two hours in a fume hood.

The loading dye was mixed with various amounts of PCR II product depending on the amount of DNA in the sample. Based on the visual examination of the EtBr-stained agarose gel the following amounts of DNA were loaded on DGGE gel (Table 11).

Table 11. The approximate amount of DNA (ng) in every lane in the DGGE gel in conventional raw milk (C) and in organic raw milk (O). The samples are in the order they were loaded on the gel. R: reference.

C	R	C1D0	C1D4	C2D0	C2D4	R	C3D0	C3D4	C4D0	C4D4	R	C5D0	C5D4	C6D0	C6D4	R
ng	175	300	450	300	450	175	300	450	300	450	175	300	450	300	450	175

O	R	O1D0	O1D4	O2D0	O2D4	R	O3D0	O3D4	O4D0	O4D4	R	O5D0	O5D4	O6D0	O6D4	R
ng	175	300	300	300	300	175	300	216	450	300	175	450	300	300	216	175

Two different loading dyes were used and their identical behaviour was tested beforehand. Organic raw milk PCR II products were loaded to DGGE polyacrylamide gel using Bio-Rad's 2 x gel loading dye included in the electrophoresis reagent kit. Conventional raw milk PCR II products were loaded with self-prepared 2 x loading dye.

<u>2 x gel loading dye</u>
2% Bromophenol blue (Sigma, St. Louis, Missouri, USA), final concentration 0,05%
2% Xylene cyanol FF (Sigma, St. Louis, Missouri, USA), final concentration 0,05%
100% Glycerol (Fluka Biochemika, Buchs, Switzerland), final concentration 70%
dH_2O

When 2 x loading dyes were mixed with PCR II products, the sample contained 35% glycerol. We also tested 6 x Orange Loading Dye (Fermentas, Burlington, Ontario, Canada) that contained 60% glycerol and 5 x Glycerol Gel Loading Dye (Amresco, Solon, Ohio, USA) that contained 30% glycerol. When mixed with the PCR II products, the preceding had a final concentration of 10% glycerol and the latter only 6%. These concentrations were not enough to drag the sample to the bottom of the well.

The running buffer (1 x TAE) was pre-warmed to 60ºC before inserting the gel into the buffer chamber and warmed again to 60ºC before loading the samples. Conventional and organic PCR II products were electrophoresed on two different gels however both had the same sample order on the gel (Table 11). The day 0 (D0) and day 4 (D4)

amplicons from each sample (C1 - C6 and O1 - O6) were placed next to each other, and the reference was placed in every fifth well.

The samples were run on 70 V for 21 hours. The voltage was run through the gel for 15 minutes before starting the stirrer in the buffer tank to prevent mixing of the samples. After the run was completed the gel was dyed for 40 minutes in dark with 30 µl of 10 000 x SYBR Gold nucleic acid stain (Invitrogen, Eugen, Oregon, USA) in 300 ml of 1 x TAE-buffer. SYBR Gold is very sensitive and can detect as few as 35 pg of double stranded DNA (dsDNA) on polyacrylamide gel compared to 290 pg of dsDNA dyed with EtBr and 46 pg of dsDNA dyed with SYBR Green II (Tuma et al. 1999). The gel was transferred to UV-table and photographed using UVIpro System (UVItec Ltd. Cambridge, United Kingdom) which includes darkroom, camera and software.

4.8.3 Analysis of DGGE profiles

Images were analysed using GelCompar® II programme (version 5.1, Applied Maths NV, St-Martens-Latem, Belgium). GelCompar® II was used to normalise and compare DGGE profiles from conventional and organic raw milks from day 0 and day 4. Gels were matched and normalised using reference that was described earlier.

The similarity among different samples was calculated using Pearson’s correlation coefficient and the clustering was done with the unweighted pair-group method using arithmetic averages (UPGMA). Also maximum parsimony cluster analysis was performed with bootstrap value 1000.

5 Results

5.1 Microbiological study and antimicrobial resistance

5.1.1 Total counts and proportion of psychrotrophic bacteria

The total counts were determined both on plate count agar (PCA) plates and Mueller-Hinton plates without antibiotics (MH). The total counts of initial flora revealed that 10 out of 12 samples (Figure 4) were of excellent quality (quality class E) according to the Finnish Association of Milk Hygiene's standards. Class E raw milk contains less than $5*10^4$ cfu/ml. Sample O4D0 was of lower quality only when the counts were determined on MH plate, and for sample O5D0 this was observed for both types of plates. Differences between MH and PCA were lower than one log-unit in all except one case (O4D0).

The initial amount of psychrotrophic bacteria was in most of the samples lower than the total counts, as can be expected. In all the conventional raw milk samples the count was approximately 10^3 cfu/ml or less (Figure 5). In organic raw milk samples O1D0 - O3D0 the count was of the same level with conventional raw milk samples. Samples O4D0 - O6D0 on the other hand had noticeably higher psychrotrophic counts.

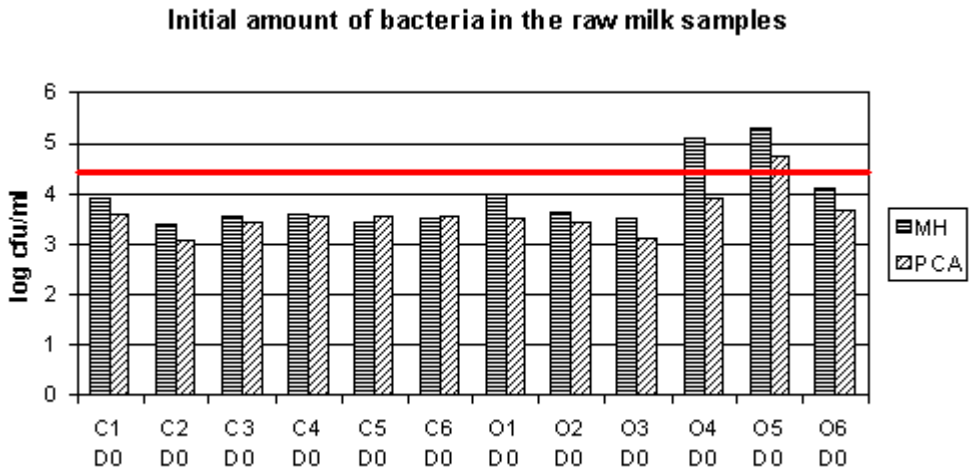

Figure 4. Initial amount of bacteria in conventional and organic raw milk samples incubated at 30ºC for 3 days on Mueller-Hinton plates without antibiotics (MH) and on plate count agar plates (PCA). The red line indicates the limit for quality class E (< $5*10^4$ cfu/ml).

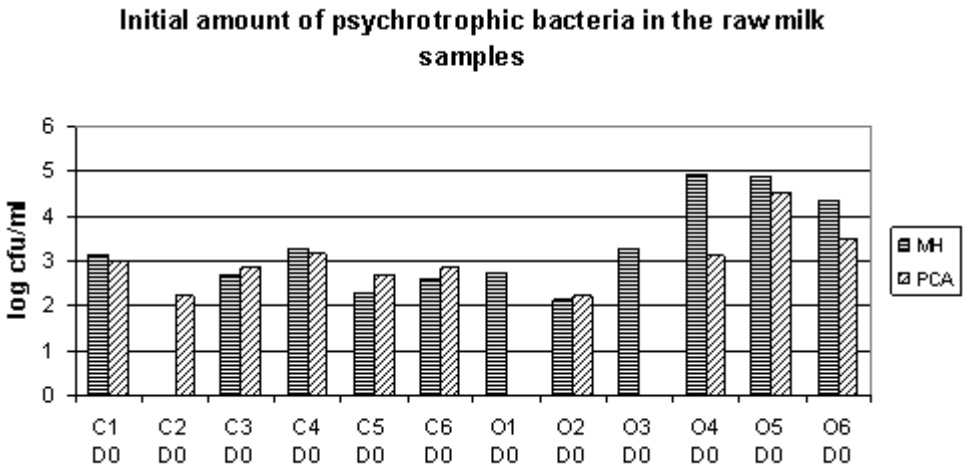

Figure 5. Initial amount of psychrotrophic bacteria in conventional and organic raw milk samples incubated at 7ºC for 10 days on Mueller-Hinton plates without antibiotics (MH) and on plate count agar plates (PCA).

After four days of cold storage, the number of bacteria had risen by 0,7 - 2,9 log-units when calculated on MH-plates. On MH plates the highest numbers were observed for samples O4D4 and O5D4 (~$2*10^7$ cfu/ml) and the lowest for C1D4 (~$4*10^4$ cfu/ml) (Figure 6). On PCA plates the counts were again lower than on MH, except for sample O6D4, and the difference was lower than one log-unit in all, except for one sample (O2D4).

The psychrotrophic counts had also risen during the cold storage by 2,2 - 3,6 log-units when calculated on MH-plates (Figure 7), the average being 2,9 log-units (sample C2 was excluded from the average since the count from day 0 psychrotrophs was not successful, Figure 2). The conventional raw milk samples and organic samples O1 - O3 still had lower counts than samples O4 - O6, but the difference was not as noticeable as in the initial counts (Figure 5).

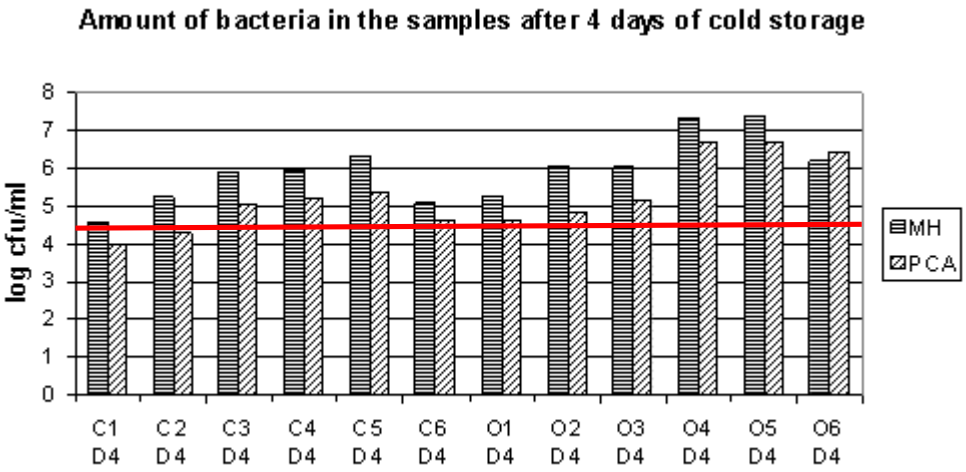

Figure 6. Amount of bacteria as obtained at 30ºC incubation for 3 days on Mueller-Hinton plates without antibiotics (MH) and on plate count agar (PCA) plates in conventional and organic raw milk samples after 4 days of cold storage at 4ºC. The red line indicates the limit for quality class E (< $5*10^4$ cfu/ml).

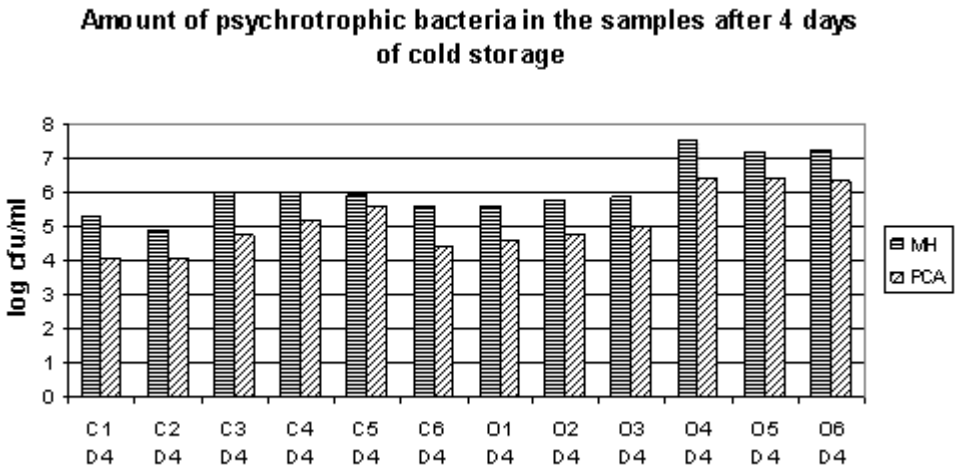

Figure 7. Amount of psychrotrophic bacteria as obtained at 7ºC incubation for 10 days on Mueller-Hinton plates without antibiotics (MH) and on plate count agar (PCA) plates in conventional and organic raw milk samples after 4 days of cold storage at 4ºC.

The proportion of psychrotrophs was calculated by comparing the cfu/ml values from MH plates grown at 7ºC for 10 days to those grown at 30ºC for 3 days. If the psychrotrophic count was higher than the total count, the proportion of the psychrotrophs was estimated to be 100%. The proportion of psychrotrophic bacteria increased notably from day 0 to day 4 (Figure 8). In samples C1, C2, C3, C5, C6, O1

and O2 the initial proportion of psychrotrophs in the raw milk bacterial community was less than 20% but after 4 days of cold storage (at 4ºC) it was more than 40% in all the samples. The proportion of psychrotrophs reached 100% in half of the samples (C1, C3, C6, O1, O4 and O6). Samples C4 and O3 - O6 had high proportion (39 - 100%) of psychrotrophs already before the beginning of the cold incubation.

The initial proportion (on day 0) of psychtrotrophs from the total population was on average 31,5%. If the samples O4 - O6 were excluded, the average percentage on day 0 was 18,5%. The average after the cold incubation was 80,6%.

High initial value of psychrotrophs for samples O4 - O6 can be explained by the fact that the raw milk samples were one day older than the rest of the organic samples. The high initial value in C4 compared to other conventional samples, and in O3 compared to O1 and O2 cannot be explained by the available facts.

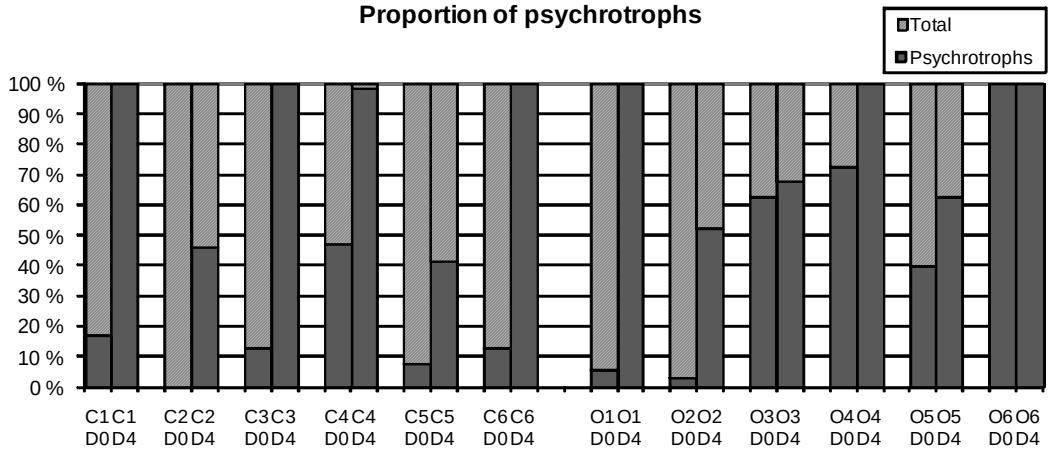

Figure 8. Proportion (%) of psychrotrophs from the total bacterial counts calculated from Mueller-Hinton (MH) plates in conventional and organic raw milk samples at day 0 (D0) and day 4 (D4).

5.1.2 Antimicrobial resistance in bacterial populations

In conventional raw milk samples, the antimicrobial resistance seemed to be more prevalent than in organic raw milk samples. The proportion of resistant bacteria across all sampling days and antimicrobial classes used in this study was 9,6% in conventional and 6,9% in organic raw milk samples (Table 12).

Table 12. The proportion of resistant bacteria (%) to different antimicrobial agents (gentamycin G, ceftazidime C, levofloxacin L and trimethoprim-sulfamethoxazole TS) before (D0) and after (D4) the cold storage, at two different incubation temperatures (30ºC and 7ºC) for conventional and organic raw milk.

		G	C	L	TS	Average
Conventional	D0 30ºC	8,4%	34,7%	2,8%	20,0%	16,5%
raw milk	D0 7ºC	21,0%	20,2%	6,6%	13,4%	15,3%
	D4 30ºC	1,1%	5,2%	0,1%	5,4%	2,9%
	D4 7ºC	0,9%	5,3%	0,1%	8,0%	3,6%
						9,6%
Organic	D0 30ºC	3,7%	18,8%	0,5%	9,3%	8,1%
raw milk	D0 7ºC	4,4%	26,9%	0,5%	7,1%	9,7%
	D4 30ºC	0,2%	1,0%	0,1%	7,0%	2,1%
	D4 7ºC	0,1%	2,9%	0,1%	27,2%	7,6%
						6,9%

The highest proportion of resistance in conventional raw milk bacteria was seen at day 0 for both incubation temperatures. The average resistance in the bacterial populations was, across all antimicrobials, concentrations and samples, 16,5% at 30ºC and 15,3% at 7ºC (Table 12). At day 4 the average resistance was 2,9% at 30ºC and 3,6% at 7ºC. The resistance in the conventional raw milk samples was highest at day 0 to ceftazidime at 30ºC and at 7ºC (34,7% and 20,2%, respectively), to gentamycin at 7ºC (21,0%) and to trimethoprim-sulfamethoxazole at 30ºC (20,0%). The bacteria were most susceptible to levofloxacin with the resistances being less than 7% on day 0 and day 4 for both incubation temperatures.

The differences in the proportion of resistant bacteria between day 0 and day 4 were seen especially for samples C2 and C5 (Figure 9). High resistance was noticeable among isolates from C2 at day 0 at 30ºC and from C5 at day 0 at 7ºC. C5 contained less than 10% psychrotrophs at day 0 (Figure 8) but there was still a high proportion of resistance among these bacterial isolates.

Bacteria showed some resistance to all antimicrobials in conventional raw milk samples C1 - C5, and to all except levofloxacin in sample C6 (Figure 9). For samples C1, C3, C4 and C6 the proportion of resistant bacteria was higher in the lower ceftazidime concentration (CII) as expected, but from sample C5 bacteria showed considerably high resistance to the higher ceftazidime concentration (CI). In sample C2 the resistance was as high for both antibiotic concentrations (CI and CII).

The resistance was high to both concentrations of trimethoprim-sulfamethoxazole (TSI and TSII) for samples C2 and C5. For sample C3 the resistance was over 40% on day 0 at 7ºC (Figure 9). Some resistance to gentamycin was detected in all samples but the resistance was especially high for sample C5 on day 0 at 7ºC. High proportions of bacteria were susceptible to levofloxacin (L), except for sample C5.

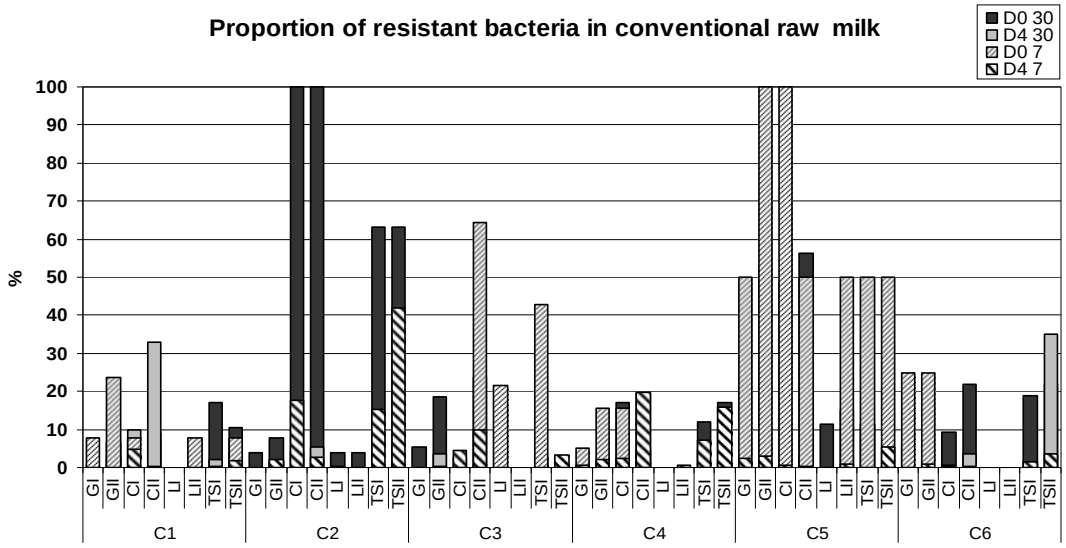

Figure 9. Proportion of bacteria resistant to different antimicrobials (GI: gentamycin 16 mg/l, GII: gentamycin 4 mg/l, CI: ceftazidime 32 mg/l, CII: ceftazidime 8 mg/l, LI: levofloxacin 8 mg/l, LII: levofloxacin 2 mg/l, TSI: trimethoprim-sulfamethoxazole 1:19; 8 mg/l for trimethoprim and 152 mg/l for sulfamethoxazole, TSII: trimethoprim-sulfamethoxazole 1:19; 4 mg/l for trimethoprim and 76 mg/l for sulfamethoxazole) in conventional raw milk samples C1 - C6 at day 0 (D0) and at day 4 (D4). The plates were incubated at 30ºC and at 7ºC for 3 and 10 days, respectively.

The proportion of resistant bacteria was more evenly distributed between days and incubation temperatures in organic than in conventional raw milk samples (Table 12). The average resistance in the bacterial populations was, across all the antimicrobials, concentrations and samples, 8,1% on day 0 at 30ºC, 9,7% on day 0 at 7ºC and 7,6% on day 4 at 7ºC. On day 4, at 30ºC, the resistance was lower (2,1%). For individual antimicrobials, bacteria showed high resistance only to ceftazidime on day 0 at both incubation temperatures (26,9% at 7ºC and 18,8% at 30ºC) and to trimethoprim-sulfamethoxazole on day 4 following the 7ºC incubation (27,2%). Bacteria were most susceptible to levofloxacin, the same way as in conventional raw milk samples.

The resistance to TS was highest in samples O1 - O3 on day 4 at 7ºC and in sample O6 on day 0 at 30ºC (Figure 10). High proportions of bacteria were resistant to ceftazidime on day 0 at 7ºC in samples O1, O5 and O6 and in sample O3 on day 0 at 30ºC. The resistance was very low to levofloxacin in all organic raw milk samples and low (< 15%) to gentamycin in all samples except O6.

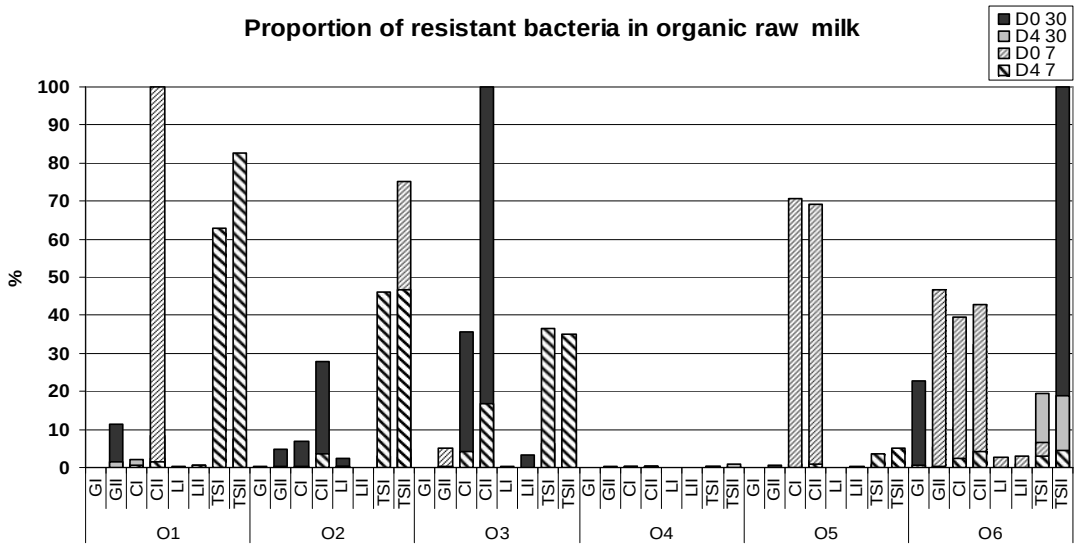

Figure 10. Proportion of bacteria resistant to different antimicrobials (GI: gentamycin 16 mg/l, GII: gentamycin 4 mg/l, CI: ceftazidime 32 mg/l, CII: ceftazidime 8 mg/l, LI: levofloxacin 8 mg/l, LII: levofloxacin 2 mg/l, TSI: trimethoprim-sulfamethoxazole 1:19; 8 mg/l for trimethoprim and 152 mg/l for sulfamethoxazole, TSII: trimethoprim-sulfamethoxazole 1:19; 4 mg/l for trimethoprim and 76 mg/l for sulfamethoxazole) in organic raw milk samples C1 - C6 at day 0 (D0) and at day 4 (D4). The plates were incubated at 30ºC and at 7ºC for 3 and 10 days, respectively.

Among the considered raw milk samples the proportion of resistant bacteria seemed to decrease during the four days cold storage. From Table 12 it can be seen that when the proportions of resistant bacteria at day 0 and day 4 are compared within an incubation temperature, the proportion decreases. This can be seen for all individual antimicrobials and also in the averages from all antimicrobial agents. The only exception is the incubation at 7ºC in TS-containing plates, where the proportion of resistant bacteria increased from 7,1% on day 0 to 27,2% on day 4 in organic raw milk samples.

5.2 Changes in bacterial populations

5.2.1 DNA extraction from raw milk samples

Norgen Biotek's Milk Bacterial DNA Isolation Kit did not give the result suggested by the manufacturer when the protocol was followed nor did it after modifications. The manufacturer argues that PCR products from bacterial DNA can be visualized on ethidium bromide (EtBr) stained agarose gel if the sample contains 10^1 or more cfu/ml. In this case the band is dim but visible. DNA extraction and the following PCR conditions were tested from raw milk samples that contained bacteria ranging from $5*10^2$ cfu/ml to 10^9 cfu/ml (Table 13). The PCR products were not visible on EtBr-agarose if the sample contained 10^6 cfu/ml or less. A positive signal was gained if the samples contained $3*10^8$ cfu/ml or 10^9 cfu/ml. A bacterial load of 10^7 was not tested.

The tests that the manufacturer had performed were done with samples spiked with various amounts of *E. coli* DH5α and the resulting PCR amplicon with *E. coli* specific primers was around a size of 550 bp. The manufacturer has also done some tests on mastitis milk with various degrees of infection (Tom Hunter, Norgen Biotek Corp., email message to the author, 15 April 2009).

Finnzymes' PathoProof™ Mastitis PCR Assay's DNA extraction protocol was followed as recommended by the manufacturer and no modifications were made. Also with this kit the first round of PCR seemed negative on EtBr-agarose, even when samples carried up to 10^5 cfu/ml (Table 14), but the following PCR II produced a clear band with EtBr-staining. The manufacturer does not give any claims about the bacterial cell numbers that can be successfully extracted and used as PCR template.

Table 13. Successfulness of nested-PCR (PCR I and PCR II) after extraction of bacterial DNA with Norgen Biotek's Milk Bacterial DNA Isolation Kit from raw milk samples with various amounts of bacterial cells.

	cfu/ml									
	$5*10^2$	$2*10^3$	$1*10^4$	$2*10^4$	$1*10^5$	$2*10^5$	$5*10^5$	$1*10^6$	$3*10^8$	$1*10^9$
PCR I	-	-	-	-	-	-	-	-	+	+
PCR II	+	+		+		+				

- = PCR product not seen on EtBr-stained agarose gel
+ = PCR product seen on EtBr-stained agarose gel
empty = not tested

Table 14. Successfulness of nested-PCR (PCR I and PCR II) after extraction of bacterial DNA with Finnzymes' PathoProof™ Mastitis PCR Assay from raw milk samples with various amounts of bacterial cells.

	cfu/ml				
	$2*10^3$	$4*10^3$	$5*10^3$	$2*10^4$	$1*10^5$
PCR I	-	-	-	-	-
PCR II	+	+	+	+	+

- = PCR product not seen on EtBr-stained agarose gel
+ = PCR product seen on EtBr-stained agarose gel

5.2.2 DGGE profile analysis of conventional raw milk samples

In conventional raw milk samples from day 0, PCR amplicons were spread along the whole length of the denaturing gradient indicating that there was a strong variability in GC-content of the amplicons and that the community present in the samples was diverse (Figure 11). Some of the dominant bands that were present at day 0 were absent at day 4. C1D0 and C5D0 had a same dominant band on the bottom of the lane and this band could not be seen at day 4 in either of the samples. There was an intense band present C1D0 and C5D0 at the position of the reference band Ba. (*Bacillus cereus*). This band was not present at day 4 indicating a very low number of this species. Disappearance of a band means that the relative abundance of that species is so low that it cannot be detected anymore on DGGE, not that the species is totally absent. Appearance of a band means that the species was present also in the initial population but the number was too low to be detected.

At day 4 the community structure was less diverse since the bands were located mostly on the top of the DGGE gel (Figure 11). This indicates that the PCR amplicons from the community contained more AT-rich areas than at day 0. One major band was present in all samples from day 4. The band was located at the top of the gel which indicates a low GC-content. This band could be seen at day 0 in sample 3 (C3D0) and very dimly in sample 4 (C4D0). The number of cells has been very low at the beginning of the analysis, but after four days of cold storage this species became one of the most dominant in the community. There were also major bands present in all samples from day 4 at the level between reference bands L.i. and A.j. (*Listeria innocua* and *Acinetobacter johnsonii*). Samples C1D4 and C6D4 had three intense bands, samples C2D4, C3D4 and C5D4 had two and sample C4D4 had one.

Most of the bands from day 0 samples were very faint. GelCompar® II programme could not recognise all the bands that were seen on gel. The programme detected 10 - 27 bands and 10 - 12 bands from conventional raw milk samples day 0 and day 4 respectively (Table 15). For samples C1, C5 and C6 the reduction in the number of bands from day 0 to day 4 was clear which means a lower diversity on day 4. Samples C2, C3 and C4 seemed also more diverse on day 0 than day 4 when the DGGE gel was inspected, but the programme could only recognise 10 - 12 bands on both days in those samples.

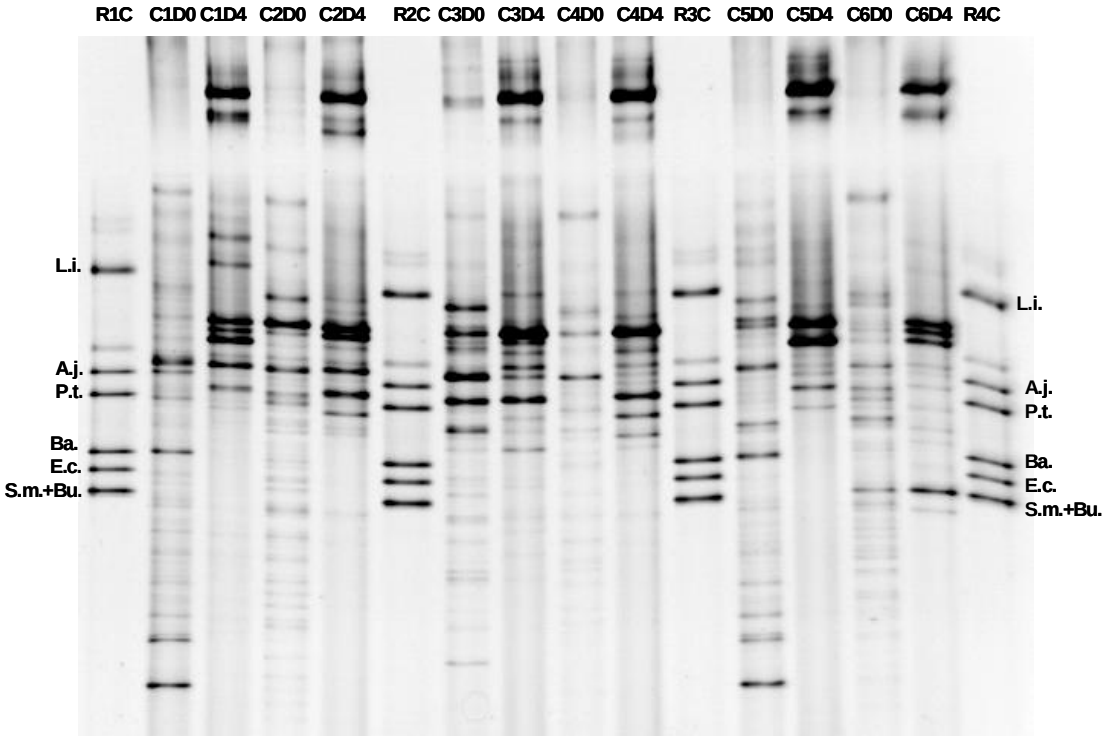

Figure 11. DGGE gel picture of conventional raw milk samples.
R1C-R4C: reference standards include the following species: L.i.: *Listeria innocua*, A.j.: *Acinetobacter johnsonii*, P.t.: *Pseudomonas tolaasii*, Ba.: *Bacillus cereus*, E.c.: *Escherichia coli*, S.m.: *Stenotrophomonas maltophilia* and Bu.: *Burkholderia cepacia*.

Table 15. Number of bands detected by GelCompar® II programme in different samples.

Sample	Bands	Sample	Bands
C1D0	21	O1D0	26
C1D4	12	O1D4	16
C2D0	10	O2D0	24
C2D4	11	O2D4	15
C3D0	10	O3D0	19
C3D4	12	O3D4	20
C4D0	11	O4D0	12
C4D4	10	O4D4	11
C5D0	22	O5D0	11
C5D4	10	O5D4	10
C6D0	27	O6D0	18
C6D4	12	O6D4	12

5.2.3 DGGE profile analysis of organic raw milk samples

Organic raw milk samples O1 - O3 showed similar community structure at day 0 and day 4 as the conventional raw milk samples. At day 0 the population was more diverse and the bands were spread along the whole length of the gel (Figure 12). Some bands were clear, some very faint. O1D0 and O2D0 both had a band on the same position as reference Ba. (*Bacillus cereus*) but the bands were not visible anymore at day 4. At day 4, the bands were located at the top of the gel and a few very intense bands were present. In samples O1D4 and O2D4 the major band was located at the top of the gel, like in all conventional raw milk samples. The band could also be seen on sample O3D4 but it was not as intense.

Samples O4 - O6 showed less change in the profile after the four days cold storage (Figure 12). In O4 and O6 few GC-richer bands that could be seen at day 0 were not present anymore at day 4 but otherwise the profiles seemed very similar. O5D0 and O5D4 profiles were almost identical, only the intensity of some bands changed. The intense band at the top of the denaturing gel in all conventional raw milk samples and in organic samples O1 - O3 from day 4 was not present in sample O4. In O5D0, O5D4 and O6D0 the band could be seen faintly. There was one intense band located also between references L.i. and A.j. (*Listeria innocua* and *Acinetobacter johnsonii*) in samples O3D4, O4D0, O4D4, O5D0, O5D4, O6D0 and O6D4. Samples O5 and O6 both had intense band also just above A.j. (*Acinetobacter johnsonii*).

In organic raw milk samples GelCompar® II programme detected 26 - 11 bands at day 0 and 20 - 10 bands at day 4 (Table 15). The faintest bands that could be seen on the gel picture were not recognised by the programme.

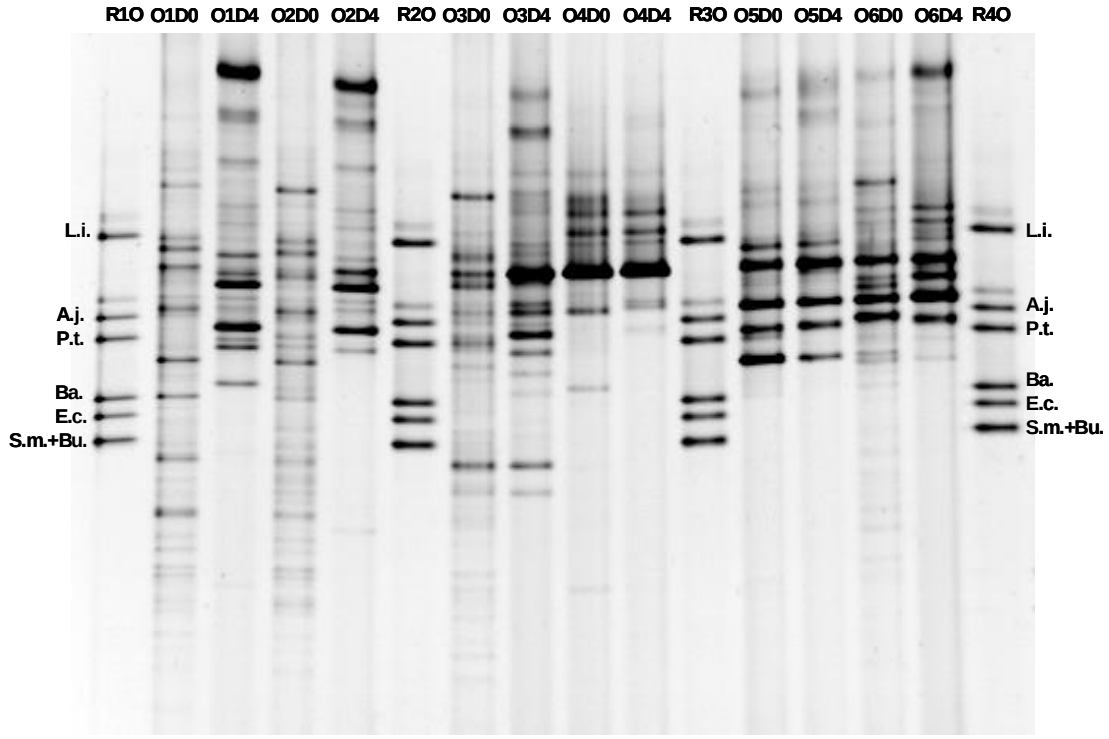

Figure 12. DGGE gel picture of organic raw milk samples.
R1O-R4O: reference standards include the following species: L.i.: *Listeria innocua*, A.j.: *Acinetobacter johnsonii*, P.t.: *Pseudomonas tolaasii*, Ba.: *Bacillus cereus*, E.c.: *Escherichia coli*, S.m.: *Stenotrophomonas maltophilia* and Bu.: *Burkholderia cepacia*.

The reference standard showed extra bands with *Burkholderia cepacia* (Bu.) and *Acinetobacter johnsonii* (A.j.) (Figures 11 and 12). The extra band from Bu. was visible above A.j. and the two extra bands from A.j. could be seen above L.i.. The origin of the extra bands was verified with DGGE where the bacterial species used in the reference were run on separate lanes (data not shown). References *Stenotrophomonas maltophilia* (S.m.) and *Burkholderia cepacia* (Bu.) could not be separated with the 35- 70% gradient used in this study.

5.2.4 Cluster analysis with Pearson's correlation and UPGMA

From the cluster analysis the same fact could be seen as from DGGE gel pictures; there was a clear difference between day 0 and day 4 community fingerprints. Day 0 and

day 4 of all the conventional samples and organic samples O1 - O3 were clearly separated so that samples from day 0 were closely clustered and so were day 4 samples (Figure 13). In fact, day 4 samples formed two clusters: one with samples C5D4, C6D4, C3D4, C4D4 and C2D4 and another with samples O1D4, O2D4 and C1D4. Within these clusters the similarity values ranged from 35,2% to 96%. The similarity value of 96% was between samples C3D4 and C4D4. The day 0 cluster was formed by C2D0, O3D0, O1D0, O2D0 C3D0 and C4D0. Within day 0 cluster similarity values ranged from 56,9% to 77,2%. The clustering was not dependent at all on the origin of the raw milk although this was hypothesized to have an impact on sample clustering so that the conventional raw milk samples would be clustered separately from organic samples.

Organic samples O4 - O6, however, formed pairs at day 0 and day 4 (Figure 13). O6D0 and O6D4, O5D0 and O5D4 and O4D0 and O4D4 share 75%, 88,5% and 87,5% similarity, respectively. These pairs also formed a distinct cluster that also had sample O3D4 in it.

The cophenetic correlation value gives an estimation of the faithfulness of each branch. The bigger the number the more reliable the branching is (Applied Maths NV 2008). The branching between individual samples had in almost all cases the cophenetic value of 100 (Figure 13). The main clusters (day 0, day 4 and O4 - O6 cluster) had a cophenetic value ranging from 75 - 92.

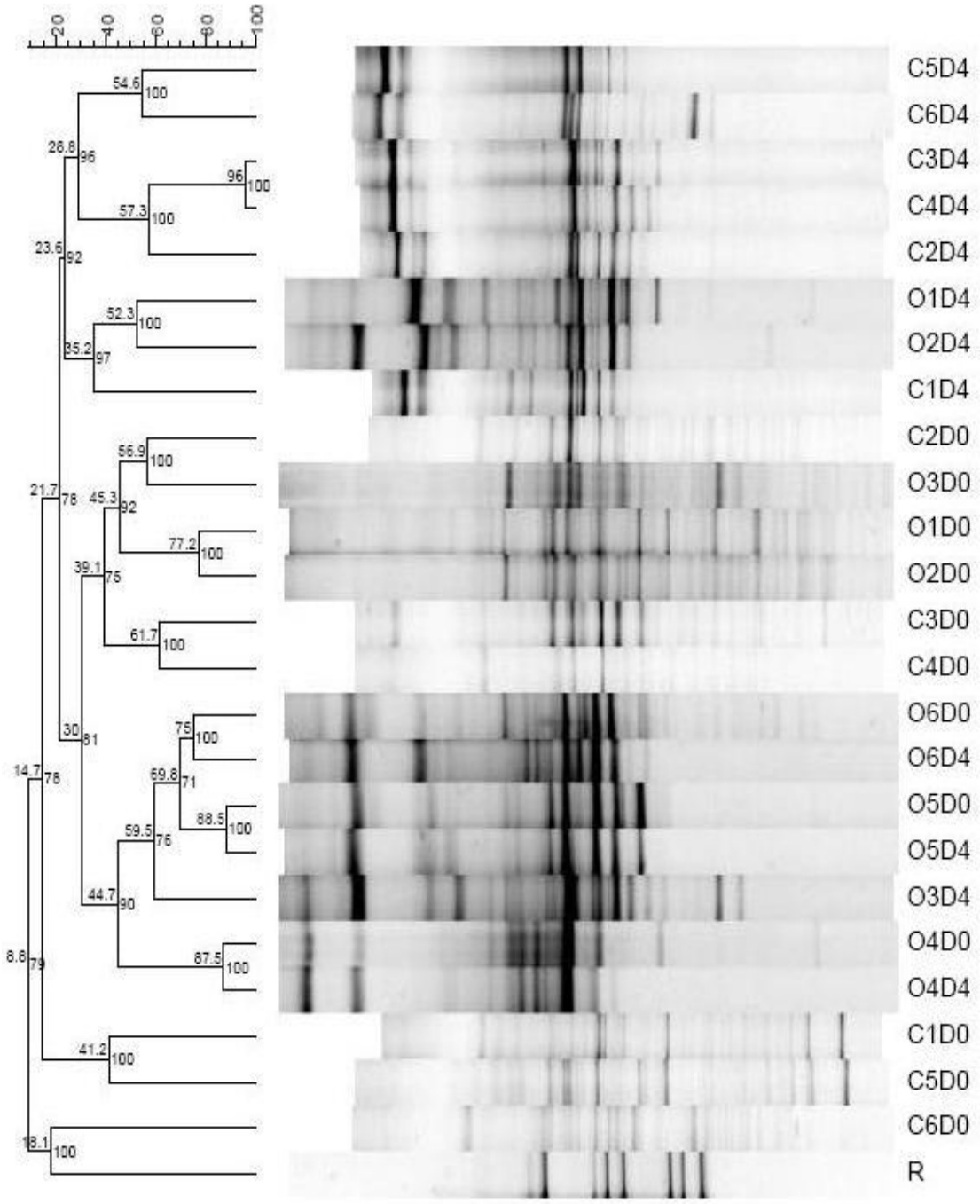

Figure 13. Dendrogram of DGGE profiles of bacterial communities in conventional and organic raw milks at day 0 (D0) and at day 4 (D4). Cluster analysis was based on Pearson's correlation index (0.0% - 100.0%) and the unweighted pair-group method with arithmetic averages (UPGMA). The numbers depicted with one decimal are the similarity values of every branch based on Pearson's correlation and UPGMA and the numbers in the fork branches are cophenetic correlation values.

5.2.5 Similarity values of the samples

The similarity values between individual samples were calculated with GelCompar® II program (version 5.1, Applied Maths NV, St-Martens-Latem, Belgium) using Pearson's correlation. Pairwise comparison between day 0 and day 4 within samples showed that similarity values were highest in samples O4, O5 and O6 (Table 16). These samples showed the least change in community structure. C2 and C3 showed higher similarity values than the rest of the conventional samples. Similarities were lowest with samples O1, O2 and C1. In these latter samples the change in community structure was the greatest during the four day cold storage.

When all other comparisons, than comparison within an individual sample between day 0 and day 4 were inspected, the highest similarity values (≥ 50%) were within day 0 samples and within day 4 samples in 20 out of 24 cases (Appendix 2).

Table 16. Similarity values (%) between day 0 and day 4 profile fingerprints from conventional and organic samples calculated with Pearson's correlation.

Sample	C1	C2	C3	C4	C5	C6	O1	O2	O3	O4	O5	O6
Similarity (%)	8,9	52,5	41,2	27,7	11,3	20,5	0,0	2,6	42,0	87,5	88,5	75,0

In the reference profiles run with conventional raw milk samples the similarities varied from 96,0 - 99,0% (Appendix 2). In reference profiles run with organic milk samples the corresponding values were 90,3 - 97,4%. When the comparison of the reference profiles was made between the gels the similarities were as low as 84,5%. Samples O2D0, C1D0 and O1D0 showed the highest similarities with all the references. In O2D0 similarities with the references varied between 16,1 - 20,1%, in C1D0 7,5 - 14,7% and in O1D0 9,8 - 13,3%. Because these samples were the most congruent with the references, we can expect that their population contained also some of the reference species (Table 8) or their close relatives. The rest of the samples showed similarity values lower than 10% with all the references. All the samples that showed 0% similarity with the reference fingerprints were day 4 samples from both conventional and organic sources.

6 Discussion

6.1 Microbiological study

The raw milk samples used in this study were of different age when the analysis started at day 0 and at day 4. Conventional samples were about 2 days old at day 0 and 6 days old when sampling was done at day 4 (Table 2). Organic raw milk samples O4 - O6 were 3 days old and samples O1 - O3 were 4 days old when the first set of analysis began (day 0). At day 4 organic samples were 7 and 8 days old, respectively. Griffiths et al. (1987) stated that the initial psychrotroph counts reflect to a large degree the age of the raw milk. This could also be seen in this study as the organic raw milk samples O4 - O6 had 1 - 3 log-units higher initial psychrotroph counts than the rest of the samples (Figure 5). The rise in the psychrotrophic numbers during the 4 days cold storage at 4ºC was on average 2,9 log-units. This is in agreement with the study done by Banks et al. (1988). They stored farm bulk milk for 4 days at 2ºC and 6ºC and the psychrotroph counts rise during the storage on average 1,8 to 3,5 log-units, respectively. Griffiths et al. (1988) stored raw milk in the similar conditions as Banks et al. (1988) and the rise in psychrotroph counts was a bit higher: 2,8 log-units at 2ºC and 3,9 log-units at 6ºC. Higher numbers were probably due to the origin of the raw milk since the samples were a mix from farm bulk tank and creamery silo.

The initial proportion of the psychrotrophs from the total counts at day 0 ranged from 0% to 100% (Figure 8). The average proportion of the psychrotrophs at day 0 was 31,5% and if the organic raw milk samples O4 - O6 were excluded the average was 18,5%. The latter value is in agreement with the study done by Hantsis-Zacharov & Halpern (2007). They examined the proportion of culturable psychrotrophs from raw milk samples from Israeli farms and the average percentage was 14,7% ± 6,4%.

To make a reliable comparison between conventional and organic raw milk, the age of raw milk samples should have been the same. On the other hand, the samples used in this study suggested that the age of the milk does not have a strong impact on the bacterial population after a certain age has been reached.

The International Dairy Federation recommends the use of plate count agar (PCA) for standard plate counts. We determined the total counts on PCA and Mueller-Hinton (MH) plates. The PCA plates were prepared with drop method and the MH plates with pour method. When the bacterial numbers were high, the estimation of colony forming

units from the drops on the PCA plates was very hard and higher dilutions should have been used. One has to consider if the drop method gives accurate results in samples where the bacterial numbers were high. Munsch-Alatossava et al. (2007) have studied the difference between drop and spread methods on PCA plates and found out that the counts were 0,3-log units lower with the drop method. In this study the difference was somewhat bigger, but two different media, PCA and MH, were used, thus the comparison to the study by Munsch-Alatossava et al. (2007) is not straight forward.

6.2 Antimicrobial resistance

The hypothesis, that the bacteria in organic raw milk are more susceptible to antimicrobials than the bacteria in conventional raw milk, seemed to hold true, although more samples need to be studied to get statistically significant results. The cattle is treated for mastitis with the same antimicrobials in both types of dairy farming; the difference in the resistance and susceptibility of the bacteria might be a consequence from the forbiddance of preventative medication for organic cattle or the differential housing conditions of the cows. Also the longer precautionary time before the milk is accepted for food production after the antibiotic treatment might have an effect to the lower resistance in the organic raw milk bacteria.

In conventional raw milk samples the resistance was highest to ceftazidime on day 0 at 30ºC (34,7%) (Table 12). High resistance could also be observed on day 0 at 7ºC and in organic raw milk samples in both incubation temperatures on day 0. This can be explained by the fact that ceftazidime belongs to the β-lactam class, which comprises antibiotics that are most frequently used in Finland to treat mastitis. Munsch-Alatossava and Alatossava (2007) tested two concentrations of ceftazidime on raw milk isolates and found out that 26 out of 42 isolates were resistant to lower concentration (4 mg/l) and 4 isolates also to higher concentration (32 mg/l) of ceftazidime. For trimethoprim-sulfamethoxazole the resistance among isolates was even higher since 30 out of 42 isolates were resistant to the concentration of 2 mg/l. Munsch-Alatossava, Xheng and Alatossava noticed the high resistance to trimethoprim-sulfamethoxazole also in organic raw milk samples by disc diffusion test (unpublished data, discussion with Patricia Munsch-Alatossava 7.4.2009). The reason for this is not known but the same trend can be also found in this study. The resistance to TS in organic raw milk bacteria was the highest (27,2%) on day 4 at 7ºC and in conventional raw milk on day 0 at both incubation temperatures (Table 12).

Bacteria were most susceptible to levofloxacin and gentamycin in both raw milk types. The only exception was the high resistance (21,0%) to gentamycin in conventional raw milk samples on D0 7ºC. Resistance of raw milk isolates to gentamycin was also noticed by Munsch-Alatossava and Alatossava (2007): 11 out of 42 isolates were resistant to 8 mg/l of gentamycin. Levofloxacin is not used in Finland to treat mastitis, but the class it represents (fluoroquinolones) is occasionally used. Gentamycin belongs to the class of aminoglycosides which is used sometimes to treat mastitis caused by gram positive bacteria.

Altogether, the resistance to the considered antimicrobials decreased during four days of cold storage. The only exception was the organic raw milk psychrotrophs incubated on TS-containing plates. The level of resistance increased from 7,1% on day 0 to 27,2% on day 4 (Table 12). This also confirms the result discussed above about a high resistance to TS within psychrotrophic isolates from raw milk samples (Munsch-Alatossava, Xheng and Alatossava, unpublished data, discussion with Patricia Munsch-Alatossava 7.4.2009).

The stability of the antimicrobials used in this study is not known, but it can be assumed that some reduction in the potency of the antimicrobials might have occurred during the incubations. Long incubation time of psychrotroph plates may have led to lowered concentration of antibiotics after ten days. Fang and Pyörälä (1996) noticed that milk decreases the efficiency of sulfonamides. This could be due to binding of sulfonamides to milk proteins, which can partly explain the high proportion of resistant bacteria on TS plates. The raw milk samples were diluted tenfold but some loss of potency might have happened.

The number of samples used in this study was limited and more samples should have been studied to obtain statistically significant results.

6.3 DNA extraction, PCR and DGGE

The optimization of the DNA extraction was somewhat problematic. Even though the manufacturers' protocols were followed, no product could be discerned on EtBr-stained agarose gel after the first PCR. Norgen Biotek, the manufacturer of the Milk Bacterial DNA Isolation Kit, argues that the PCR from as few as 10 bacterial cells can be visualized on EtBr-agarose gel. The manufacturer has tested the efficiency of the extraction from milk that has been spiked with various amounts of *E. coli* cells. It could be more advantageous to use traditional methods in the DNA extraction from raw milk

since the extraction columns used by commercial kits might be easily blocked by the raw milk components. Ramesh et al. (2002) avoided the problems caused by milk fat and proteins by using diethyl ether and chloroform to remove fat and sodium dodecyl sulphate (SDS) and NaOH to reduce the inhibitory effect of proteins on PCR.

In the DGGE gel pictures, there were intense bands on both conventional and organic day 4 samples, which could have indicated that there were a few dominant species after cold storage (Figures 11 and 12). Intense bands could have also included several species that were not separated enough in the gradient. Narrowing of the gradient from 35 - 70% to e.g. 35 - 45% could help to separate the intense bands seen on the top of the gels. When a narrower gradient is used, PCR amplicons can migrate on a longer distance on the gel and better resolution in the pictures is gained. Raw milk bacteria seemed to have a variable GC-content in their V3-region and surrounding areas because the PCR amplicons spread along the 35 - 70% gradient. This means that if a narrower gradient is used, two overlapping gels should be run to include all the bands in the analysis. For two overlapping gels gradients 35 - 55% and 50 - 70 % could be used. The use of one gel only with a wide gradient can lead to underestimation of number of species. Lafarge et al. (2004) attained better separation of the raw milk bacterial species by using two different separation techniques. The bacteria with low GC-content genomes (< 55%) were separated with temporal temperature gel electrophoresis (TTGE) and medium- or high GC genomes (> 55%) were separated by DGGE.

Another source of an error comes from the universal primers used to amplify the rDNA fragments. Amplification of all bacterial DNA present in the samples could have led to underestimation of biodiversity because, according to Vallaeys et al. (1997), some related or even non-related species can co-migrate on the gel and though are not separated. Also the limitation of the DGGE method to detect species, which are present in low numbers, can be a source of an error. Only major populations, i.e. those representing ~1% of the target organisms in terms of relative proportion, are displayed in the DGGE profiles (Muyzer et al. 1993, Murray et al. 1996). This means that some minor populations might not have been taken into account in the DGGE profile analysis.

Besides the risk of underestimation of the number of bacterial species there is also a risk for overestimation. One source for overestimation comes from the intragenomic sequence variation of the 16S rDNA (Dahllöf et al. 2000). The formation of PCR artefacts (e.g. chimeras) can also lead to overestimation of the number of species (von Wintzingerode et al. 1997). There is a possibility that GelCompar® II took also into

account those bands that were sequence variants of one species or PCR artefacts. The reference standard showed extra bands with *Burkholderia cepacia* and *Acinetobacter johnsonii*. The extra bands might have represented intragenomic variance or a PCR artefact. If the source was a PCR reaction, some extra bands are supposedly also present in the bands originating from the raw milk bacterial DNA, since the raw milk samples were amplified with the same PCR components and cycling parameters as reference strains used in the reference. We could have tested longer final extension time in the PCR reaction to eliminate the double bands, since Janse et al. (2004) noticed that longer final extension, up to 30 minutes, decreased the amount of double bands significantly in the DGGE analysis.

6.3.1 Cluster analysis

The cluster analysis of raw milk population profiles showed that the origin of the raw milk did not impact the clustering. The age of the raw milk had much stronger impact and the profiles were clustered according to the age of the milk. Organic raw milk samples O4 - O6 did not show much change in the community profile after the four days cold storage (Figure 12). Since these samples were already at the beginning of the analysis (day 0) 4 days old, it seemed that the community composition did not change after the raw milk was 4 days old. All the other samples, which were 2 - 3 days old at the beginning of the analysis, showed clear changes in the community structure during the four day cold storage (Figures 11 and 12).

In the cluster analysis of the DGGE gel, the similarity values between reference profiles were 96,0 - 99,0% and 90,3 - 97,4% on the gels that contained conventional raw milk samples and organic raw milk samples, respectively. The similarity values between reference profiles from two different gels were as low as 84,5% (Appendix 2). This implies that the reproducibility of the gels might not have been very good and the results, which were obtained from the comparison between gels, have to be questioned. Also the comparison of the lanes within a gel had some margin of error.

The cluster analysis gives a simplified view of a complex data set. Simplification results in the loss of information and every data set presented in the form of dendrogram will contain errors (Applied Maths NV 2008). The uncertainty of the branching can be estimated with various statistical methods, and in this study this was done with cophenetic correlation. The correlation showed that the innermost branches were very reliable, since the value was always 100%. The outer branches had lower value and

could have arranged differently if the clustering analysis had been performed many times.

Unweighted pair-group method with arithmetic averages (UPGMA) results in just one dendrogram but there are also other equally good alternatives. This is the case especially with data sets that contain identical similarity values. Identical values are more frequent with categorical and binary datasets, like one used in this study, than with quantitative measurements registered as decimal numbers (Applied Maths NV 2008). The cluster analysis and the resulting dendrogram cannot thereby be regarded as a sole possibility for branching pattern.

7 Conclusions

In this study we showed that the PCR-DGGE method is an efficient tool to analyse changes in bacterial populations in raw milk and that cold (4ºC) storage has an evident effect in the population composition. High similarity values between day 0 and day 4 in organic samples O4 - O6 means that there was not much change in bacterial community structure after the raw milk has reached a certain age. In this study the community did not change much after the raw milk was 4 days old.

As hypothesized, antimicrobial resistance was more prevailing in conventional than, in organic raw milk. The proportion of resistant bacteria decreased during the cold storage. Only in the trimethoprim-sulfamethoxazole treatment the proportion of resistant psychrotrophic bacteria increased.

In the future statistical tests will be performed for the results of the current study. More samples will be handled and included in the statistical tests to reach statistically more significant results. Also more repetitions will be made from one single sample. The testing for the multiresistance of single isolates, which were picked up from the plates, will be performed. Further optimization for the PCR-DGGE method will be done. Elimination of the double bands from DGGE gels and standardization of the denaturing gel preparation steps is needed to reach good reproducibility and to enable reliable comparisons between gels. Sequencing of DNA from the bands, which were cut out from both DGGE gels, will be performed.

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