ISSN 0355-1180

UNIVERSITY OF HELSINKI

Department of Food and Environmental Sciences

EKT Series 1494

CHARACTERISATION OF ANTIBIOTIC-RESISTANT PSYCHROTROPHIC BACTERIA IN RAW MILK

Bhawani Shankar Chamlagain

Helsinki 2010

HELSINGIN YLIOPISTO — HELSINGFORS UNIVERSITET — UNIVERSITY OF HELSINKI

Tiedekunta/Osasto — Fakultet/Sektion — Faculty	Laitos — Institution — Department
Faculty of Agriculture and Forestry	Department of Food and Environmental Sciences

Tekijä — Författare — Author
Bhawani Shankar Chamlagain

Työn nimi — Arbetets titel — Title
Characterisation of antibiotic-resistant psychrotrophic bacteria in raw milk

Oppiaine —Läroämne — Subject
Food Sciences (Food Safety)

Työn laji — Arbetets art — Level	Aika — Datum — Month and year	Sivumäärä — Sidoantal — Number of pages
M. Sc. Thesis	December 2010	54

Tiivistelmä — Referat — Abstract

Dynamics of raw milk associated bacteria during cold storage of raw milk and their antibiotic resistance was reviewed, with focus on psychrotrophic bacteria. This study aimed to investigate the significance of cold storage of raw milk on antibiotic-resistant bacterial population and analyse the antibiotic resistance of the Gram-negative antibiotic-resistant psychrotrophic bacteria isolated from the cold-stored raw milk samples.

Twenty-four raw milk samples, six at a time, were obtained from lorries that collected milk from Finnish farms and were stored at 4°C/4 d, 6°C/3 d and 6°C/4 d. Antibiotics representing four classes of antibiotics (gentamicin, ceftazidime, levofloxacin and trimethoprim-sulfamethoxazole) were used to determine the antibiotic resistance of mesophilic and psychrotrophic bacteria during the storage period. A representative number of antibiotic-resistant Gram-negative isolates retrieved from the cold-stored raw milk samples were identified by the phenotypic API 20 NE system and a few isolates by the 16S rDNA gene sequencing. Some of the isolates were further evaluated for their antibiotic resistance by the ATB PSE 5 and HiComb system.

The initial average mesophilic counts were found below 10^5 CFU/mL, suggesting that the raw milk samples were of good quality. However, the mesophilic and psychrotrophic population increased when stored at 4°C/4 d, 6°C/3 d and 6°C/4 d. Gentamicin- and levofloxacin-resistant bacteria increased moderately ($P < 0.05$) while there was a considerable rise ($P < 0.05$) of ceftazidime- and trimethoprim-sulfamethoxazole-resistant population during the cold storage. Of the 50.9 % (28) of resistant isolates (total 55) identified by API 20 NE, the majority were *Sphingomonas paucimobilis* (8), *Pseudomonas putida* (5), *Sphingobacterium spiritivorum* (3) and *Acinetobacter baumanii* (2). The analysis by ATB PSE 5 system suggested that 57.1% of the isolates (total 49) were multiresistant. This study showed that the dairy environment harbours multidrug-resistant Gram-negative psychrotrophic bacteria and the cold chain of raw milk storage amplifies the antibiotic-resistant psychrotrophic bacterial population.

Avainsanat — Nyckelord — Keywords
Raw milk, psychrotrophic bacteria, antibiotic resistance, multiresistant

Säilytyspaikka — Förvaringsställe — Where deposited
Viikki Campus Library

Muita tietoja — Övriga uppgifter — Further information
EKT Series 1494. Public 14.12.2011 Funding: University of Helsinki

PREFACE

This study was carried out at the Department of Food and Environmental Sciences, division of Dairy Technology, Faculty of Agriculture and Forestry, of the University of Helsinki. I am highly indebted to University of Helsinki for providing a wonderful opportunity to study my Master degree in Food Sciences and awarding me an International student grant.

My sincere gratitude goes to my supervisor, Patricia Munsch-Alatossava, PhD, for her untiring guidance during the work and creating such an enjoyable working environment throughout the work. I would also like to thank Professor Tapani Alatossava for arranging the working possibility and for providing the financial grant for the study period. I thank both of you for your valuable comments and suggestions during the writing process.

My sincere thank also goes to Professor Per Saris for his role as responsible Professor for my thesis. His suggestions and guidance during the study were valuable.

I would like to thank my friend Qiao Shi for her help during the work and thanks also goes to my classmates Flora Agalga, Göker Gurbuz, Marta Nogueroles Moya and Xiaoyan Liu for creating such a wonderful friendship. Many thanks to Kevin Deegan for being such a helpful coordinator of the MScFood program.

Finally, I am highly indebted to my family members for their continuous motivation and inspiration during my stay in Finland.

Helsinki, Finland

Bhawani Shankar Chamlagain

TABLE OF CONTENTS

1 INTRODUCTION

Generally low temperature storage (at 4-6°C) is the main method of preserving raw milk in farms and processing plants. However, this kind of storage practice is not enough to control the growth of psychrotrophic bacteria which are able to grow well below 7°C. It is well recognized that the growth of many Gram-negative psychrotrophic bacteria (such as *Pseudomonas*, *Acinetobacter* or *Aeromonas*) cause considerable defects in the product quality such as flavor defects, emulsion degradation and gelation in the products by their heat resistant extracellular enzymes lipases and/or proteases (Zall 1990; Barbano et al. 2006; Hantsis-Zacharov and Halpern 2007). In addition, a few studies reported that a significant proportion of these problematic psychrotrophic bacteria showed extensive resistance to many functional classes of antibiotics and the level of multiple antibiotic resistant traits increased along the cold chain of raw milk storage and transportation (Straley et al. 2006; Munsch-Alatossava and Alatossava 2007).

Since last few decades, antibiotic resistance in bacteria has been increasingly recognized as a worldwide clinical and public health problem (Levy 2002). The increased use of antibiotics, both in human or animal medicine, is considered as a prime factor in spreading the antibiotic resistance problem (Aarestrup 1999; Hawkey and Jones 2009). However, the contribution of agricultural use of antibiotics on the emergence of antibiotic-resistant bacteria cannot be underestimated; especially the increased antibiotic resistance in enteric bacteria is concerned (Levy and Marshall 2004). Silbergeld et al. (2008) in a review claimed that the use of antimicrobial drugs in agriculture is the major driving force in spreading the antimicrobial resistance worldwide citing four evidences: the agriculture is the largest user of antimicrobials worldwide, the antimicrobial drugs in agriculture are used mostly in subtherapeutic levels, every clinical class of drugs has been employed in agriculture, and the antimicrobial-resistant pathogens are exposed to human via animal food products and are also disseminated to the environment.

In the growing concern of antibiotic resistance in food-related bacteria, many studies were undertaken in recent years to assess the antibiotic resistance of bacteria in agricultural products such as raw milk (Citak et al. 2005; Straley et al. 2006; Munsch-Alatossava and Alatossava 2007), cheese (Valenzuela et al. 2009), raw vegetables (Boehme et al. 2004), ground meat products (White et al. 2001), poultry and their products (Sackey et al. 2001; Ahmed et al. 2009) and so on. These studies reported that a significant proportion of

isolates recovered from the food products demonstrated extensive resistance to antibiotics. The resistant bacteria through food products can transfer the resistance genes to the intestinal flora of humans and the commensal flora can be a reservoir of resistance genes for pathogenic bacteria (van den Bogaard 2000; Aarestrup et al. 2008). The antibiotic resistance in raw milk associated bacteria is of significant importance as raw milk is still consumed directly in many farm families, their employees and nearby families in many parts of the world, including the United States (Shiferaw et al. 2000; Oliver et al. 2009). Although pasteurization of raw milk is enough to kill the pathogens and many contaminants, however, the risk from the recontamination of pasteurized milk by contaminating bacteria (including the psychrotrophic bacteria) cannot be ignored (Eneroth et al. 1998).

This study aimed to assess the significance of cold storage of raw milk on the antibiotic-resistant bacterial population, analyse the antibiotic resistance of resistant psychrotrophic Gram-negative isolates recovered from the cold-stored raw milk samples and identify these isolates by the phenotypic API 20 NE system and a few isolates by the genetic 16S rDNA gene sequencing.

In the first part of the thesis, the scientific literature on raw milk psychrotrophic bacteria and the antibiotic resistance in food-associated bacteria, particularly milk-associated bacteria, is reviewed. In the second part of the thesis, the methods of determining antibiotic-resistant bacteria in raw milk during the cold storage period and analysing the antibiotic resistance of isolates recovered from the milk samples are explained. In the final sections, the results of the effect of cold storage of raw milk on the antibiotic-resistant bacterial population are discussed, including the antibiotic resistance analysis by the ATB PSE 5 system.

2 LITERATURE REVIEW

2.1 Bacteria in raw milk

Cow milk is an excellent growth medium for microorganisms since it contains most of the nutrients such as carbohydrates, proteins, fats, vitamins and minerals required for the growth of microorganisms, combined with higher water activity. Microorganisms enter the raw milk in a variety of ways such as from the environment (water, soil, vegetation, bedding materials), the udder and the milking and storage environments. Thus the initial microbial population in raw milk is highly variable and largely influenced by the type and level of contamination during milking. For example, bedding material, untreated water, soil and vegetation have been reported as the major sources of psychrotrophs; soil for Coliforms and the bedding material for spore formers. Similarly, mastitis pathogens like *Staphylococcus* spp., *E. coli* contaminate the milk within the udder due to its infection by these pathogens. In addition, milking system, including milking machines, pipelines, bulk tanks and bulk tankers could be potential sources of contamination of the milk (Bramley and McKinnon 1990; Hayes and Boor 2001).

A diverse group of bacteria can be found in raw milk, both Gram-positives and Gram-negatives. Gram-positive genera that reported occurring in raw milk include *Micrococcus*, *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Lactococcus*, *Bacillus*, *Clostridium*, *Lactobacillus*, *Listeria*, *Corynebacterium*, *Arthrobacter* or *Brevibacterium*. Similarly, Gram-negative genera that can be detected in raw milk are *Campylobacter*, *Pseudomonas*, *Stenotrophomonas*, *Acinetobacter*, *Alteromonas*, *Flavobacterium*, *Alcaligenes*, *Brucella*, *Escherichia*, *Citrobacter*, *Salmonella*, *Enterobacter*, *Yersinia* or *Aeromonas* (Gilmour and Rowe 1990). Raw milk could be a potential source of human pathogens such as *Campylobacter jejuni*, Shiga-toxin-producing *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp., enterotoxigenic *Staphylococcus aureus*, *Yersinia enterocolitica*, *Mycobacterium bovis*, *Brucella* spp. or *Coxiella burnetti* (Jayarao and Henning 2001; Oliver et al. 2009).

2.2 Psychrotrophic bacteria during raw milk storage and transportation

The cooling preservation of raw milk has enabled large scale collection, storage and processing of raw milk days after milking. Despite the variable initial bacterial population in raw milk, the refrigerated storage of milk after milking has a significant effect on the type and number of bacteria in the cold-stored raw milk. Although refrigeration can largely reduce the growth of mesophilic bacteria, however, it has consistently shown that the cold storage supports the growth of psychrotrophic bacteria, a group of bacteria that can grow at 7°C or less and become the main cause of spoilage of raw milk at low temperature storage (Bramley and McKinnon 1990; Hayes and Boor 2001; Kumaresan et al. 2007).

Particularly, the temperature of refrigerated storage has a significant effect in the storage quality of raw milk as is the initial amount of psychrotrophic bacteria: microbiologically good quality milk can be stored for 48 h at 4°C avoiding any significant change in its quality (Guinot-Thomas et al. 1995). Lafarge et al. (2004) reported that the increase in psychrotrophic bacteria occurred within 24 hours of cold storage and noted a significant variation in bacterial population among raw milk samples. Rasolofo et al. (2010) found that the psychrotrophic count remained stable up to 3 days storage at 4°C while it increased considerably when continued to store up to 7 days. However, these researchers observed that the psychrotrophic bacteria rose to a significant level when the milk was stored at 8°C within 3 days. In another study in India, Kumaresan et al. (2007) reported that reducing the storage temperature from 7°C to 2°C decreased the growth of psychrotrophic bacteria significantly and recommended to store the raw milk at 2°C before the milk is processed.

Psychrotrophs, both Gram-positives and Gram-negatives, have been reported in the refrigerated milk; the majority of them belong to the genera *Pseudomonas*, *Aeromonas*, *Acinetobacter*, *Alcaligenes*, *Achromobacter*, *Enterobacter*, *Flavobacterium*, *Klebsiella*, *Bacillus*, *Clostridium*, *Microbacterium*, *Micrococcus* and *Corynebacterium*, including coliforms such as *Escherichia*, *Enterobacter*, *Citrobacter* and *Klebsiella* (Zall 1990; Hayes and Boor 2001). However, Gram-negative *Pseudomonas* is the predominant psychrotrophic bacteria identified in raw milk (Bramley and McKinnon 1990; Uraz and Çitak 1998; Munsch-Alatossava and Alatossava 2006; Hantsis-Zacharov and Halpern 2007; Ercolini et al. 2009; Rasolofo et al. 2010).

2.3 Characteristics of raw milk psychrotrophs

Many Gram-negative psychrotrophs in raw milk stored at refrigerated temperature have shown to produce extracellular heat-stable proteolytic and lipolytic enzymes. Although the Gram-negative psychrotrophs are killed during pasteurization, these heat-resistant enzymes are not inactivated at normal pasteurization and ultra-high temperature treatment (UHT) of raw milk and these enzymes after the heat treatment degrade the milk and milk products (Bramley and McKinnon 1990; Hayes and Boor 2001).

Psychrotrophs expressing both proteolytic and lipolytic characteristics are *Pseudomonas* spp., *Achromobacter* spp., *Acinetobacter* spp., *Serratia marscescens* while some are only proteolytic, for example, *Flavobacterium* spp. (Jayarao and Wang 1999; Hayes and Boor 2001; Hantsis-Zacharov and Halpern 2007). However, *Pseudomonas* spp. account about a half of the total flora among the Gram-negative psychrotrophic bacteria in raw milk (Bramley and McKinnon 1990; Hayes and Boor 2001; Johnson 2001).

Proteases cause bitterness through protein hydrolysis and lipases impart rancid flavor due to milk fat hydrolysis. The psychrotrophs and their proteolytic and lipolytic enzymes in raw milk are considered primarily responsible for the spoilage of refrigerated milk and milk products (Zall 1990; Barbano et al. 2006; Hantsis-Zacharov and Halpern 2007). Particularly, Wang and Jayarao (2001) observed that proteinases rather than lipases were primarily responsible for the spoilage of raw milk by *Pseudomonas fluorescens* at the refrigerated temperatures. Wiedmann et al. (2000) reported that 58% of 66 *Pseudomonas* isolates tested demonstrated protease and lipase activity.

2.4 Antibiotic resistance in bacteria

2.4.1 Antibiotics

Antibiotics are defined as a group of natural microbial products or synthetic chemical compounds that inhibit the growth of and even kill bacteria. Natural antibiotics are produced by both bacteria or fungi and act by blocking some essential cell processes in other bacteria. Man-made antibiotics also target some of the vital cell mechanisms in

bacteria. In addition, another category of antibiotics, i.e. semi-synthetic antibiotics, are the chemically modified form of the natural antibiotics to enhance the effectiveness of natural antibiotics. The first natural antibiotic isolated in pure form was penicillin and it came into clinical practice in the 1940s. Antibiotics are grouped into different classes (Table 1) based on their chemical structures: such as β-lactams, aminoglycosides, macrolides, ketolides, tetracyclines, glycopeptides and others, and based on their mode of actions: such as inhibitors of bacterial cell wall synthesis, inhibitors of protein synthesis, disruptors of cell membranes and inhibitors of nucleic acid synthesis (Walsh 2003).

Table 1. Major antibiotics grouped according to their mechanisms of action and their chemical structures (Levy and Marshall 2004).

Mechanism of action	Antibiotic families
Inhibition of cell wall synthesis	Penicillins; cephalosporins; carbapenems; daptomycin; monobactams; glycopeptides
Inhibition of protein synthesis	Tetracyclines; aminoglyocides; oxazolidonones; streptogramins; ketolides; macrolides; lincosamides
Inhibition of DNA synthesis	Fluoroquinolones
Competitive inhibition of folic acid synthesis	Sulfonamides; trimethoprim
Inhibition of RNA synthesis	Rifampin/ansamycins
Other	Metronidazole

Beta-lactams (penicillins, cephalosporins, cephamycins, monobactams, and carbapenems), glycopeptides and other antibiotics inhibit the biosynthesis of peptidoglycan, a rigid polymer in cell wall that resists a high osmotic pressure inside the cell. In contrast, aminoglycosides and tetracyclines complexes with the conserved sequences of the 16S rRNA of the 30S ribosomal subunit while macrolides, ketolides, lincosamides and chloramphenicol bind to the sequences of the 23S rRNA of the 50S ribosomal unit thus affecting the protein biosynthesis (Mascaretti 2003). Antibiotics like fluroquinolones act on deoxyribonucleic acid (DNA) gyrase and topoisomerase IV and prevent synthesis of DNA and ribonucleic acid (RNA). On the other hand, sulfonamides (for example sulfamethoxazole) and trimethoprim interferes in the folic acid metabolism in bacteria that indirectly affects the nucleic acid synthesis. Sulfamethoxazole blocks the enzyme dihydropteroate synthase in folic acid synthesis while trimethoprim inhibits the enzyme dihydrofolate reductase that supplies the pyridime thymidylate for the DNA biosynthesis (Walsh 2003).

2.4.2 Antibiotic resistance

When antibiotics were first introduced in clinical practices in the 1940s, they were virtually effective against pathogenic bacteria. However, the effectiveness of the antibiotics was greatly reduced due to the emergence of the antibiotic-resistant bacteria sooner or later after their introduction (Walsh 2003; Aminov 2009). Several new antibiotics, targeting essential physiological or metabolic functions of the bacterial cell, were introduced in the last several decades but bacteria responded each time by evolving themselves as resistant strains (Barbosa and Levy 2000; Levy and Marshall 2004).

Several factors have been implicated for the emergence of the antibiotic-resistant bacteria and their spreading in the environment. In general, there is a consensus that the increased use of antibiotics is the prime factor for the increased resistance in pathogenic bacteria (Levy and Marshall 2004). Not only the antibiotic use in human medicine has been linked to the elevated antibiotic resistance in bacteria, the use of antibiotics in veterinary medicine, agriculture, aquaculture, horticulture and other human activities have been identified as the other driving forces in escalating the problem (Aarestrup 1999; Barbosa and Levy 2000; Aminov 2009).

The antibiotics are used for food animal production in one of the following situations: treatment of infectious diseases, metaphylactics, prophylactis, and growth promotion (Aarestrup 2005). Quantity as much as half of the total antibiotics produced worldwide is used for food animal production, primarily as growth promoter and prophylactic agents (Aarestrup 1999). Although the use of antibiotics as growth promotion has been banned in the European Union (EU), however, it is being continuously used in other parts of the world (Levy and Marshall 2004). This selective pressure on the environment is not insignificant for the development of antibiotic resistance in enteric bacteria in animals, considering the fact that animals frequently harbor human pathogens in their intestinal tract such as *Salmonella*, *Campylobacter*, *Yersinia*, *Listeria* and enterohaemorrhagic *E. coli* (Aarestrup 1999; Levy and Marshall 2004).

Silbergeld et al. (2008) in a review claimed that the antimicrobial drugs use in agriculture is the major driving force in increasing the antimicrobial resistance in bacteria worldwide citing four evidences: the agriculture is the largest user of antimicrobials worldwide, the antimicrobial drugs in agriculture are used mostly in subtherapeutic levels, every clinical

class of drugs has been employed in agriculture, and the antimicrobial-resistant pathogens are exposed to human via animal food products and are also disseminated to the environment. An intervention on antibiotics use in agriculture brought a major reduction in the antibiotic resistance in food isolates. For example, the ban of antibiotics use for growth promotion in most countries during the 1990s resulted in a significant reduction in the antibiotic resistance in food animals and animal food products (Aarestrup et al. 2008).

A bacterial strain is defined as resistant strain to an antibiotic when the strain is able to grow at the lowest concentration of the antibiotic that usually inhibits the growth of the wild strain belonging to the same species. This lowest concentration of the antibiotic that effectively prevents the growth of a particular strain is called minimum inhibitory concentration (MIC). The antibiotic resistance may be either intrinsic or acquired resistance. The intrinsic resistance is caused by structural or functional characteristics inherited to a bacterial group (that includes species, genus or even to a higher level) such as the low affinity of the antibiotics to the target, the inability of the antibiotics to enter the bacterial cell, removal of the antibiotics from the cell by chromosomally encoded active exporters, inactivation of antibiotics by innate enzymes and other mechanisms (Guardabassi and Courvalin 2006).

Unlike intrinsic resistance, the acquired resistance is inherited to only some strains of a particular genus or species. However, the acquired resistance possesses a serious problem as it causes the emergence and spread of resistance to the usually susceptible bacteria. This mobile nature of acquired resistance can easily disseminate among bacteria of morphologically and ecologically distinct groups (horizontal transfer) through mobile genetic elements such as bacteriophages, plasmids, naked DNA or transposons. In addition, mutation on genetic elements can also enable bacteria to acquire the resistance to a particular antibiotic (Levy and Marshall 2004). The process of acquisition of resistance genes can be through transduction in which bacteriophages mediates the transfer of DNA having resistant determinants into the host cell or the transformation which involves the uptake of plasmids or naked DNA from the environment. Another important process of acquiring resistance is the conjugation that is the transfer of plasmids or chromosomal DNA by cell-to-cell contact. The conjugation has been regarded as by far the most important mechanism in spreading the antibiotic resistance, even between distinct taxonomic and ecological groups of bacteria (Barbosa and Levy 2000). The horizontally transferable plasmids that contain some specific genetic structures such as complex

transposons or integrons enhance the distribution of resistance genes between bacteria (Walsh and Fanning 2008).

2.4.3 Mechanisms of bacterial antibiotic resistance

Several mechanisms have been proposed by which bacteria evolved to overcome the detrimental effects of antibiotics in the surrounding environment. The bacteria may restrict the entry of antibiotics by modifying the cell membrane composition, by reducing the uptake or exporting the antibiotics from the cell by active-efflux. In addition, alteration of the antibiotics by cellular enzymes may be another way of getting resistance to antibiotics. The bacterial cell may also respond by altering the affinity of the antibiotics to the target or by overexpression of the target (Mascaretti 2003).

Inactivation of antibiotics by enzymes is the main mechanism of resistance to β-lactams, aminoglycosides, and phenicols. β-lactamases are the most important drug-inactivating enzymes that hydrolyze the β-lactam ring of penicillins, cephalosporins and carbapenems. On the other hand, the aminoglycoside-altering enzymes interfere catalyze the transfer reaction that restricts the binding of the antibiotic to ribosomes. However, enzymatic destruction of antibiotics has not been observed in the synthetic class of antibiotics: the sulfamethoxazole-trimethoprim, the fluroquinolones or the oxazolidines (Walsh 2003).

Specific-drug-resistance (SDR) efflux pumps are the primary mechanism of resistance against tetracyclines, other multiple-drug-resistance (MDR) pumps play important roles for the multiple resistance against antimicrobials such as β-lactams, macrolides and fluoroquinolones (Walsh 2003; Guardabassi and Courvalin 2006). Multidrug resistance can be acquired by bacteria through accumulation of genes in R plasmids or transposons that encode resistance to specific antibiotics and/or by the MDR pumps that can export more than single drug type (Nikaido 2009). This R plasmid with multidrug resistance genes may be transferred to another bacterial species quickly either by conjugation, transformation or transduction and hence the new species can be resistant to several antibiotics. In this way, the pathogenic bacteria can become multidrug resistance (Mascaretti 2003).

2.4.4 Antibiotic resistance in food-related bacteria

The role of the food chain as a source of antimicrobial-resistant pathogens, including *Salmonella* and *Campylobacter*, is of significant consequences to the public health (Mølbak 2004). The use of antibiotics in food animals production, such as for growth promotion and prophylactic reason, is of significant importance for the increased resistance in zoonotic bacteria such as *Salmonella, Campylobacter, Listeria,* enterohaemorrhagic *E. coli* (Aarestrup 1999; Sørum and L'Abée-Lund 2002; Levy and Marshall 2004). The antibiotics used in animal husbandry are also of great significance as many of these antibiotics are used in the human medicine and the resultant antibiotic-resistant bacteria can spread the resistance phenomenon worldwide through the global trade of animal foods (Aarestrup et al. 2008). Similarly, commensal bacteria, that interact with zoonotic bacteria through the food chain, are also of increased concern as these bacteria might act as reservoirs of antibiotic resistance genes that can be transferrable to pathogenic bacteria of humans (van den Bogaard et al. 2000; Wang et al. 2006; Hawkey and Jones 2009). The agricultural practices provide a significant impact on the dissemination of antibiotic resistance elements in the environment, including the use of antibiotics in the fruits and vegetables production. The application of antibiotics in animal husbandry and the subsequent isolation of the antibiotic-resistant bacteria in the production chain suggests, in many cases, a direct relationship while it may not be the case when the reduced use of antibiotics in organic farming brings the reduction of the antibiotic resistance on the pathogenic bacteria (Wright 2010).

In the growing concern of antibiotic resistance in food-related bacteria, more and more studies were undertaken in recent years to assess the antibiotic resistance of bacteria isolated from food products such as raw milk (Citak et al. 2005; Straley et al. 2006; Munsch-Alatossava and Alatossava 2007), cheese (Valenzuela et al. 2009), raw vegetables (Boehme et al. 2004), ground meat products (White et al. 2001) and poultry (Sackey et al. 2001). The majority of these studies were focused on assessing the prevalence of antibiotic resistance in enteric bacteria; however few of them also considered non-enteric bacteria.

In a study on the prevalence of antibiotic-resistant bacteria in vegetables and seed sprouts, Boehme et al. (2004) reported that the antibiotic-resistant bacteria as high as 10^8 CFU/g were observed in seed sprouts and many of these isolates were multidrug-resistant. Compared with seed sprouts the common vegetables were less contaminated with

antibiotic-resistant bacteria. Like Sackey et al. (2001) reporting on the prevalence of multiresistant enteropathogenic bacteria in poultry in Ghana, a widespread multidrug resistance has been reported in *E. coli* and *Salmonella* isolated from retail raw chicken products in Japan; 40.6% of 69 *E. coli* isolates and all 10 *Salmonella* isolates studied were multidrug-resistant (Ahmed et al. 2009). These isolates showed multidrug resistance against antibiotics such as ampicillin, streptomycin, spectinomycin, kanamycin, tetracycline, trimethoprim-sulfamethoxazole, nalidixic acid, cefoperazone, cephalothin, cefoxitin, and ciprofloxacin. The authors concluded that the retail chicken meat could be of significant importance in spreading the multidrug-resistant bacteria.

2.4.5 Antibiotic resistance of raw milk-associated bacteria

The raw milk-associated bacteria have shown various levels of resistance against many antibiotics of clinical significance. Although many studies focused on pathogens in raw milk of public health significance, however, few studies also considered antibiotic resistance on raw milk associated other bacteria. Bulk tank milk (BTM) was used to monitor the antimicrobial resistance in dairy farms and suggested that the monitoring of the antimicrobial resistance of *E. coli* in bulk milk can reasonably provide the overview of antimicrobials use in the farm (Berge et al. 2007). The prevalence of multidrug resistant *E. coli* and *Salmonella* in BTM was reported being 23%; the frequency of isolating *E. coli* was more frequent than *Salmonella*. Citak et al. (2005) studied the antibiotic resistance of enterococci isolated from raw milk and reported that the predominant enterococci were *E. faecalis* (54.2%) and *E. faecium* (29.0%). These isolates demonstrated extensive resistance to antibiotics such as ampicillin, ciprofloxacin, erythromycin, gentamicin, imipenem, streptomycin, tetracycline and vancomycin. Similarly, enterococci isolated from the raw cow's milk and cheeses derived from such milk showed resistance against many antibiotics such as ciprofloxacin, levofloxacin, erythromycin, tetracycline but all were sensitive to ampicillin, chloramphenicol and gentamicin (Valenzuela et al. 2009).

The prevalence of Gram-negative bacteria in bulk tank milk was studied by Straley et al. (2006). Among 54 bulk tank milk samples (obtained from six farms for a period of nine months) analysed, 46 (85%) of the samples were positive for Gram-negative bacteria. The amount of these bacteria in bulk tank milk varied widely among farms (12-1310 CFU/mL) and *Pseudomonas* spp. were the dominant non-coliform Gram-negative bacteria that

showed extensive resistance to antibiotics considered in the study. The antibiotic-resistant *Pseudomonas* spp. are of significant importance in dairy industries because they grow at low temperature and have the potential of forming biofilms in the bulk tank. In addition, other Gram-negative bacteria detected in this study were belonging to the genera *Acinetobacter*, *Citrobacter*, *Enterobacter,* and to *E. coli*. These bacteria were resistant to first, second and third generation of cephalosporins but were susceptible to fourth generation of cephalosporins, fluoroquinolones (enroflaxacin) and aminoglycosides (gentamicin). This study suggested that bulk tank milk could be a major source of antimicrobial-resistant Gram-negative bacteria (Straley et al. 2006).

A more detailed study of the antibiotic resistance of psychrotrophic bacteria during cold storage and transportation of raw milk was carried out by Munsch-Alatossava and Alatossava (2007). The study reported that a significant proportion of psychrotrophic bacteria spoiling raw milk in Finland exhibited multidrug resistance and the multiresistant traits increased along the cold chain of raw milk. Among the 60 isolates (recovered from farms, trucks or silos) analysed by an in vitro antibiotic resistance/susceptibility testing system (the ATB® PSE strips), the proportion of resistant isolates to at least five antibiotics were half and 60% of the isolates were multiresistant (resistant to more than two classes of antibiotics). The frequency of multidrug-resistant psychrotrophic bacteria was rising along the cold chain of raw milk storage and transportation (i.e. from farms to trucks and from trucks to silos). The isolates were mostly resistant to β-lactams but the majority was susceptibile to non-β-lactams such as aminoglycosides. The most of the isolates showed resistance to ticarcillin and ticarcillin-clavulonic acid while the proportion of isolates resistant to aztreonam of the β-lactams was rising from farm to truck and from truck to silo raw milk samples. Higher percentages of isolates were susceptible to piperacillin, piperacillin-tazobactam and ceftazidime. On the other hand, the proportion of colistin- and cotrimoxazol-resistant isolates was increased along the cold chain of raw milk; from 53.8% to 70.6% (colistin) and from 42.3% to 82.3% (cotrimoxazol) respectively for the farm isolates and the silo isolates.

Many of these isolates were assigned to be *Pseudomonas fluorescens*, *P. aeruginosa*, *Stenotrophomonas maltophilia*, *Acinetobacter* spp., and *Burkholderia* spp. Most of the isolates identified as *P. fluorescens* showed resistance to the majority of the β-lactams and to the folate pathway inhibitor antibiotics (cotrimoxazole); however, they were mostly susceptible to aminoglycosides. The *P. aeruginosa* isolates were susceptible to all of the

antibiotics of the ATB® PSE strip except cotrimoxazole. On the other hand, *S. maltophilia* exhibited extensive resistance whereas *Burkholderia* spp. were mostly susceptible to aminoglycosides and ciprofloxacin (Munsch-Alatossava and Alatossava 2007).

2.5 Identification of bacteria from raw milk

Both phenotypic and genotypic methods have been employed in the identification of microbial communities in raw milk. The morphological, physiological and biochemical features of a bacterial cell are utilized to identify a bacterial isolate in the phenotypic system, such as API and BIOLOG (Vandamme et al. 1996). API 20 NE system was used in some studies to monitor the non-fermenting Gram-negative rods in raw milk (Holm et al. 2004; Munsch-Alatossava and Alatossava 2006). Despite limitations, direct sequencing of entire or parts of the bacterial 16S rRNA has become a powerful molecular phylogenic technique that can be applied to identify a strain to the species level using the polymerase chain reaction (PCR) and a set of appropriate primers (Vandamme et al. 1996). This technique is of particular importance as it allows the identification of both culturable and unculturable bacteria (Hanage et al. 2006).

Among genetic methods, both culture-dependent and –independent techniques are utilized in the identification of bacterial communities in raw milk and milk products such as in cheese (Delbès et al. 2007). The traditional culture-dependent methods are usually time consuming and provide an incomplete picture of the bacterial composition. Culture-independent methods such as denaturing gradient gel electrophoresis (DGGE), temporal temperature gradient electrophoresis (TTGE), single strand conformation polymorphism (SSCP) that utilize molecular fingerprinting techniques have been employed to study the bacterial diversity in raw milk (Ogier et al. 2002; Lafarge et al. 2004; Verdier-Metz et al. 2009).

In recent years, the 16S rRNA gene sequencing has been increasingly applied at identifying both mesophilic and psychrotrophic bacteria in raw milk (Delbès et al. 2007; Ercolini et al. 2009). In addition, a few studies combined the culture-dependent method and the 16S rRNA sequencing to assess the microbial dynamics in raw milk (Hantsis-Zacharov and Halpern 2007; Rasolofo et al. 2010). Similarly, other genotypic methods such as ribotyping (Wiedmann et al. 2000) and DNA-microarray (Giannino et al. 2009)

have been employed for the characterisation of *Pseudomonas* spp. and other microbial communities in raw milk.

Wiedmann (2000) reported a good identification of *Pseudomonas* isolates to the species level by API 20 NE system and noted that BIOLOG system did not provide reliable identification to the species level. Wang and Jayarao (2001) characterised *Pseudomonas fluorescens* isolated from bulk tank milk both by phenotypic (API 20 NE) and genotypic (16S-23S PCR ribotyping) methods and reported that API 20 NE system can satisfactorily differentiate different strains. In another study by Munsch-Alatossava and Alatossava (2006), both API 20 NE and BIOLOG systems enabled only partial identification of the psychrotrophic bacteria spoiling the raw milk in Finland and suggested to complement these identification systems with genotypic techniques.

2.6 Analysis of antibiotic resistance

The susceptibility of bacteria to antibiotics can be analysed by phenotypic and genotypic techniques. The phenotypic methods are based on the ability of an antibiotic to inhibit the growth of bacteria under specified growth conditions. Some of the phenotypic methods of antimicrobial susceptibility testing are agar dilution, broth dilution and disk diffusion and E-test (Schwarz et al. 2010). Genotypic methods such as PCR based detection of resistance genes and restriction fragment length polymorphism analysis is increasingly used for evaluating the antibiotic resistance in bacteria (Walsh 2003).

Broth dilution is one of the earliest antimicrobial susceptibility testing methods that involve testing of an antibiotic at different concentrations in tubes with a standardised bacterial suspension ($1\text{-}5\times10^5$ CFU/mL) after overnight incubation. The lowest concentration of an antibiotic that prevents the growth of the bacterial strain is the minimum inhibitory concentration (MIC). Although this method provides the MIC value, the method is tedious and is of manual nature so that there is possibility of errors. However, a modification of the tube method is achieved like in the broth microdilution method that uses disposable trays that contain varying concentration of antibiotics to be tested in the wells of the trays (Jorgensen and Ferraro 2009).

On the other hand, the antimicrobial diffusion methods based on discs containing antibiotics or plastic strips impregnated with antibiotics (E-test) are widely used in vitro antibiotic susceptibility testing. The size of the diameter of the zone of inhibition when an antibiotic disc is placed on the surface of inoculated (test organism) Mueller-Hinton agar plate and incubated at optimum temperature for 16-24 h classifies the test bacterium as susceptible, intermediate or resistant based on the criteria set by the Clinical and Laboratory Standards Institute or CLSI (Jorgensen and Ferraro 2009). The E-test strips contain antibiotics in a continuous gradient, which when placed on the culture smeared surface of a Mueller-Hinton agar plate and incubated gives the MIC value of the antibiotic for the test culture. The MIC value is the corresponding concentration in the strip at the intersection point of the lowest part of the elliptical zone of inhibition and the test strip. The E-test provides the susceptibility in terms of MIC value rather than the category results and has been reported as more reliable as other antimicrobial susceptibility testing methods (Baker et al. 1991). A similar method to E-test based on the gradient diffusion principle, the HiComb MIC test, is available for the determination of MIC value for microorganisms against a particular antibiotic and has been used for the antibiotic susceptibility testing of clinical isolates based on MICs criteria (Menezes et al. 2008).

Broth microdilution method was used on the study of antimicrobial susceptibility of clinical non-enteric Gram-negative bacilli collected worldwide during 1997 to 2003 using the breakpoints established by CLSI (Sader and Jones 2005). Similarly, the microdilution based method was employed for the analysis of antimicrobial resistance of the Gram-negative bacteria in raw milk (Straley et al. 2006) and enterobacteria in agricultural food products (Boehme et al. 2004). On the other hand, agar diffusion method using discs containing antibiotics were extensively used to determine the antimicrobial resistance of the bacteria isolated from different food products, including raw milk (Citak et al. 2005; Ahmed et al. 2009; Koluman et al. 2009; Nam et al. 2010). A semi-automatic method ATB PSE 5 has been used for the in vitro antimicrobial susceptibility testing of psychrotrophic bacteria isolated from raw milk samples (Munsch-Alatossava and Alatossava 2007). This phenotypic method categorizes an isolate into susceptible, intermediate or resistant to antibiotics under consideration.

3 EXPERIMENTAL RESEARCH

3.1 Aims of the study

Apart from the widely recognized spoilage potential of raw milk during cold storage by psychrotrophic Gram-negative bacteria, few studies reported that these problematic bacteria are also resistant to antibiotics of many classes and the antibiotic resistance traits on these bacteria increased along the cold storage and transportation. The main objectives of this study were to assess the significance of the cold chain of raw milk storage on the antibiotic resistant bacterial population and analyse the antibiotic resistance of resistant Gram-negative psychrotrophic bacteria by phenotypic ATB PSE 5 system. The study also aimed to identify these antibiotic-resistant bacterial isolates by phenotypic system (API 20 NE) and a few isolates by 16S rDNA gene sequencing.

3.2 Materials and methods

3.2.1 Raw milk samples collection and storage

Altogether 24 raw milk samples were analysed in this study. The milk samples were received from milk tankers that collected milk from Finnish farms during the period of May to August and in November 2009. Six milk samples were studied at a time, thus the analysis was performed in four separate experiments. After aseptically collecting the milk in sterile sample bottles, the raw milk samples were brought to the laboratory in an insulated box. Immediately after receiving the first set of milk samples were stored at 6°C for 3 days, the second set at 6°C for 4 days and at 4°C for 4 days each for the third and the fourth experiments. The storage temperature and the time period were selected such that the normal storage condition of the raw milk storage (4-6°C) can be represented in this study. In each experiment, the total counts and the amount of antibiotic resistant bacteria, both mesophilic and psychrotrophic, were determined at the beginning and at the end of respective storage periods. In addition, an intermediate analysis was conducted in the fourth experiment-samples stored at 4°C for 4 days- after two days of storage.

3.2.2 Determination of antibiotic-resistant bacteria in milk

The antibiotic-resistant bacteria present in the raw milk samples were analysed according to the dilution method recommended by the European Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical Microbiology and Infectious Diseases (EUCAST 2000). Antibiotics representing four classes were considered; gentamicin (G) of aminoglycosides, ceftazidime (C) of β-lactams, levofloxacin (L) of quinolones and trimethoprim-sulfamethoxazole (TS) of folate synthesis inhibitors, to determine the antibiotic-resistant bacterial population in raw milk based on their ability to develop into visible colonies after incubation.

Two concentrations of antibiotics were evaluated for each sample of milk: the higher at four times the minimum inhibitory concentration (MIC) for gentamicin and ceftazidime and two times the MIC for levofloxacin and TS, and the lower concentration was the MIC for the respective antibiotics. The MICs (μg/mL) for G, C, L and TS were 4, 8, 2 and 4 (T) & 76 (S) respectively (EUCAST 2000) for the bacterial genera of concern. Antibiotics were added to the sterile Mueller-Hinton (MH) media after cooling to about 50°C in a water-bath. The media containing antibiotics was then poured into pre-labeled sterile petri dishes on a level surface and allowed to set and dry at the room temperature until the agar surface was dry. In addition, the MH agar plates without antibiotics were also prepared to determine the total bacterial population.

A 50 μL of the milk sample (after adequate dilution) was spread on the surface of the MH agar media plates containing antibiotics, including on the surface the plates without antibiotics by spread plating technique to determine the total and antibiotic-resistant counts. The first six milk samples were analysed in duplicates while the rest of the analysis was performed in quadruplicates for each milk sample. For each milk sample, one set of plates was incubated at 30°C for 2-3 d while the other set was incubated at 7°C for 10 d to determine the mesophilic and psychrotrophic population respectively. After respective incubation period, the total and antibiotic-resistant visible colonies were counted and reported as CFU/mL.

3.2.3 Selection and purification of isolates

Some distinct colonies on the antibiotic containing plates stored at 7°C for 10 day, i.e. the antibiotic-resistant psychrotrophs, were carefully isolated and were purified on plate count agar (PCA) plate by the streaking method. It was aimed to represent the isolates from all four types of antibiotic plates, considering the colony characteristics such as shape, size or colour. Several successive purifications were performed until a pure isolate was obtained. The pure isolates were subcultured in agar slant and also stored at -20°C in Luria-Bertani (LB) + 30% glycerol medium. Only Gram-negative isolates were considered for identification and antibiotic resistance study.

3.2.4 Identification of isolates

API 20 NE

The Gram-negative isolates were characterised by API 20 NE system (BioMérieux, Marcy-l'Etoile, France), according to the manufacturer's instructions. The fresh purified culture (18-24 h old) was transferred with a loop into a 5 ml of 0.85% saline solution and the suspension was adjusted to 0.5 McFarland. 200 µL of the suspension was added to the API AUX Medium and was homogenized with a pipette, preventing the bubbles generation. The saline suspension was distributed into the tubes of tests NO_3 to PNPG, avoiding bubble formation. On the other hand, tubes and cupules of the assimilation tests GLU to PAC were filled with the API AUX Medium suspension till flat or slightly convex meniscus. The cupules of the tests GLU, ADH and URE were filled with mineral oil until a concave meniscus was formed. The boxes were closed and incubated at 30°C for 24 h, after which the results were noted as per the instructions on specific tests with the help of the Reading Table provided with the kit. A further 24 hours of incubation was continued for all tests except the first three and the results were recorded in the result sheet. The profiles were analysed with the apiweb^{TM} database V7.0 (BioMérieux).

16S rDNA gene sequencing

The genomic DNA of the six Gram-negative isolates considered in this identification by 16S rDNA gene sequencing was isolated according to manufacturer's instructions

(Promega, Madison, WI, USA). A 700-bp fragment of the 16S rDNA gene including the V3 region was amplified according to the procedure described by Ogier et al. (2002) using primers W01 (5´-AGA GTT TGA TC[AC] TGG CTC-3´) and W012 (5´-TAC GCA TTT CAC C[GT]C TAC A-3´). The PCR products were analysed by 1% agarose gel electrophoresis to confirm their purity and the lengths comparing with a standard having DNA of defined fragments (Fermentas, Helsinki, Finland). The PCR products were sequenced at the Biocenter (University of Helsinki) and the sequences were submitted to GenBank for the identification.

3.2.5 Antibiotic resistance analysis

ATB PSE 5 Strips

The ATB PSE 5 strips (BioMérieux, Marcy-l'Etoile, France) were used to evaluate the antibiotic resistance/susceptibility of the isolates as per the manufacturer's instructions. The strip contained 15 antibiotics in cupules at a single or two concentrations and two pairs of cupules for control. The antibiotics were representative of five classes; β-lactams includes penicillins (ampicillin-sulbactam (FAM), ticarcillin (TIC), ticarcillin-clavulonic acid (TCC), piperacillin (PIC), piperacillin-tazobactam (TZP)), cephems (cefepime (FEP), ceftazidime (CAZ)), and carbapenems (imipenem (IMI), meropenem (MERO)). Similarly, the aminoglycosides comprise amikacin (AKN), gentamicin (GEN) and tobramycin (TOB) while ciprofloxacin (CIP) represents the quinolones class. Colistin (COL) is a polycationic peptide and the cotrimoxazol (TSU) is a folate pathway inhibitor. The concentrations of the antibiotics in the ATB PSE 5 strips (µg/mL) were: FAM, 8/4 to 16/8; TIC, 16 to 64; TCC, 16/2 to 64/2; PIC, 16 to 64; TZP, 16/4 to 64/4; FEP, 8 to 16; IMI, 4 to 8; MERO, 4 to 8; CAZ, 8 to 16; AKN, 16 to 32; GEN, 4 to 8; TOB, 4 to 8; CIP, 1 to 2; COL, 2 and TSU, 2/38.

In case of two concentrations, the isolates were considered susceptible (S) (no growth or turbidity at either concentrations), intermediate (growth and turbidity only in lower concentration) and resistant (growth and turbidity at both concentrations). On the other hand, the isolates were categorized as sensitive or resistant in case of single antibiotic concentration. An isolate resistant to more than two classes of antibiotics was categorized as a multiresistant.

The same bacterial suspension prepared in API 20 NE (0.5 McFarland) was used for ATB PSE 5 antibiotic resistance study. The suspension (10 µL) was transferred into an ampule of ATB medium and homogenized, avoiding the formation of bubbles. 135 µl of this medium was inoculated into each cupules, the strip was covered with the lid and finally incubated at 30°C for 18-24 h in aerobic conditions.

HiComb MIC test

Since ATB PSE 5 system provides the MICs of the isolates in range, the specific MIC for a particular antibiotic was determined by HiComb MIC test system (HiMedia, Mumbai, India) following manufacturer's instructions. A liquid culture of the test isolate was prepared by inoculating the isolate in Tryptone Soy Broth (5 mL) and incubating at 25-30°C for 6 h till a light to moderate turbidity was developed. A sterile cotton swab was soaked into the inoculum and the excess liquid was drained by pressing against the upper inside wall of the inoculum tube. Then the isolate laden swab was streaked uniformly on the agar surface of the Muller-Hinton plate, by turning the plate at 60 degree angle for three times. The antibiotic laden strip was placed on the surface of the agar plate after slightly drying the plate for 5-15 minutes, facing the higher concentration towards the side of the plate. The plates were then incubated at 25-30°C depending on the optimum growth temperature of the isolate for 24-48 h. After incubation, the intersection of the elliptical zone of inhibition and the comb-like projection of the strip was the corresponding MIC value of the isolate for that particular antibiotic.

3.2.6 Statistical analysis

The change in average (total and antibiotic-resistant) mesophilic and psychrotrophic bacterial population during the cold storage of raw milk samples was statistically evaluated by one-way analysis of variance (ANOVA) and the comparison of the respective mesophilic and psychrotrophic counts was performed with an independent-samples T-test using SPSS Statistics 17.0 (SPSS Inc., Chicago, Illinois, USA). P-values less than 0.05 were considered statistically different.

3.3 Results

3.3.1 Effect of cold storage of raw milk on bacterial population

The change on mesophilic and psychrotrophic bacterial population during the cold storage of raw milk is shown in Figure 1. The average mesophilic counts (log CFU/mL) of 24 raw milk samples considered in this study on day 0 were 4.01±0.46 while psychrotrophic counts were 3.49±0.59. There was a significant ($P < 0.05$) difference among the 24 milk samples in both the initial mesophilic and psychrotrophic population and also the mesophilic population was significantly ($P < 0.05$) higher than the psychrotrophs.

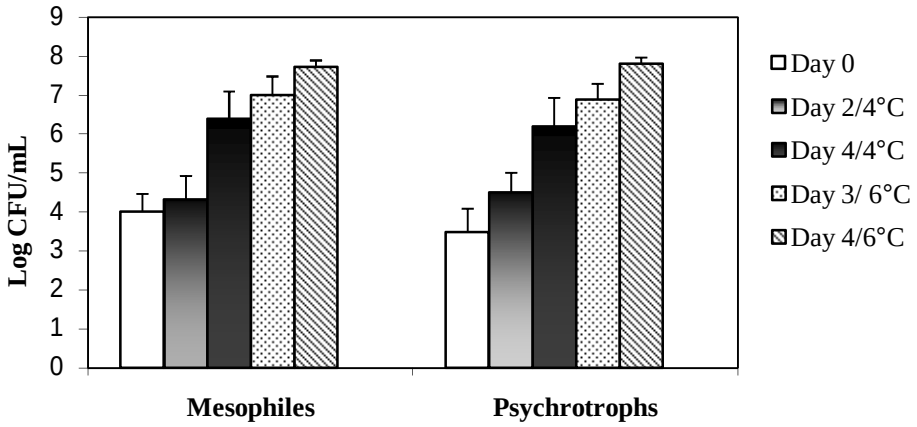

Figure 1. Shift of mesophilic and psychrotrophs population (log CFU/mL) at different storage temperatures (4 or 6°C) and time (0 to 4 days) of raw milk. The corresponding figures were an average of 24 raw milk samples for day 0 and were of 6 milk samples for other days, analysed between May to November 2009.

An increase of the mesophilic and psychrotrophic population (log CFU/mL) to 4.33±0.61 and to 4.51±0.50 respectively was observed when the milk samples were stored at 4°C for 2 days. Upon continue storage up to 4 days at 4°C the mesophilic count reached to 6.40±0.69 whereas the psychrotrophic count increased to 6.20±0.73. This increase in both the mesophilic and psychrotrophic population after 4 days storage at 4°C was significantly ($P < 0.05$) higher than the respective initial population. Only insignificant difference ($P = 0.115$) was found on the mesophilic counts between day 0 and day 2, the rests were statistically significant ($P < 0.05$) between each storage period.

On the other hand, both the mesophilic and psychrotrophic counts (log CFU/mL) exceeded the bacterial population of 4°C/4 days storage within 3 days when the milk was stored at 6°C. The population further increased to 7.73±0.16 (mesophilic) and 7.81±0.16 (psychrotrophic) when the milk samples were stored for 4 days. Both the mesophilic and psychrotrophic populations were significantly ($P < 0.05$) different on day 0, day 3 and day 4 storage at 6°C. No significant difference (independent samples t-test; $P = 0.08$) was found between the mesophilic and psychrotrophic counts after day 4 storage of milk samples at 6°C.

3.3.2 Effect of cold storage of raw milk on antibiotic-resistant bacterial population

The raw milk samples were analysed to determine the resistant bacterial population, both mesophilic and psychrotrophic, against antibiotics gentamicin (G), levofloxacin (L), ceftazidime (C), and trimethoprim-sulfamethoxazole (TS) after their respective storage period. The average antibiotic-resistant counts (log CFU/mL) during the cold storage of raw milk against these four antibiotics (belonged to four antibiotic classes) are shown in Figure 2 (Figures 2a for G, 2b for L, 2c for C and 2d for TS), and the range of resistant counts during the storage period along with statistical results are presented in Table 2. An increased antibiotic-resistant population, both mesophilic and psychrotrophic, was found during the storage of raw milk; however, the extent of rise in numbers was antibiotics dependent: bacteria resistant to gentamicin and levofloxacin increased moderately compared to the considerable rise in ceftazidime and trimethoprim-sulfamethoxazole-resistant bacteria at the end of storage periods.

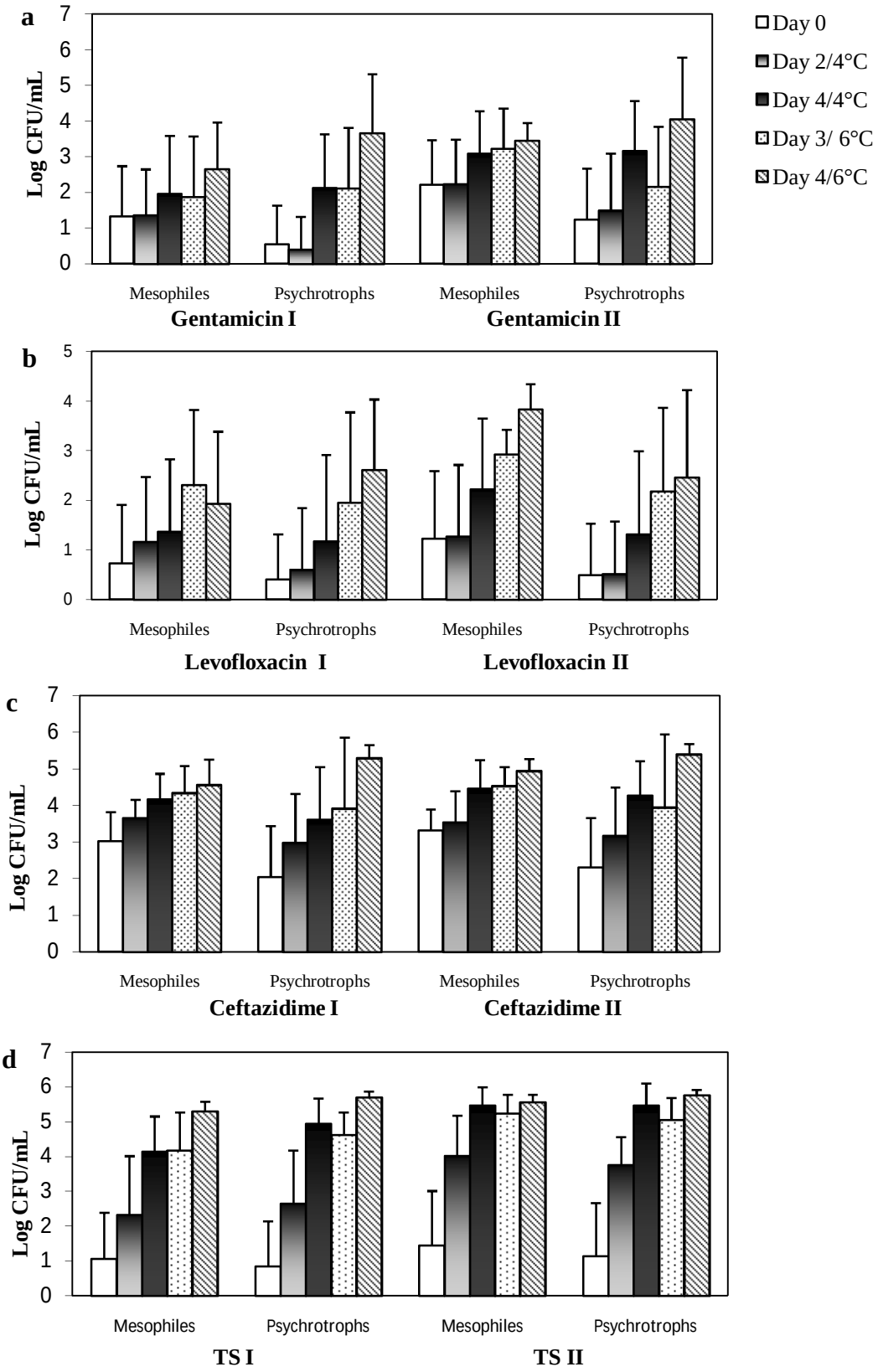

Figure 2. Shift of antibiotic-resistant mesophilic and psychrotrophic bacteria (log CFU/mL) in raw milk during the cold storage (at 4 and 6°C). G, gentamicin (2a); L, levofloxacin (2b); C, ceftazidime (2c); and TS, trimethoprim-sulfamethoxazole (2d). I and II account for the higher and lower antibiotics concentrations: I, four times the MIC for G & C and two times the MIC for L & TS ; II, the MIC (4, 8, 2 & 4/76 µg/mL for G, C, L & TS, respectively).

Table 2. Antibiotic-resistant (G, gentamicin; L, levofloxacin; C, ceftazidime; TS, trimethoprim-sulfamethoxazole) bacterial population* during the cold storage of raw milk.

Antibiotic-resistant/storage condition		0 d/4°C		2 d/4°C		4 d/4°C		3 d/6°C		4 d/6°C	
		Average	Range	Average	Range	Average	Range	Average	Range	Average	Range
GI-resistant	Mesophiles	1.33[a]	0.00-4.36	1.36[a]	0.57-2.02	1.96[a]	0.57-3.98	1.87[a]	0.00-3.75	2.65[b]	0.00-3.54
	Psychrotrophs	0.54[a]	0.00-2.41	0.40[a]	0.00-0.69	2.13[b]	0.00-5.07	2.11[b]	0.00-4.09	3.66[c]	0.69-5.86
GII-resistant	Mesophiles	2.22[a]	0.00-4.48	2.23[a]	1.15-3.29	3.09[b]	1.98-4.02	3.22[b]	1.50-4.08	3.45[b]	2.80-4.07
	Psychrotrophs	1.23[a]	0.00-4.50	1.49[a]	0.00-2.85	3.17[b]	1.15-6.00	2.16[a]	0.00-3.66	4.05[b]	0.57-5.84
LI-resistant	Mesophiles	0.73[a]	0.00-3.44	1.17[a]	0.00-2.11	1.37[a]	0.00-3.67	2.32[b]	0.00-3.88	1.94[b]	0.73-3.46
	Psychrotrophs	0.40[a]	0.00-2.72	0.61[a]	0.00-1.34	1.18[ab]	0.00-5.21	1.96[bc]	0.00-4.28	2.62[c]	0.00-3.65
LII-resistant	Mesophiles	1.22[a]	0.00-3.66	1.28[a]	0.57-2.10	2.23[b]	0.65-4.63	2.93[b]	2.45-3.58	3.84[c]	3.25-4.50
	Psychrotrophs	0.49[a]	0.00-3.44	0.52[a]	0.00-1.72	1.32[ab]	0.00-5.26	2.19[bc]	0.00-3.87	2.47[c]	0.00-4.43
CI-resistant	Mesophiles	3.02[a]	1.15-4.31	3.66[b]	3.21-4.11	4.17[c]	3.34-5.70	4.35[c]	3.26-5.25	4.56[c]	3.33-5.20
	Psychrotrophs	2.04[a]	0.00-4.09	2.99[b]	1.22-4.20	3.62[b]	1.15-5.42	3.92[b]	0.00-5.78	5.30[c]	4.83-5.91
CII-resistant	Mesophiles	3.32[a]	1.15-4.50	3.54[a]	2.62-4.31	4.47[b]	3.60-6.60	4.53[bc]	3.76-5.22	4.95[c]	4.48-5.26
	Psychrotrophs	2.30[a]	0.00-4.47	3.17[b]	1.70-4.30	4.27[c]	2.80-6.01	3.95[bc]	0.00-5.78	5.40[d]	5.15-5.92
TSI-resistant	Mesophiles	1.06[a]	0.00-3.68	2.32[b]	1.27-4.50	4.14[c]	3.01-5.14	4.17[c]	2.60-5.52	5.30[d]	5.02-5.70
	Psychrotrophs	0.84[a]	0.00-3.61	2.65[b]	1.95-3.82	4.94[c]	3.60-6.23	4.62[c]	3.73-5.72	5.70[d]	5.50-5.86
TSII-resistant	Mesophiles	1.45[a]	0.00-4.39	4.01[b]	2.51-5.00	5.47[c]	4.57-6.44	5.24[c]	4.26-5.82	5.56[c]	5.43-5.87
	Psychrotrophs	1.13[a]	0.00-4.88	3.76[b]	2.79-4.74	5.47[c]	4.64-6.41	5.05[c]	4.03-5.91	5.75[c]	5.52-5.98

* Average of quadruplicates except duplicates in case of 3 d/6°C.

[a,b,c,d]Dissimilar superscripts along the row shows the average respective antibiotic-resistant log CFU/ml counts were significantly higher ($P < 0.05$) during the storage of the raw milk samples.

The increase of gentamicin-resistant mesophilic bacteria (log CFU/mL) from 1.33 to 1.96 at higher concentration (GI, 16 μg/mL) was insignificant ($P > 0.05$) after four days of storage at 4°C. The resistant counts were 1.87 log CFU/mL on day 3 when stored at 6°C and rose to 2.65 log CFU/ml after 4 days which were significantly higher ($P < 0.05$) than the initial gentamicin-resistant mesophilic bacteria. On the other hand, the corresponding gentamicin-resistant psychrotrophic bacteria (log CFU/mL) were only 0.54 at the beginning, increased to 2.13 after 4 days storage at 4°C while they became 3.66 during the storage of the raw milk samples at 6°C. This rise of gentamicin-resistant psychrotrophs was statistically significant ($P < 0.05$) in 4 days storage of raw milk at 4°C with initial count and storage at 6°C for 4 days increased the gentamicin-resistant psychrotrophs significantly higher than the average counts at 4°C. However, the average resistant mesophiles at lower concentration (log CFU/mL) of gentamicin (GII, 8 μg/mL) rose significantly ($P < 0.05$) from 2.22 to 3.09 within 4 days of storage at 4°C but this mesophilic population did not differ significantly ($P < 0.05$) when stored at 6°C for 4 days (3.45 log CFU/mL). Likewise, the numbers of gentamicin-resistant psychrotrophic bacteria at lower concentration of gentamicin significantly increased ($P < 0.05$) from 1.23 to 3.17

log CFU/mL while no significant difference was found between the 4°C storage and at 6°C storage in 4 days (4.05 log CFU/mL).

Levofloxacin-resistant mesophilic and psychrotrophic population (log CFU/mL) at higher concentration of levofloxacin (LI, 4 µg/mL) were 1.37 and 1.18 at 4°C and were 1.94 and 2.62 at 6°C respectively after the 4 days storage of the raw milk samples from the initial counts of 0.73 and 0.40. Statistically the increase in number of levofloxacin-resistant mesophiles from the counts of day 0 was insignificant ($P > 0.05$) on day 4 at 4°C but the counts were significantly higher ($P < 0.05$) when the milk samples were stored at 6°C. The levofloxacin-resistant psychrotrophs were significantly higher on day 4 than on day 3 at 6°C while the corresponding mesophiles were insignificant ($P > 0.05$). However, the average counts of levofloxacin-resistant mesophilic bacteria at lower concentration (LII, 2 µg/mL) on day 4 was significantly higher ($P < 0.05$) than on day 3 at 6°C.

The initial average ceftazidime-resistant mesophilic bacterial counts (log CFU/mL) at higher concentration of ceftazidime (CI, 32 µg/mL) were 3.02, rose significantly ($P < 0.05$) to 4.17 on day 4 at 4°C but were statistically similar ($P > 0.05$) with the counts on day 4 at 6°C. However, ceftazidime-resistant psychrotrophs counts were much higher (5.30 log CFU/ml) at 6°C than at 4°C on 4^{th} day storage of raw milk samples. At lower concentration of ceftazidime (CII, 8 µg/mL), resistant mesophilic bacteria increased significantly ($P < 0.05$) from 3.32 to 4.47 on day 4 at 4°C but were significantly lower than the respective counts at 6°C (4.95 log CFU/mL). Similarly, ceftazidime-resistant psychrotrophic counts at 6°C were significantly higher than those counts in the milk samples stored at 4°C on day 4.

In case of higher concentration of (8/152 µg/mL) trimethoprim-sulfamethoxazole (TSI), the average TS-resistant mesophilic bacteria (log CFU/mL) rose significantly ($P < 0.05$) within 2 days storage at 4°C and become 4.14 on day 4. The corresponding counts at 6°C were considerably higher than the counts of 4°C during the same storage period of 4 days. The trend was similar with the TS-resistant psychrotrophs; however, the counts on day 4 at 6°C were at 5.70 compared with 5.30 of TS-resistant mesophiles. In contrast, the average mesophilic and psychrotrophic bacteria at lower concentration (TSII, 4/76 µg/mL) at 4°C on day 4 were statistically similar ($P > 0.05$) with the counts of day 4 at 6°C storage.

3.3.3 Identification of the isolates

Of the 67 antibiotic-resistant isolates considered for identification, including nine isolates from organic milk samples from a previous study conducted at the Dairy Technology division of the University of Helsinki, about 18% (12) were Gram-positive. The result of the identification of the remaining 82% (55) Gram-negative isolates by API 20 NE system and 16S rDNA gene sequencing is presented in Table 3, together with their source of origin, colony characteristics and the best growth temperature.

Identification by API 20 NE

Among the 55 Gram-negative antibiotic-resistant isolates analysed by API 20 NE, 25 (45.5%) isolates were identified either to an acceptable, good or very good identification level: eight isolates identified as *Sphingomonas paucimobilis*, five as *Pseudomonas putida*, three as *Sphingobacterium spiritivorum*, two as *Acinetobacter lwoffii* (7AB3, 7AB4) and one each as *Acinetobacter baumannii/calcoaceticus* (9AB4), *Acinetobacter junii/johnsonii* (2AB5) *Chryseobacterium indologenes* (5AB1), *Chryseobacterium meningosepticum* (3AB5), *Pseudomonas fluorescens* (2AB1), *Stenotrophomonas maltophilia* (3AB3), *Weeksella virosa/Empedobacter brevis* (O4h). In addition, three isolates could be identified to the genus level; two to genus *Pseudomonas* (4Ab4, 3AB1), and one to genus *Acinetobacter* (6AB2). The identification remained doubtful, low discriminated or unacceptable for the remaining 27 isolates: some of these isolates were proposed to be *Sphingobacterium spiritivorum*, *Sphingomonas paucimobilis*, *Stenotrophomonas maltophilia*, *Pasteurella pneumotropica* or *Pseudomonas* spp.

Identification by 16S rDNA gene sequencing

The result of the six Gram-negative isolates studied by 16S rDNA gene sequencing to supplement the identification by API 20 NE is presented in Table 3. Three isolates (2Ab6, 2AB1, 5AB3) were identified as *Pseudomonas* spp., two isolates (7AB3, 9AB4) as *Acinetobacter* and one isolate (9AB2) was suggested to be *Sporocytophaga* or *Flavobacterium*.

Table 3. Identification of the antibiotic-resistant psychrotrophic isolates by API 20 NE (phenotypic) and 16S rDNA gene sequencing (genotypic).

Isolate	Isolate source	Colony characteristics	Best growth at		**API 20 NE**			**16S rDNA gene sequencing**
			25°C	30°C	API 20 NE Numerical profiles	ID%	Result	Closest relative in GenBank database
2Ab2	LI	CW/regular		+	0056110		Unac. profile	
2Ab5	TSI	CW/regular		+	0014404		Doub. profile	
2Ab6	TSI	CW/regular		+	0346412		Unac. profile	***Pseudomonas***
2Ab7	TSII	CW/regular		+	0346410		Unac. profile	
4Ab1	GII	CW/regular		+	0420000	64.5	*Brevundimonas vesicularis* low disc.	
4Ab4	TSI	CW/regular		+	0347454		Genus ***Pseudomonas***	
5Ab1	GI	CW/irregular		+	0466304	97.5	***Sphingobacterium spiritivorum*** G	
1AB1	LI	Yellow	+		0466300	84.3	*Sphingobacterium spiritivorum* low disc.	
1AB2	CII	Yellow	+		0462304	98.7	***Sphingobacterium spiritivorum*** G	
1AB3	GII	Yellow		+	1230000	84.0	*Pasteurella pneumotropica* doubtful	
1AB4	GI	CW/regular	+		0462300	89.7	*Sphingobacterium spiritivorum* low disc.	
1AB5	LI	Yellow	+		0466300	84.3	*Sphingobacterium spiritivorum* low disc.	
1AB6	CI	Yellow	+		0466300	84.3	*Sphingobacterium spiritivorum* low disc.	
2AB1	TSI	CW/regular		+	0146414	82.0	***Pseudomonas fluorescens*** Acc.	***Pseudomonas* spp.**
2AB2	LII	CW/regular	+		0447300	99.8	***Sphingomonas paucimobilis*** VG	
2AB4	GI	CW/regular		+	0466700	98.5	***Sphingomonas paucimobilis*** G	
2AB5	CI	CW/regular	+		0000011	91.3	***Acinetobacter junii/johnsonii*** G	
3AB1	TSI	CW/regular		+	0146456	74.3	Genus ***Pseudomonas***	
3AB2	CI	CW/regular		+	0224462		Unac. profile	
3AB3	CI	CW/irregular	+		0456141	96.6	***Stenotrophomonas maltophilia*** G	
3AB4	LII	CW/regular	+		1660000		Not valid	
3AB5	GI	Pink	+		0456200	93.7	***Chryseobacterium meningosepticum*** G	
4AB2	GII	Yellow		+	2410000	93.1	*Chryseobacterium indologenes* low disc.	

Table 3. Continued

4AB3	CII	Yellow	+		0462400	98.9	***Sphingomonas paucimobilis*** G	
4AB4	TSII	CW/regular		+	0341451	97.7	***Pseudomonas putida*** G	
5AB1	GII	Yellow		+	2610000	99.7	***Chryseobacterium indologenes*** VG	
5AB3	TSII	CW/irregular	+		0141415	99.1	***Pseudomonas putida*** VG	***Pseudomonas* spp.**
5AB4	TSI	CW/regular	+		0141455	99.1	***Pseudomonas putida*** VG	
6AB1	TSI	CW/regular		+	0346416		Unac. profile	
6AB2	CI	CW/regular	+		0000001		Genus ***Acinetobacter***	
6AB3	CII	CW/regular		+	1476000		Unac. profile	
6AB5	LI	CW/irregular		+	0452101	88.0	*Stenotrophomonas maltophilia* low disc.	
7AB3	CI	CW/regular	+		0000010	91.8	***Acinetobacter lwoffii*** G	***Acinetobacter***
7AB4	CI	CW/regular	+		0000010	91.8	***Acinetobacter lwoffii*** G	
8AB1	LII	Yellow	+		0462300	89.7	*Sphingobacterium spiritivorum* low disc.	
8AB2	LI	CW/regular	+		0456011		Unac. profile	
8AB3	TSI	CW/regular		+	0140457	99.6	***Pseudomonas putida*** VG	
8AB4	TSI	CW/regular		+	0140457	99.6	***Pseudomonas putida*** VG	
9AB1	GI	Yellow	+		1463753	85.3	*Pseudomonas luteola* low disc.	
9AB2	GI	Yellow	+		1463335	99.9	***Sphingomonas paucimobilis*** VG	***Sporocytophaga*/ *Flavobacterium***
9AB3	CI	Yellowish white	+		0043331		Unac. profile	
9AB4	CI	CW/regular		+	0040453	90.5	***Acinetobacter baumannii/calcoaceticus*** G	***Acinetobacter***
10AB1	GI	Yellow/irregular	+		0442300	55.0	*Sphingomonas paucimobilis* doubtful	
10AB2	GII	Yellow/regular	+		1463300	99.8	***Sphingomonas paucimobilis*** VG	
10AB4	LI	CW/regular	+		0456150		Unac. profile	
11AB2	GII	Yellow	+		1463726	99.9	***Sphingomonas paucimobilis*** VG	
11AB4	CII	CW/regular	+		0446700	98.0	***Sphingomonas paucimobilis*** G	
12AB1	GI	Golden yellow	+		1467720	92.1	*Sphingomonas paucimobilis* low disc.	
12AB2	GI	Golden yellow	+		1463304	97.6	***Sphingomonas paucimobilis*** G	

Table 3. Continued

O1g	CII	White	+		0466300	97.5	***Sphingobacterium spiritivorum*** G
O1k	LII	Orange	+		1467340	69.5	*Rhizobium radiobacter* low disc.
O2a	LII	CW	+		0446100	56.4	*Sphingomonas paucimobilis* low disc.
O2g	LII	CW		+	0466300	84.3	*Sphingobacterium spiritivorum* low disc.
O3e	LI	CW		+	0446300	64.0	*Sphingomonas paucimobilis* doubtful
O4h	LII	CW	+		0050400	81.3	***Weeksella virosa/Empedobacter brevis*** Acc.

G= gentamicin, C= ceftazidime, L= levofloxacin, TS= trimethoprim-sulfamethoxazole. I represents higher concentration (4 times MIC for G & C; 2 times MIC for L & TS) and II represents lower concentration of antibiotics at MICs (µg/mL) [G= 4; C= 8; L= 2; TS= 4/76]. CW= creamy white. In API 20 NE: VG, very good; G, good; Acc., acceptable; low disc. = low discriminated; unac. profile = unacceptable profile; doub. profile = doubtful profile. Bold text indicates the isolates were identified at least to genus level.

3.3.4 Antibiotic resistance analysis (ATB PSE 5 strip)

The detailed profile of the in vitro antibiotic resistance of 49 Gram-negative isolates (Table 3) by ATB PSE 5 system is shown in Table 4 and the percentage of susceptible, intermediate and resistant isolates against each antibiotic is presented in Figure 3. Among the beta-lactams, the isolates showed highest resistance (71.4%) with ceftazidime (CAZ), followed by ampicillin (FAM) and cefepime (FEP) which were both at 51.0%, ticarcillin (TIC) at 44.9%, ticarcillin-clavulanic acid (TCC) at 34.7%, piperacillin (PIC) at 30.6%, piperacillin-tazobactam (TZP) and imipenem (IMI) both at 28.6% whereas only 26.5% of the isolates were resistant to meropenem (MERO). About half of the isolates evaluated showed resistance against the aminoglycosides class of the antibiotics: 46.9% were resistant to amikacin and gentamicin, and 57.1% against tobramycin. On the other hand, a majority of the isolates (69.4%) were found to be resistant with poly-peptide antibiotic colistin, however, only 44.9% isolates were resistant to folate pathway inhibitor antibiotics cotrimoxazol (trimethoprim-sulfamethoxazole). In addition, a significant number of isolates demonstrated resistance to the lower concentration of TIC (26.5%), TCC (16.3%), PIC (20.4%) and FEP (14.3%) of the ATB PSE 5 strip.

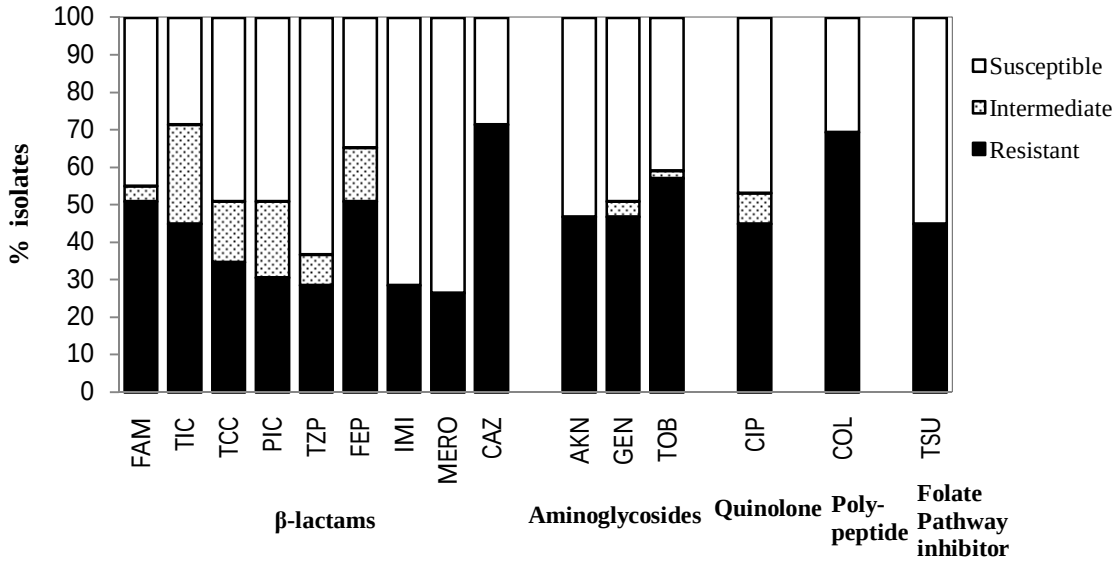

Figure 3. Percentage of isolates susceptible, intermediate and resistant to 15 antibiotics, representing five classes of antibiotics, of the ATB PSE 5 system. FAM, ampicillin-sulbactam; TIC, ticarcillin; TCC, ticarcillin-clavulonic acid; PIC, piperacillin; TZP, piperacillin-tazobactam; FEP, cefepime; CAZ, ceftazidime; IMI, imipenem; MERO, meropenem; AKN, amikacin; GEN, gentamicin; TOB, tobramycin; CIP, ciprofloxacin; COL, colistin; TSU; trimethoprim-sulfamethoxazole.

When the resistance of the isolates against the 15 antibiotics of the ATB PSE 5 system were compared (Figure 4), 25.5 % isolates were found to be resistant to all of the 15 antibiotics, 15% isolates to 7 antibiotics and all together 59.6% isolates were resistant to more than 4 antibiotics. Two of the 49 isolates studied were sensitive to all the antibiotics of the ATB PSE 5 strip. The number of isolates resistant to three or more classes of antibiotics (i.e. multiresistant) was observed to be 28 (59.7%) [Figure 5a]: 12 were resistant to 5 classes, 8 isolates each to 3 and 4 antibiotic classes.

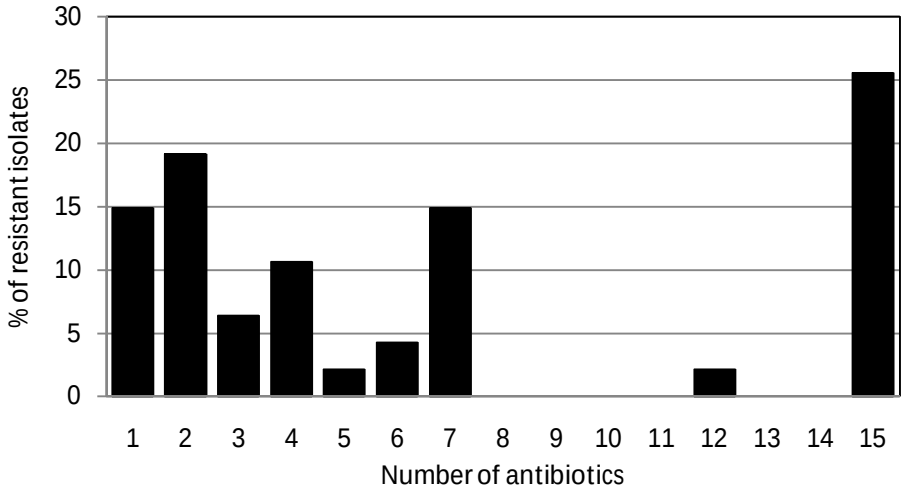

Figure 4. Percentages of isolates that demonstrated resistance against the number of antibiotics (ranging from 1 to 15), as obtained by the ATB PSE 5 system.

The number of multiresistant isolates according to ATB PSE 5 system when compared with their source of isolation (whether gentamicin-, ceftazidime-, levofloxacin- or trimethoprim-sulfamethoxazole-resistant) is shown in Figure 5b. Among the 28 multiresistant isolates, 12 out of 14 (85.7%) were levofloxacin-resistant isolates, 11 out of 12 (91.6%) were gentamicin-resistant isolates, and 5 out of 12 (41.6%) were ceftazidime-resistant isolates. However, none of the 11 trimethoprim-sulfamethoxazole-resistant isolates were multiresistant. Two (O1k, O4h) of the non-multiresistant levofloxacin-resistant isolates were retrieved from organic milk samples (from a previous study performed at Dairy Technology division, University of Helsinki); both of them were resistant to two classes of antibiotics.

Table 4. Results of the antibiotic susceptibility testing of the isolates by ATB PSE 5 system, the isolates were retrieved from agar plates containing antibiotics derived from raw milk samples.

Classes	β-lactams									Amino-glycosides			Quinolone	Polypeptide	Folate pathway inhibitor
Antibiotic/isolates	FAM	TIC	TCC	PIC	TZP	FEP	IMI	MERO	CAZ	AKN	GEN	TOB	CIP	COL	TSU
Sphingomonas paucimobilis															
2AB2	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
2AB4	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
4AB3	S	I	S	R	I	S	S	S	R	S	S	I	R	R	S
9AB2	S	I	S	S	S	I	S	S	R	S	S	R	S	R	S
10AB2	S	R	S	S	S	I	S	S	R	R	R	R	I	R	S
11AB2	I	R	S	I	I	R	S	S	R	R	R	R	S	R	S
11AB4	S	S	S	S	S	R	S	S	R	S	S	S	S	S	S
12AB2	S	R	S	S	S	R	S	S	R	S	I	R	S	R	S
Pseudomonas putida															
4AB4	R	I	I	S	S	S	S	S	S	S	S	S	S	S	R
5AB3	R	I	I	S	S	I	S	S	S	S	S	S	S	S	R
5AB4	R	I	I	S	S	S	S	S	S	S	S	S	S	S	R
8AB3	R	R	R	S	S	S	S	S	S	S	S	S	S	S	R
8AB4	R	R	R	S	S	I	S	S	S	S	S	S	S	S	R
Pseudomonas spp.															
3AB1	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Pseudomonas fluorescens															
2AB1	R	I	I	S	S	S	S	S	S	S	S	S	S	S	S
Sphingobacterium spiritivorum															
5Ab1	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
1AB2	S	I	S	I	S	R	S	S	R	R	R	R	R	R	S
8AB1	S	S	S	I	S	R	S	S	R	R	R	R	R	R	S
Acinetobacter lwoffii															
7AB3	S	S	S	I	S	S	S	S	R	S	S	S	S	S	S
7AB4	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S
Acinetobacter baumannii/calcoaceticus															
9AB4	R	R	R	S	S	S	S	S	S	S	S	S	S	S	R
Chryseobacterium meningosepticum															
3AB5	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Weeksella virosa/Empedobacter brevis															
O4h	S	S	S	S	S	R	S	S	R	S	S	S	I	R	S

Table 4. Continued

Stenotrophomonas maltophilia															
3AB3	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Unidentified															
2Ab2	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
2Ab5	R	I	I	S	S	S	S	S	S	S	S	S	S	S	S
2Ab6	R	I	I	S	S	S	S	S	S	S	S	S	S	S	R
2Ab7	R	I	I	S	S	S	S	S	S	S	S	S	S	S	S
1AB1	S	S	S	I	S	R	S	S	R	R	R	R	R	R	S
1AB3	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
1AB4	S	I	S	I	S	R	S	S	R	R	R	R	R	R	S
1AB5	S	S	S	I	S	R	S	S	R	R	R	R	R	R	S
1AB6	S	S	S	I	I	R	S	S	R	R	R	R	R	R	S
3AB2	S	S	S	S	S	S	S	S	R	S	S	S	S	R	S
3AB4	S	S	S	S	S	R	S	S	R	S	S	R	R	R	S
6AB1	R	I	I	S	S	S	S	S	S	S	S	S	S	S	R
6AB3	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
6AB5	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
8AB2	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
9AB1	S	R	S	S	S	I	S	S	R	S	I	R	S	R	S
9AB3	S	S	S	I	S	S	S	S	S	S	S	S	S	S	S
10AB1	I	R	S	I	I	I	S	S	R	R	R	R	I	R	S
10AB4	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
12AB1	S	I	S	S	S	I	S	S	R	S	S	R	S	R	S
O1g	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
O1k	S	S	S	S	S	S	S	S	R	S	S	S	I	R	S
O2a	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
O2g	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
O3e	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

FAM, ampicillin-sulbactam; TIC, ticarcillin; TCC, ticarcillin-clavulonic acid; PIC, piperacillin; TZP, piperacillin-tazobactam; FEP, cefepime; CAZ, ceftazidime; IMI, imipenem; MERO, meropenem; AKN, amikacin; GEN, gentamicin; TOB, tobramycin; CIP, ciprofloxacin; COL, colistin; TSU; trimethoprim-sulfamethoxazole. S, susceptible; I, intermediate; R, resistant.

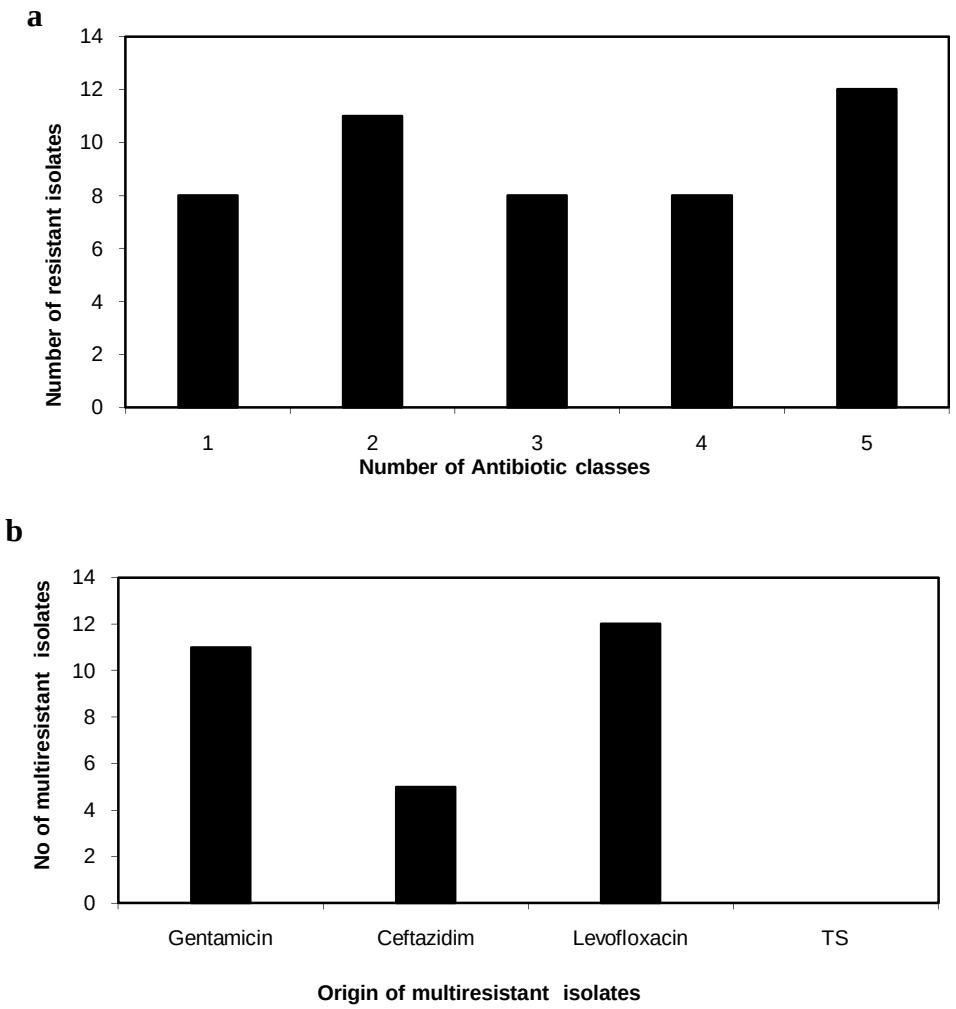

Figure 5. a) Distribution of isolates resistant against number of antibiotic classes (ranging from 1 to 5); b) number of multiresistant isolates considering their origin (resistant either against gentamicin, ceftazidime, levofloxacin or trimethoprim-sulfamethoxazole (TS), as obtained by ATB PSE 5 system.

Table 4 also lists the antibiotic resistance profile of the 27 isolates identified by API 20 NE and 16S rDNA gene sequencing as obtained from ATB PSE 5 system. Among eight isolates identified by API 20 NE as *Sphingomonas paucimobilis*, two (2AB2, 2AB4) isolates were resistant to all the 15 antibiotics of the ATB PSE 5 strip while the remaining six isolates showed varying levels of susceptibility: all were resistant to ceftazidime but were sensitive to imipenem, meropenem and trimethoprim-sulfamethoxazole while five were resistant to colistin. On the other hand, five isolates identified as *Pseudomonas putida* were mostly sensitive to the majority of the antibiotics except that they were resistant to the β-lactam antibiotic ampicillin and to the folate pathway inhibitor trimethoprim-sulfamethoxazole. However, one isolate (3AB1) assigned as *Pseudomonas* spp. by API 20 NE had demonstrated resistance to all antibiotics of the ATB PSE 5 strip while another isolate 2Ab6 identified as *Pseudomonas* by 16S rDNA gene sequencing was only resistant to ampicillin and cotrimoxazol. Contrary to 3AB1, the isolate 2AB1 identified as

Pseudomonas fluorescens was susceptible to aminoglycosides, quinolone, poly-peptide and folate pathway inhibitor antibiotics and was only resistant to ampicillin-sulbactam and to the lower concentration of TIC and TCC of β-lactams.

Similarly, one (5Ab1) of the three isolates identified as *Sphingobacterium spiritivorum* was resistant to all the antibiotics of the ATB PSE 5 strip whereas the other two isolates (1AB2, 8AB1) were susceptible to β-lactams (except cefepime and ceftazidime) and trimethoprim-sulfamethoxazole, but were resistant to amikacin, gentamicin, tobramycin, colistin and ciprofloxacin. Isolate 9AB4 found to be *Acinetobacter baumannii/calcoaceticus* by API 20 NE showed resistance only to ampicillin, ticarcillin and ticarcillin-clavulonic acid of β-lactams and trimethoprim-sulfamethoxazole of the folate synthesis inhibitor. However, isolates 3AB3 and 3AB5 identified as *Stenotrophomonas maltophilia* and *Chryseobacterium meningosepticum* respectively were resistant to all 15 antibiotics of the ATB PSE 5 system.

3.3.5 MIC determination by HiComb system

The MIC values for the 11 isolates determined by HiComb system for antibiotics gentamicin, ceftazidime, levofloxacin and trimethoprim-sulfamethoxazole (TS) is shown in Table 5. All of the isolates had MIC values of 0.25 μg/mL or less for the antibiotic levofloxacin, while the MICs for other antibiotics varied considerably depending on the isolates origin. TS-resistant isolates (2Ab6, 2AB1, 5AB3, 8AB3) had MIC value for antibiotic TS higher than 240 μg/mL, while MICs for gentamicin varied between 0.128 to 0.256, and for ceftazidime between 1.024 to 2.048 (5AB3) μg/mL. On the other hand, the MIC for ceftazidime-resistant isolates (4AB3, 7AB3, 9AB4, 11AB4) varied widely for the same antibiotic: the MIC values for ceftazidime were 0.256 (7AB3), 1.024 (AB3, 9AB4), and more than 256 μg/mL (11AB4); the MIC values were below 0.256 μg/mL for gentamicin while it was above 240 (4AB3) and others had less than 0.5 μg/mL for trimethoprim-sulfamethoxazole. However, the gentamicin-resistant isolates had MIC values that ranged from 2.048 (10AB2) to 4.096 μg/mL (9AB2, 11AB2) for gentamicin; all isolates had MIC values higher than 256 μg/mL for ceftazidime whereas the MIC values ranged between 30 to 240 μg/mL (9AB2, 60; 10AB2, 30 and 11AB2 >240) for trimethoprim-sulfamethoxazole.

Table 5. Minimum inhibitory concentration (MIC) against antibiotics gentamicin (G), ceftazidime (C), levofloxacin (L) and trimethoprim-sulfamethoxazole (TS) of 11 isolates as obtained by HiComb system.

Isolate	Source of isolate[a]	MIC (μg/mL)			
		Gentamicin	Ceftazidimee	Levofloxacin	Trimethoprim-sulfamethoxazole
2Ab6	TSI	0.128	1.024	0.25	>240
2AB1	TSI	0.128	1.024	0.25	>240
4AB3	CII	0.256	1.024	0.25	>240
5AB3	TSII	0.128	2.048	0.25	>240
7AB3	CI	<0.064	0.256	<0.005	0.5
8AB3	TSI	0.256	1.024	0.25	>240
9AB2	GI	4.096	256	0.25	60
9AB4	CI	0.064	1.024	0.25	<0.001
10AB2	GII	2.048	>256	0.25	30
11AB2	GII	4.096	>256	0.25	>240
11AB4	CII	<0.064	>256	0.25	<0.001

[a] Isolates resistant to respective antibiotics at I= higher (G=16; C=32; TS=8/152) and at II= lower (MICs; G= 4; C= 8; TS= 4/76) concentrations (μg/mL).

3.4 Discussion

3.4.1 Effect of cold storage of raw milk on bacterial population

The average mesophilic bacterial population of 23 raw milk samples was below 10^5 CFU/mL, suggesting that the milk samples were of good quality (EC 2004). However, one sample had the initial mesophilic counts of 5.13 log CFU/mL. The milk samples considered in this study were received from milk lorries that collected milk from bulk tank in the farms, the time the milk samples remained at cold temperature before the sampling period was unknown. A wide variation of the initial mesophilic population among 24 raw milk samples that ranged from 3.17 to 5.13 log CFU/mL may indicate varying levels of farm hygiene, milking and storage practices (Bramley and McKinnon 1990). Of the many factors affecting the initial bacterial population in raw milk, the cold storage of raw milk after milking might have a significant role for the initial higher bacterial counts in some of the milk samples (Hayes and Boor 2001).

Both the average mesophilic and psychrotrophic bacteria remained below 10^5 CFU/mL during two days storage at 4°C but increased considerably within four days of storage. However, the significant rise of psychrotrophic bacteria within two days suggests that the cold chain favors the growth of psychrotrophs. Lafarge et al. (2004) using temporal temperature gel electrophoresis (TTGE) and denaturing gradient gel electrophoresis (DGGE) investigated the bacterial dynamics during refrigerated storage of raw milk and reported that the rise of psychrotrophic population observed within 24 h of raw milk storage at 4°C. According to Rasolofo et al. (2010), the rise of psychrotrophic bacteria was noticed soon after three days storage of untreated raw milk at 4°C. This is in good agreement with our observation that storing the raw milk beyond 3 days at 4°C could result in a significant rise of psychrotrophic bacteria. When the raw milk was stored at 6°C, the psychrotrophic counts already reached the level of 4°C on day 4 within 3 days and increased further afterwards. The initial mesophilic population was significantly higher than the psychrotrophic population but no significant difference was observed in four days storage at 6°C. This was in agreement with previous reporting that the psychrotrophic bacteria dominate the bacterial population during refrigerated storage of raw milk (Cousin 1981; Eneroth et al. 1998; Hayes and Boor 2001; Holm et al. 2004; Munsch-Alatossava and Alatossava 2006).

3.4.2 Effect of cold storage of raw milk on antibiotic-resistant bacterial population

Many studies had been undertaken to study the bacterial population in raw milk or bulk tank milk (Guinot-Thomas et al. 1995; Jayarao and Wang 1999; Jayarao and Henning 2001; Lafarge et al. 2004; Hantsis-Zacharov and Halpern 2007; Ercolini et al. 2009). However, few studies focused the analysis of antibiotic-resistant bacterial counts in raw milk (Straley et al. 2006) and the evaluation of antibiotic resistance of raw milk associated bacteria during cold storage and transportation (Munsch-Alatossava and Alatossava 2007). This study investigated the significance of cold storage on the antibiotic-resistant bacterial population in raw milk.

A significant rise of resistant bacterial population (Figure 2; Table 2) against all four antibiotics tested (gentamicin, levofloxacin, ceftazidime and trimethoprim-sulfamethoxazole, TS), that represented four major classes of antibiotics (aminoglycosides, quinolones, β-lactams and folate pathway inhibitors, respectively), was observed during the cold storage of raw milk samples at 4°C and at 6°C. A wide variation on the resistant counts was observed among raw milk samples before or after the storage period. Gentamicin- and levofloxacin-resistant bacterial counts, both mesophilic and psychrotrophic, increased moderately (average counts, 1.94 to 4.05 CFU/mL) compared to a considerable rise of ceftazidime- and trimethoprim-sulfamethoxazole-resistant population (average counts, 4.95 to 5.75 log CFU/mL) during the storage period.

At both concentrations of gentamicin (Figure 2a), the average resistant psychrotrophic population increased significantly ($P < 0.05$) only after day 4 storage at 4°C which were statistically insignificant with the resistant counts of day 3 when the milk samples were stored at 6°C but the counts on day 3 were significantly lower than of the day 4 at 6°C. Similarly, no significant rise of levofloxacin-resistant psychrotrophic counts (Figure 2b) was found on day 4 storage of raw milk at 4°C. However, the levofloxacin-resistant psychrotrophs counts (average 2.47-2.62 CFU/mL) were below the gentamicin-resistant counts (3.66-4.05 CFU/mL) on day 4 of the milk samples stored at 6°C.

On the other hand, both ceftazidime- and TS-resistant psychrotrophic populations (Figure 2c, 2d) were significantly higher from the initial resistant counts within 2 days storage of raw milk samples at 4°C. The counts of day 4 at 4°C did not differ significantly with the counts of day 3 at 6°C and were significantly lower than the respective population of day 4

at 6°C. Interestingly, the TS-resistant mesophilic and psychrotrophic counts on day 4 at 4°C and on day 4 at 6°C were statistically similar, suggesting that the majority of the TS-resistant mesophilic bacteria in raw milk during this storage period were TS-resistant psychrotrophs since most of the psychrotrophic bacteria in raw milk can grow at up to 32°C (Hantsis-Zacharov and Halpern 2007).

Straley et al. (2006) reported that bulk tank milk could be a significant source of antimicrobial-resistant Gram-negative bacteria and observed a considerable variation between farms and within milk samples from the same farms. However, they considered only three antibiotics (tetracycline from the inhibitor of protein synthesis, and ampicillin and ceftiofur from inhibitor of cell wall synthesis) to determine the antibiotic-resistant population. The average ampicillin-resistant Gram-negative counts (ranged between 0 - 17,000 CFU/mL) were higher than tetracycline- resistant counts (ranged between 0 to 1265 CFU/mL) while average ceftiofur-resistant counts were in between (ranged 0-14,000 CFU/mL) and the authors noted that these antibiotics were commonly used for treating dairy cattle. Our result (that included both the Gram-positive and Gram-negative bacteria) of the initial mesophilic ceftazidime-resistant counts in raw milk (ranged between 1.15-4.50 log CFU/mL) were in the range as that of ampicillin-resistant counts, however, resistant counts against gentamicin (from the same protein synthesis inhibitor class as that of tetracycline) in our study were higher than that reported by the Straley et al. (2006) study.

3.4.3 Identification of the antibiotic-resistant isolates

The antibiotic-resistant isolates were recovered from agar plates containing antibiotics used for determining resistant psychrotrophic bacteria from the cold-stored raw milk samples at 4°C/4 d and at 6°C/3-4 d. Phenotypic identification of these resistant isolates by API 20 NE system enabled the identification to only 45.5% (25 isolates) to the species level; however, three isolates were identified to the genus level in addition. Other 27 isolates remained doubtful, low discriminated or unacceptable; many were proposed to be similar to the identified groups of isolates. Despite limited numbers of isolates considered for identification in this study, the API 20 NE system, designed to identify Gram-negative rods, did not reliably identified the isolates.

Munsch-Alatossava and Alatossava (2006) were able to identify only 49.2% of the raw milk-associated psychrotrophic bacteria by API 20 NE system. These isolates were evaluated for their antibiotic resistance by ATB® PSE system and reported that significant proportion of these isolates were multidrug resistant (Munsch-Alatossva and Alatossava 2007). The authors pointed out that the low level of identification by API 20 NE might be due to its absolute reliance on the phenotypic features, the Gram-reaction based approach, the limited number of tests in the strip and the limited databases. In a study of the non-fermenting Gram-negative clinical isolates, Bosshard et al. (2006) also reported that the level of identification to species level by API 20 NE was limited to 54% and suggested that 16S rRNA gene sequencing could be a better choice if proper identification to species level is required.

Of the 6 isolates considered for 16S rDNA gene sequencing, 4 were identified similar to that by API 20 NE, one unacceptable profiled by API 20 NE was identified as *Pseudomonas* and another proposed as *Sphingomonas paucimobilis* by API 20 NE system was suggested to be *Sporocytophaga* or *Flavobacterium*. Although only a few isolates were analysed by 16S rDNA gene sequencing, it can be of significance as the gene sequencing can complement the API 20 NE for the identification of the Gram-negative psychrotrophic bacteria in raw milk (Munsch-Alatossava and Alatossava 2006).

Eight isolates were assigned to *Sphinogomonas paucimobilis* by API 20 NE and all of these isolates were grown in agar plates that contained gentamicin, levofloxacin and ceftazidime demonstrating the resistance against these antibiotics. This bacterial species have been reported as an opportunistic pathogen and the infection may lead to bacteremia/septicaemia (Ryan and Adley 2010). *Sphingomonas* spp., including *Sphingomonas paucimobilis*, was recovered from the drinking water system in Finland and Sweden and majority of the isolates studied were able to grow at 5°C and were capable of forming biofilm in water pipelines (Koskinen et al. 2000). The water used in the dairy farms could be a potential source of this bacterial species in raw milk. *Pseudomonas putida* was the second largest species identified in this study (5 isolates out of 25) and interestingly, all were resistant to trimethoprim-sulfamethoxazole. This species accounted for about 12% of the total Gram-negative bacteria in bulk tank milk samples in a study by Jayarao and Wang (1999).

On the other hand, *Pseudomonas fluorescens* has been identified as the dominant spoilage species in refrigerated raw milk (Jayarao and Wang 1999; Holm et al. 2004; Munsch-

Alatossava and Alatossava 2006). However, only one *P. fluorescens* isolate that was resistant to trimethoprim-sulfamethoxazole was accurately identified in this study, suggesting that this primary spoilage bacterium of raw milk was sensitive to other three antibiotics (gentamicin, levofloxacin, and ceftazidime) tested. Three of the isolates identified as *Sphingobacterium spiritivorum*, including one isolate (01g) resistant to ceftazidime from an organic milk sample considered in a previous study at the Dairy Technology division of the University of Helsinki. Hantsis-Zacharov and Halpern (2007) reported that this species and other species from the class *Sphingobacteria* were detected in all seasons of the milking but were in low numbers.

Both *Acinetobacter lwoffii* (2 isolates; 7AB3, 7AB4) and *Stenotrophomonas maltophilia* (1 isolate, 3AB3) species identified in this study were also reported in bulk tank milk samples by Jayarao and Wang (1999). In addition, Munsch-Alatossva and Alatossava (2006) also detected 3 out of 67 isolates from raw milk samples using API 20 NE and BIOLOG as *S. maltophilia*. This species from genus *Stenotrophomonas* has been reported as an opportunistic nosocomial pathogen (Berg et al. 1999). One isolate identified as *Acinetobacter baumannii/calcoaceticus* in the present study was also identified in 17 out of 225 isolates recovered from mastitic raw milk samples in the Republic of Korea and many of them were multidrug resistant (Nam et al. 2010).

.

3.4.4 Antibiotic resistance of the isolates

Among 55 Gram-negative isolates considered in this study, 49 were analysed for their antibiotic resistance with an in vitro ATB PSE 5 system. Since the ATB PSE 5 system does not provide the MIC values for antibiotics, consequently 11 isolates were also analysed by HiComb system to determine the specific MIC value for the antibiotics used in this study. Due to the limited number of isolates analysed for their antibiotic resistance in this study, it might be difficult to compare with other findings and a majority of these studies concerned the clinical isolates and only a few of them evaluated the antibiotic resistance in raw milk-associated psychrotrophic bacteria.

The proportion of isolates demonstrated resistant ranged from 26.5% against meropenem to as high as 71.4% to ceftazidime (Figure 3) of the ATB PSE 5 strips. 59.7% isolates were resistant to three or more classes of antibiotics (Figure 5a), thus they were categorized as

multiresistant isolates. Since several antibiotics of β-lactams class were included in the semi-automated ATB PSE 5 strips, the isolates also showed considerable variation from 26.5% to 71.4% while isolates demonstrating resistant against aminoglycosides ranged narrowly from 46.9 % to 57.1%. The number of resistant isolates to poly-peptide antibiotic colistin was higher than the folate pathway inhibitor trimethoprim-sulfamethoxazol.

The isolates identified as *S. paucimobilis* showed a considerable variation of the susceptibility against antibiotics of the ATB PSE 5 strips. However, all of these isolates were resistant to ceftazidime, 7 out of 8 isolates to colistin and 3 out of 8 were resistant to ciprofloxacin. However, only 25%, 7.1%, and 10.7% of the clinical isolates of *S. paucimobilis* collected worldwide (1997-2003) were resistant to ceftazidime, ciprofloxacin and trimethoprim-sulfamethoxazole (Sader and Jones 2005). On the other hand, *P. putida* isolates were all sensitive to ceftazidime and colistin but were resistant against trimethoprim-sulfamethoxazole. Interestingly, the only isolate identified as *P. fluorescens* (2AB1) was sensitive to all antibiotics of the ATB PSE strip except it was resistant to ampicillin and intermediate resistant to ticarcillin. However, Munsch-Alatossava and Alatossava (2007) reported that only one of the 27 isolates of *P. fluorescens* was sensitive to trimethoprim-sulfamethoxazole and all were sensitive to aminoglycosides. *Pseudomonas* spp. were shown extensive resistance against several antibiotics and were of concern as this group of bacteria can grow at the low temperatures and can form biofilms in the bulk milk tank (Straley et al. 2006).

Three isolates identified as *Sphingobacterium spiritivorum* were resistant to all aminoglycosides, ciprofloxacin, colistin and trimethoprim-sulfamethoxazole of the ATB PSE 5 strips whereas the isolate (3AB5) *Chryseobacterium meningosepticum* was entirely resistant to all antibiotics of the strip and it was the same case with only isolate identified as *Stenotrophomonas maltophilia* (3AB3). Higgins et al. (2001) in clinical isolates and Munsch-Alatossava and Alatossava (2007) in raw milk isolates of *S. maltophilia* reported an extensive resistance to many of the β-lactam antibiotics but all were sensitive to trimethoprim-sulfamethoxazole. The clinical isolates of *S. maltophilia* in Nicodemo et al. (2004) study also exhibited susceptibility to the trimethoprim-sulfamethoxazole but 40% of the 70 isolates considered were resistant to ticarcillin, a β-lactam.

On the other hand, *Acinetobacter baumannii/calcoaceticus* (one isolate, 9AB4) was sensitive to aminoglycosides, quinolone, polypeptide and many of the β-lactams but was

resistant to trimethoprim-sulfamethoxazole. Nam et al. (2010) also reported that about 90% of the 17 *A. baumanni* isolates from mastitic raw milk samples were sensitive to gentamicin and amikacin. Straley et al. (2006) also found that the *Acinetobacter* spp. were sensitive to amikacin of the aminoglycosides class. On the contrary, these isolates were sensitive to ticarcillin but the isolate (9AB4) in the present study was resistant to ticarcillin. However, two isolates (7AB3, 7AB4) identified as *Acinetobacter lwoffii* were sensitive to all antibiotics except ceftazidime, which is in agreement (except against ceftazidime) with the susceptibility profile of a clinical isolate of *Acinetobacter lwoffii* against β-lactams, gentamicin, trovafloxacin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole as reported by Higgins et al. (2001).

Interestingly, most of the gentamicin- and levofloxacin-resistant isolates demonstrated multiresistance according to the ATB PSE 5 analysis. However, the antibiotic resistance of trimethoprim-sulfamethoxazole-resistant isolates was limited to one or two classes of antibiotics and none were multiresistant. This might indicate that isolates resistant to gentamicin and levofloxacin carry multidrug-resistant genes which are usually lacking or less diverse resistance genes in case of trimethoprim-sulfamethoxazole- and ceftazidime-resistant isolates. Most of the isolates recovered from the antibiotic containing plates showed resistance to the particular antibiotic from where it was isolated, also in the ATB PSE 5 strip. However, ciprofloxacin was the quinolones class of antibiotics in the strip rather than the levofloxacin used for the isolation in this study. Despite few contradictory results, the MIC values determined with HiComb system (Table 5) also showed comparable resistance of the isolates similar to the EUCAST and the ATB PSE 5 system. Gentamicin-resistant isolate (9AB2, 11AB2) showed resistance to gentamicin, ceftazidime and trimethoprim-sulfamethoxazole whereas the MIC values for TS-resistant isolates showed that they were also resistant according to HiComb system. However, the isolates-resistant to ceftazidime (4AB3, 7AB3, 9AB4) gave the MIC value quite lower than those actually used in the isolation (8-32μg/mL).

The wide prevalence of antibiotic resistance of the isolates in our study could be the results of the wide use of broad-spectrum antibiotics and combination of antibiotics such as penicillin, ampicillin-cloxacillin, pencicillin-streptomycin for the treatment of mastitis in lactating cows in Finland. In addition, trimethoprim-sulfonamides, oxytertracycline, penispiramycin and enrofloxacin are also the available options for parenteral treatment (Pitkälä et al. 2004).

4 CONCLUSIONS

The aims of this study were to investigate the effect of cold storage of raw milk on the antibiotic-resistant bacterial population and evaluate the antibiotic resistance of the resistant isolates recovered from the cold-stored raw milk samples.

The cold storage of raw milk considerably increased the bacterial population, there was no significant difference between the mesophilic and psychrotrophic bacterial population after four days storage at 6°C, suggesting that the population was mostly of psychrotrophic nature. The average mesophilic counts were below 10^5 CFU/mL during two days storage at 4°C: reaffirms that the raw milk can be stored safely for 48 h at 4°C; however, a significant variations of the bacterial population observed among milk samples.

Although antibiotics dependent, the antibiotic-resistant bacterial population increased significantly during the cold storage of raw milk; gentamicin- and levofloxacin-resistant bacteria increased moderately whereas ceftazidime- and trimethoprim-sulfamethoxazole-resistant bacteria rose considerably. An equivalent amount of resistant bacteria were found within 3 days when the raw milk samples were stored at 6°C compared with the counts in the milk samples stored at 4°C for 4 days.

Only 50.9% of the antibiotic-resistant isolates (total 55 isolates) considered for identification were identified to very good, good and acceptable level by phenotypic API 20 NE system, including three isolates identified to genus levels. The most frequently identified were *Sphingomonas paucimobilis* (8), *Pseudomonas putida* (5), *Sphingobacterium spiritivorum* (3) and *Acinetobacter lwoffii* (2). The 16S rDNA gene sequencing identified four isolates (total six isolates) the same as by the API 20 NE system.

Of the 49 isolates considered in the antibiotic resistance study by ATB PSE 5 system, a level of resistance as high as 71.4%, 57.1%, 69.4% and 44.9% were observed for β-lactams, aminoglycosides, poly-peptide and folate pathway inhibitor class of antibiotics respectively. Among them, 59.7% were multiresistant; the levofloxacin-resistant and gentamicin-resistant isolates showed highest multidrug resistance of 85.7 and 91.6% respectively whereas none of the trimethoprim-sulfamethoxazole-resistant isolates showed

multiple resistance. On the other hand, 41.6% of the ceftazidime-resistant isolates demonstrated multidrug resistance.

This study showed that the dairy environment harbours multidrug-resistant Gram-negative psychrotrophic bacteria and the cold chain of raw milk storage amplifies the antibiotic-resistant psychrotrophic population. Although the identification remained controversial or unidentifiable for many resistant isolates considered in this study by API 20 NE system, the significant isolates identified to species level were *Sphingomonas paucimobilis*, *Pseudomonas putida*, *Sphingobacterium spiritivorum* and *Acinetobacter lwoffii*. A better identification by 16S rDNA gene sequencing for few isolates considered in this study suggests that it could be used to supplement the identification of the antibiotic-resistant Gram-negative bacteria in raw milk by API 20 NE system. Further studies are required to get a clear picture of the antibiotic resistance in milk associated bacteria in the farm and the processing plant environment.

REFERENCES

Aarestrup FM. 2005. Veterinary drug usage and antimicrobial resistance in bacteria of animal origin. Basic Clin Pharmacol Toxicol 96(4):271-81.

Aarestrup FM. 1999. Association between the consumption of antimicrobial agents in animal husbandry and the occurrence of resistant bacteria among food animals. Int J Antimicrob Agents 12(4):279-85.

Aarestrup FM, Wegener HC, Collignon P. 2008. Resistance in bacteria of the food chain: Epidemiology and control strategies. Expert Rev Anti Infect Ther 6(5):733-50.

Ahmed AM, Shimabukuro H, Shimamoto T. 2009. Isolation and molecular characterization of multidrug-resistant strains of *Escherichia coli* and *Salmonella* from retail chicken meat in Japan. J Food Sci 74(7):M405-10.

Aminov RI. 2009. The role of antibiotics and antibiotic resistance in nature. Environ Microbiol 11(12):2970-88.

Baker CN, Stocker SA, Culver DH, Thornsberry C. 1991. Comparison of the E test to agar dilution, broth microdilution, and agar diffusion susceptibility testing techniques by using a special challenge set of bacteria. J Clin Microbiol 29(3):533-8.

Barbano DM, Ma Y, Santos MV. 2006. Influence of raw milk quality on fluid milk shelf life. J Dairy Sci 89(Suppl 1):E15-19.

Barbosa TM, Levy SB. 2000. The impact of antibiotic use on resistance development and persistence. Drug Resist Updat 3(5):303-11.

Berg G, Roskot N, Smalla K. 1999. Genotypic and phenotypic relationships between clinical and environmental isolates of *Stenotrophomonas maltophilia*. J Clin Microbiol 37(11):3594-600.

Berge ACB, Champagne SC, Finger RM, Sischo WM. 2007. The use of bulk tank milk samples to monitor trends in antimicrobial resistance on dairy farms. Foodborne Pathog Dis 4(4):397-407.

Boehme S, Werner G, Klare I, Reissbrodt R, Witte W. 2004. Occurrence of antibiotic-resistant enterobacteria in agricultural foodstuffs. Mol Nutr Food Res 48(7):522-31.

Bosshard PP, Zbinden R, Abels S, Böddinghaus B, Altwegg M, Böttger EC. 2006. 16S rRNA gene sequencing versus the API 20 NE system and the VITEK 2 ID-GNB card for identification of nonfermenting Gram-negative bacteria in the clinical laboratory. J Clin Microbiol 44(4):1359-66.

Bramley AJ, McKinnon CH. 1990. The microbiology of raw milk. In: Robinson RK, Editor. Dairy microbiology: The microbiology of milk. 2nd edition. London: Elsevier Applied Science. p 163-208.

Citak S, Yucel N, Mendi A. 2005. Antibiotic resistance of Enterococcal isolates in raw milk. J Food Process Preserv 29(3-4):183-95.

Cousin MA. 1981. Presence and activity of psychrotrophic microorganisms in milk and dairy products: A review. J Food Prot 45:172-207.

Delbès C, Ali-Mandjee L, Montel M-C. 2007. Monitoring bacterial communities in raw milk and cheese by culture-dependent and -independent 16S rRNA gene-based analyses. Appl Environ Microbiol 73(6):1882-91.

[EC] European Community, Regulation 853/04. 2004. European parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin.

Eneroth A, Christiansson A, Brendehaug J, Molin G. 1998. Critical contamination sites in the production line of pasteurised milk, with reference to the psychrotrophic spoilage flora. Int Dairy J 8(9):829-34.

Ercolini D, Russo F, Ferrocino I, Villani F. 2009. Molecular identification of mesophilic and psychrotrophic bacteria from raw cow's milk. Food Microbiol 26(2):228-31.

[EUCAST] European Committee for Antimicrobial Susceptibility Testing. 2000. Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by agar dilution. Clin Microbiol Infect 6(9):509-15.

Giannino ML, Aliprandi M, Feligini M, Vanoni L, Brasca M, Fracchetti F. 2009. A DNA array based assay for the characterisation of microbial community in raw milk. J Microbiol Methods 78(2):181-8.

Gilmour A, Rowe TM. 1990. Microorganisms associated with milk. In: Robinson RK, Editor. Dairy microbiology: The microbiology of milk. 2nd edition. London: Elsevier Applied Science. p 37-75.

Guardabassi L, Courvalin P. 2006. Modes of antimicrobial action and mechanisms of bacterial resistance. In: Aarestrup FM, Editor. Antimicrobial resistance in bacteria of animal origin. Washington, DC, USA: ASM Press. p 1-18.

Guinot-Thomas P, Ammoury MA, Laurent F. 1995. Effects of storage conditions on the composition of raw milk. Int Dairy J 5(2):211-23.

Hanage WP, Fraser C, Spratt BG. 2006. Sequences, sequence clusters and bacterial species. Philos Trans R Soc Lond B Biol Sci 361(1475):1917-27.

Hantsis-Zacharov E, Halpern M. 2007. Culturable psychrotrophic bacterial communities in raw milk and their proteolytic and lipolytic traits. Appl Environ Microbiol 73(22):7162-8.

Hawkey PM, Jones AM. 2009. The changing epidemiology of resistance. J Antimicrob Chemother 64 (Suppl 1): i3-10.

Hayes MC, Boor K. 2001. Raw milk and fluid milk products. In: Marth EH, Steele JL, Editors. Applied dairy microbiology. 2nd edition. Basel, NY, USA: Marcel Dekker. p 59-76.

Higgins CS, Murtough SM, Williamson E, Hiom SJ, Payne DJ, Russell AD, Walsh TR. 2001. Resistance to antibiotics and biocides among non-fermenting Gram-negative bacteria. Clin Microbiol Infect 7(6):308-15.

Holm C, Jepsen L, Larsen M, Jespersen L. 2004. Predominant microflora of downgraded Danish bulk tank milk. J Dairy Sci 87(5):1151-7.

Jayarao BM, Henning DR. 2001. Prevalence of foodborne pathogens in bulk tank milk. J Dairy Sci 84(10):2157-62.

Jayarao BM, Wang L. 1999. A study on the prevalence of Gram-negative bacteria in bulk tank milk. J Dairy Sci 82(12):2620-4.

Johnson ME. 2001. Cheese products. In: Marth EH, Steele JL, Editors. Applied dairy microbiology. 2nd edition. Basel, NY, USA: Marcel Dekker. p 345-84.

Jorgensen JH, Ferraro MJ. 2009. Antimicrobial susceptibility testing: A review of general principles and contemporary practices. Clin Infect Dis 49(11):1749-55.

Koluman A, Akan LS, Çakiroğlu FP. 2009. Occurrence and antimicrobial resistance of enterococci in retail foods. Food Control 20(3):281-3.

Koskinen R, Ali-Vehmas T, Kämpfer P, Laurikkala M, Tsitko I, Kostyal E, Atroshi F, Salkinoja-Salonen M. 2000. Characterisation of *Sphingomonas* isolates from Finnish and Swedish drinking water distribution systems. J Appl Microbiol 89(4):687-96.

Kumaresan G, Annalvilli R, Sivakumar K. 2007. Psychrotrophic spoilage of raw milk at different temperatures of storage. J Appl Sci Res 3(11):1383-87.

Lafarge V, Ogier J-C, Girard V, Maladen V, Leveau J-Y, Gruss A, Delacroix-Buchet A. 2004. Raw cow milk bacterial population shifts attributable to refrigeration. Appl Environ Microbiol 70(9):5644-50.

Levy SB. 2002. Factors impacting on the problem of antibiotic resistance. J Antimicrob Chemother 49(1):25-30.

Levy SB, Marshall B. 2004. Antibacterial resistance worldwide: Causes, challenges and responses. Nat Med 10(Suppl 12):S122-9.

Mascaretti OA. 2003. Bacteria versus antibacterial agents: An integrated approach. Washington, DC, USA: ASM Press. 393 p.

Menezes GA, Harish BN, Sujatha S, Vinothini K, Parija SC. 2008. Emergence of vancomycin-intermediate *Staphylococcus* species in southern India. J Med Microbiol 57(7):911-2.

Mølbak K. 2004. Spread of resistant bacteria and resistance genes from animals to humans - The public health consequences. J Vet Med B Infect Dis Vet Public Health 51(8-9):364-9.

Munsch-Alatossava P, Alatossava T. 2007. Antibiotic resistance of raw-milk-associated psychrotrophic bacteria. Microbiol Res 162(2):115-23.

Munsch-Alatossava P, Alatossava T. 2006. Phenotypic characterisation of raw milk-associated psychrotrophic bacteria. Microbiol Res 161(4):334-46.

Nam H-M, Lim S-K, Kim J-M, Joo Y-S, Jang K-C, Jung S-C. 2010. In vitro activities of antimicrobials against six important species of Gram-negative bacteria isolated from raw milk samples in Korea. Foodborne Pathog Dis 7(2):221-4.

Nicodemo AC, Araujo MRE, Ruiz AS, Gales AC. 2004. In vitro susceptibility of *Stenotrophomonas maltophilia* isolates: Comparison of disc diffusion, Etest and agar dilution methods. J Antimicrob Chemother 53(4):604-8.

Nikaido H. 2009. Multidrug resistance in bacteria. Annu Rev Biochem 78:119-46.

Ogier J-C, Son O, Gruss A, Tailliez P, Delacroix-Buchet A. 2002. Identification of the bacterial microflora in dairy products by temporal temperature gradient gel electrophoresis. Appl Environ Microbiol 68(8):3691-701.

Oliver SP, Boor KJ, Murphy SC, Murinda SE. 2009. Food safety hazards associated with consumption of raw milk. Foodborne Pathog Dis 6(7):793-806.

Pitkälä A, Haveri M, Pyörälä S, Myllys V, Honkanen-Buzalski T. 2004. Bovine mastitis in Finland 2001 - Prevalence, distribution of bacteria, and antimicrobial resistance. J Dairy Sci 87(8):2433-41.

Rasolofo EA, St-Gelais D, LaPointe G, Roy D. 2010. Molecular analysis of bacterial population structure and dynamics during cold storage of untreated and treated milk. Int J Food Microbiol 138(1-2):108-18.

Ryan MP, Adley CC. 2010. *Sphingomonas paucimobilis*: A persistent Gram-negative nosocomial infectious organism. J Hosp Infect 75(3):153-7.

Sackey BA, Mensah P, Collison E, Sakyi-Dawson E. 2001. *Campylobacter*, *Salmonella*, *Shigella* and *Escherichia coli* in live and dressed poultry from metropolitan Accra. Int J Food Microbiol 71(1):21-8.

Sader HS, Jones RN. 2005. Antimicrobial susceptibility of uncommonly isolated non-enteric Gram-negative bacilli. Int J Antimicrob Agents 25(2):95-109.

Schwarz S, Silley P, Simjee S, Woodford N, van duijkeren E, Johnson AP, Gaastra W. 2010. Editorial: Assessing the antimicrobial susceptibility of bacteria obtained from animals. J Antimicrob Chemother 65(4):601-4.

Shiferaw B, Yang S, Cieslak P, Vugia D, Marcus R, Koehler J, Deneen V, Angulo F. 2000. Prevalence of high-risk food consumption and food-handling practices among adults: A multistate survey, 1996 to 1997. J Food Prot 63(11):1538-43.

Silbergeld EK, Graham J, Price LB. 2008. Industrial food animal production, antimicrobial resistance, and human health. Annu Rev Public Health 29:151-69.

Sørum H, L'Abée-Lund TM. 2002. Antibiotic resistance in food-related bacteria - A result of interfering with the global web of bacterial genetics. Int J Food Microbiol 78(1-2):43-56.

Straley BA, Donaldson SC, Hedge NV, Sawant AA, Srinivasan V, Oliver SP, Jayarao BM. 2006. Public health significance of antimicrobial-resistant Gram-negative bacteria in raw bulk tank milk. Foodborne Pathog and Dis 3(3):222-33.

Uraz G, Çitak S. 1998. The isolation of *Pseudomonas* and other Gram (-) psychrotrophic bacteria in raw milks. J Basic Microbiol 38(2):129-34.

Valenzuela AS, Omar Nb, Abriouel H, López RL, Veljovic K, Cañamero MM, Topisirovic MKL, Gálvez A. 2009. Virulence factors, antibiotic resistance, and bacteriocins in enterococci from artisan foods of animal origin. Food Control 20(4):381-5.

van den Bogaard AE, Stobberingh EE. 2000. Epidemiology of resistance to antibiotics: Links between animals and humans. Int J Antimicrob Agents 14(4):327-35.

Vandamme P, Pot B, Gillis M, de Vos P, Kersters K, Swings J. 1996. Polyphasic taxonomy, a consensus approach to bacterial systematic. Microbiol Rev 60(2): 407-38.

Verdier-Metz I, Michel V, Delbès C, Montel M-C. 2009. Do milking practices influence the bacterial diversity of raw milk? Food Microbiol 26(3):305-10.

Walsh C. 2003. Antibiotics: actions, origins, resistance. Washington, DC, USA: ASM Press. 335 p.

Walsh C, Fanning S. 2008. Antimicrobial resistance in foodborne pathogens - A cause for concern? Curr Drug Targets 9(9):808-15.

Wang HH, Manuzon M, Lehman M, Wan K, Luo H, Wittum TE, Yousef A, Bakaletz LO. 2006. Food commensal microbes as a potentially important avenue in transmitting antibiotic resistance genes. FEMS Microbiol Lett 254(2):226-31.

Wang L, Jayarao BM. 2001. Phenotypic and genotypic characterisation of *Pseudomonas fluorescens* isolated from bulk tank milk. J Dairy Sci 84(6):1421-9.

White DG, Zhao S, Sudler R, Ayers S, Friedman S, Chen S, McDermott PF, McDermott S, Wagner DD, Meng J. 2001. The isolation of antibiotic-resistant *Salmonella* from retail ground meats. N Engl J Med 345(16):1147-54.

Wiedmann M, Weilmeier D, Dineen SS, Ralyea R, Boor KJ. 2000. Molecular and phenotypic characterisation of *Pseudomonas* spp. isolated from milk. Appl Environ Microbiol 66(5):2085-95.

Wright GD. 2010. Antibiotic resistance in the environment: A link to the clinic? Curr Opin Microbiol 13(5):589-94.

Zall RR. 1990. Control and destruction of microorganisms. In: Robinson RK, Editor. Dairy microbiology: The microbiology of milk. 2nd edition. London: Elsevier Applied Science. p 115-61.