MICROBIOLOGICAL QUALITY OF COWS' MILK IN ORGANIC FARMING (PRELIMINARY REPORT)

Gundega Gulbe, **Anda Valdovska**
Latvia University of Agriculture
e-mail: gundega.gulbe85@gmail.com

Abstract
The objective of the present study was to investigate the microbiological content of cows' milk in Latvia's organic farms with a purpose to detect potential microbiological threats in milk. Samples were collected in December 2011 at 12 biological dairy farms of Latvia. Raw milk samples (N=155) obtained from cow composite milk were studied. The total mesophilic aerobic and facultative anaerobic microorganisms (MAFAM), the presence of coliforms and coagulase-positive staphylococci, count of yeasts and moulds were analysed using standard methods. Of the sampled cows 50% had a low somatic cell count (SCC) (<200,000 cells mL^{-1}), 23% - high, but 27% had a very high SCC (>500,000 cells mL^{-1}). The mean value of MAFAM in the samples with low, high and very high SCC was 4.7, 5.0 and 5.0 log10 colony forming units (cfu) mL^{-1}, respectively. The yeasts were present in 57% of milk samples with the mean concentration of 3.1 log10 cfu mL^{-1}. Moulds were found in 27% of all milk samples; their mean concentration was 4.4 log10 cfu mL^{-1}. Identified mould strains belonged to genera *Absidia*, *Aspergillus*, *Geotrichum*, *Mucor* and *Penicillium*. In cases of subclinical mastitis and latent mammary infection the most distributed mastitis pathogens were *Staphylococcus aureus*, *Micrococcus kristinae*, *Bacillus cereus* and coagulase negative staphylococci.
Key words: raw milk, microbiological quality, organic farming.

Introduction

Mastitis (an inflammation of the udder) is the most common disease affecting dairy cattle herds and the first cause of economic loss in milk production worldwide (Maréchal et al., 2011). Organic dairy farmers have identified mastitis as a major concern, mainly due to non-use of administration of long-acting intramammary antibiotics at dry-off. There is a study on mastitis that reveals that there are - more pathogenic and contagious species of mastitis causing bacteria obtained from cows on organic farms compared to milk samples collected from cows on conventional farms (Ruegg, 2009). This difference can be explained by inability of organic farmers to use effective mastitis control strategies sufficiently (Ruegg, 2009).

Council Regulation (EC) No 834/2007 of 28 June 2007 on organic production and labelling of organic products determines that for organic herd prophylaxis and treatment of ill animals, phytotherapeutic and homeopathic products may be used. Only in cases, where the above treatments do not give desired results, it is allowed to use chemically synthesised allopathic veterinary products, including antibiotics. Antibiotics should not be used at drying off and are only allowed therapeutically during lactation in case of emergency. These measures could compromise the control and treatment of clinical disease and the herd health and welfare; therefore, it is necessary to establish an effective mastitis control strategy suitable for organic farming.

The objective of this study was to investigate the microbiological content of cows' milk in Latvia's organic farms with a purpose to detect potential microbial threats in milk. Further studies will be performed with the aim to develop effective mastitis control strategy including immunization of cows with mastitis vaccine in organic dairy herds in Latvia.

Materials and Methods

The collection of raw milk samples took place in December 2011 at 12 organic dairy farms of Latvia's regions – four farms from Zemgale, three – from Vidzeme, three – from Latgale and two – from Kurzeme. The organic farms were registered by the state control institutions. Herd size varied from 7 to 277 animals in a cow-shed including six herds with 7-50 cows, four - with 51-100 cows and two herds with more than 100 cows. Fifteen lactating cows from each herd were chosen for sampling. Milk samples in the herd less than 15 cows were collected of all lactating animals. The study included various breeds (Latvian Brown, Holstein and Danish Red) as well as different varieties of cross-breeds from the first to tenth lactation.

Sampling

Milk samples were collected by trained farm personnel from a cow level (cow composite milk) during sampling procedure of milk quality monitoring according to the standard LVS 175:1999 'Sampling of raw milk'. Samples for somatic cell count (SCC) evaluation were collected in 50 mL tubes with preservative, transported to the Laboratory of milk quality of the 'Siguldas Artificial insemination and Stock breeding station' (Sigulda, Latvia) and analyses were performed according to the standard LVS EN ISO 13366-2:2007. Samples for microbiological examination were collected in sterile vacutainers, 7 mL amount (Vacutest Kima, Italy) and transported to the Laboratory of Microbiology of the Research Institute of Biotechnology and Veterinary Medicine

'Sigra' (Sigulda, Latvia) in cold chain under temperature 10 °C and frozen at -20 °C for 2-6 weeks until an examination was done. A total 155 raw milk samples were analysed.

Microbiological examination

The samples were defrosted at room temperature and serially decimal diluted with Maximum recovery dilutent (Oxoid, England) according to the standard LVS EN ISO 6887-5:2011 'Microbiology of food and animal feeding stuffs - Preparation of test samples, initial suspension and decimal dilutions for microbiological examination - Part 5: Specific rules for the preparation of milk and milk products (ISO 6887-5:2010)' and appropriate dilutions were plated on to agars.

For the enumeration of total mesophilic aerobic and facultative anaerobic microorganisms (MAFAM), Milk agar (Oxoid, England) according to standard LVS EN ISO 7218:2007 'Milk and milk products - Enumeration of colony-forming units of microorganisms - colony count technique at 30 °C' was used. Acolyte Colony counter (Synbiosis, UK) for colonies enumeration was used. For the enumeration of yeasts and moulds Sabouraud Dextrose agar (Biolife, Italia) was used according to the standard LVS ISO 21527-1:2008 'Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of yeasts and moulds - Part 1: Colony count technique in products with water activity greater than 0.95'. Plates were incubated at 22.5 °C for 10 days; subsequently, an enumeration was performed. Primary classification of moulds was based on colony characteristics (pigmentation, shape, background colour) and on microscopic examination according to Hungerford et al. (1998) and G. R. Carter and D. J. Wise (2004).

For the enumeration of bacteria and evaluation of haemolysis, we used blood agar medium containing 50 g L^{-1} sheep blood; plates were incubated for 24 h at 37 °C. If there were not present positive culture on following mediums, cultures from blood agar were identified using an identification system 'BBL Crystal Gram-positive and Enteric/Nonfermenter ID' (Becton, Dickinson and Company, USA). If on the blood agar Gram-positive bacillus was detected, colonies were transferred to a 'Bacillus cereus selective agar' (Oxoid, England). Incubation at 30 °C for 18 h and microscope examination for typical colonies of *Bacillus cereus* (*B. cereus*) was performed. Baird Parker agar with egg yolk supplement (Biolife, Italia) for the enumeration of staphylococci was used according to the standard LVS EN ISO 6888-1: 1999/A1:2003 'Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of coagulase-positive staphylococci (*S.aureus* and other species) - Part 1: Technique using Baird-Parker agar medium - Amendment 1: Inclusion of precision data'. Plates were incubated for 48 h at 37 °C. Presumptive coagulase-positive staphylococci colonies were transferred to a Brain heart infusion broth (Biolife, Italia), a Mannitol salt agar (Biolife, Italia) and tested for coagulase production using Rabbit Coagulase Plasma (Becton, Dickinson and Company, USA). *S. aureus* identification was confirmed with a diagnostic reagent 'Staphytect Plus' (Oxoid, England). Staphylococci other than *S. aureus* were identified using 'BBL Crystal Gram-positive ID' system. The isolation of Gram-negative bacteria including coliforms was carried out on Mac Conkey agar (Becton, Dickinson and Company, USA). Plates were incubated for 24 h at 37 °C. Isolated colonies were transferred to a 'Chromogenic E. coli/Coliform selective medium' (Biolife, Italia) for differentiation of *Escherichia coli* (*E. coli*) and other coliforms; tested by Reagent Stain dropper (Becton, Dickinson and Company, USA) for indole production and oxidase fermentation. For isolates not conformed to these methods, 'BBL Crystal Enteric/Nonfermenter ID' system was used.

Estimation of microbiological indices was done in accordance with the Council Regulation No 853/2004 of 29 April 2004 'Laying down specific hygiene rules for on the hygiene of foodstuffs', section IX 'Raw milk and dairy products'.

Categorisation of data

A milk sample was categorised as positive if at least one colony-forming unit of *S. aureus* or *Streptococcus agalactiae* (*S. agalactiae*) was isolated. For other microorganisms, the presence of at least three colony-forming units for positive categorisation was needed. If moderate to high growth of a major udder pathogen was found in combination with a few colony-forming units of several contaminating species, the sample would be diagnosed as positive for growth of the major udder pathogen. For the data analysis, milk secretion was categorised as Normal secretion, Disturbed secretion, Latent infection and Mastitis, according to International Dairy Federation (IDF), see Table 1. Threshold for somatic cell count estimation was 200,000 cells mL^{-1} in cow composite milk.

Table 1

Parameters for estimating milk secretion (adapted from IDF)

Bacterial culture	SCC is low	SCC is high
Negative	Normal secretion	Disturbed secretion
Positive	Latent infection	Mastitis

Using fix thresholds of 200,000 cells mL^{-1} and 500,000 cells mL^{-1}, three different somatic cell count categories were defined: low SCC<200,000, high SCC 200,000-500,000 and very high SCC>500,000.

Statistical analysis

The data were analysed using the SPSS 9.0.0 software package (SPSS Inc., Germany). Descriptive statistics including average, standard deviation and frequencies was done. To determine whether the effect of MAFAM, yeasts and moulds count was significant in explaining the variations in somatic cell count and secretion, the data were subjected to ANOVA followed by Univariate comparisons. Data are presented as mean ± standard deviation and a probability value $p<0.05$ was considered significant.

Results and Discussion

Milk quality can be estimated by count of somatic cells (SCC), mesophilic aerobic and facultative anaerobic microorganisms (MAFAM), coliforms and *S.aureus* (Nikolajeva, 2011). Milk is a complex biological fluid and by its nature, a good growth medium for many microorganisms. Because of the specific production, it is impossible to avoid contamination of milk with microorganisms; therefore, the microbiological content of milk is a major feature in determining its quality (Torkar and Teger, 2008).

Somatic cell count (SCC)

The SCC of milk is widely used to monitor udder health and milk quality (Sharif and Muhammad, 2008). SCC and bacteriological examination indicate the status of mammary gland as SCC in milk increases during intramammary infection (Harmon, 1994). Elevated SCC primarily consists of leucocytes, which include macrophages, lymphocytes and neutrophils. During inflammation, major increase in SCC is because of the influx of neutrophils into milk. Higher the SCC means greater the risk of raw milk contamination with pathogens (Sharif and Muhammad, 2008). Fifty percent of examined samples had a low SCC, 23% - high and 27% had a very high SCC. The mean value of SCC in the samples with low, high and very high SCC was 4.9, 5.5 and 6.3 log10 mL^{-1}, respectively. According to categorization of milk secretion, the mean SCC is the highest in cases of subclinical mastitis (1,293.333 or 6.1 log10 mL^{-1}), the smallest – in cases of the normal secretion (72,825 mL^{-1} or 4.9 log10 mL^{-1}), but the mean SCC of latent infection and disturbed secretion is not significantly different (Fig. 1). This means that the use of a single SCC analysis to classify quarters as uninfected or infected may not be a useful test, and bacteriological examination is strictly necessary.

C.B. Malek dos Reis et al. (2011) explain that the main factors responsible for SCC variation in mammary quarters are the occurrence of intramammar infections and the bacterial species. Milk samples with major pathogens isolation elicited higher SCC than those with minor pathogens.

Total mesophilic aerobic and facultative anaerobic microorganisms (MAFAM)

Milk may contain high bacterial numbers which form part of the product's natural microflora. The high MAFAM in some cases is related to mastitis, but not always. Mostly high MAFAM indicates milk contamination due to inadequate hygiene (Murphy, 2008). The mean value of MAFAM in the samples with low, high and very high SCC was 4.7, 5.0 and 5.0 log10 cfu mL^{-1}, respectively. As assessed by MAFAM, analyzed milk meets the Council Regulation 853/2004 requirements (maximum 5.0 log10 cfu mL^{-1}), however, it contains a large quantity of microorganisms; probably due to inadequate milking hygiene or insufficient cooling of milk and/or maintenance of cold chain thereafter.

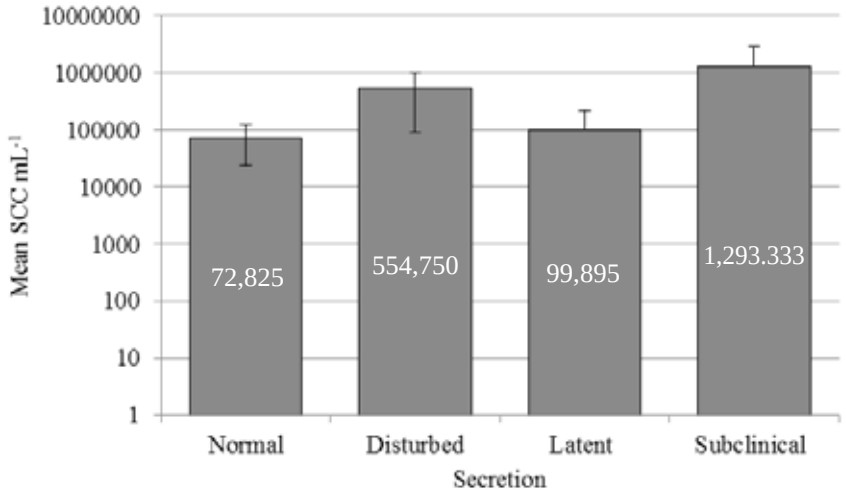

Figure 1. The mean SCC by categories of milk secretion.

Yeasts and moulds

Yeasts and moulds are normally regarded as spoilage organisms in milk. Moulds, mainly species of *Aspergillus*, *Fusarium*, and *Penicillium* can grow in milk and have ability to produce mycotoxins which can be a health hazard. Yeast spoilage is not a hazard to health (Douglas Goff, 1995). In rare cases moulds and yeasts can cause mastitis in cows, especially after the prolonged use of antibiotics (Britt, 1998). In our study the yeasts were present in 57% of milk samples with the mean concentration of 3.1 log10 cfu mL^{-1}. Moulds were found in 27% of samples and their mean concentration was 4.4 log10 cfu mL^{-1}. Correlation between SCC and count of yeast and moulds in milk was not established. For one half (n=21) of isolated moulds microscopic examination was performed and identified mould strains belonged to genera *Absidia* (n=4), *Aspergillus* (n=3), *Geotrichum* (n=1), *Mucor* (n=9) and *Penicillium* (n=4). There is the potential hazard from production of mycotoxins by moulds of genus *Aspergillus* and *Penicillium*, isolated of milk in this study.

Presence of *Enterobacteriaceae*

Enterobacteriaceae is a group of microorganisms which includes several that cause primary infections of the human gastrointestinal tract (Fox, 2010). In our study bacteria from genus *Enterobacteriaceae* were found in 11.6% of milk samples. tThis group includes *Serratia spp*., *Pseudomonae fluorescens* and coliform bacteria – *E. coli*, *Klebsiella oxytoca* and other undifferentiated. Researchers (Haguingan et al., 2010; Dadkhah et al., 2011) have found that udder infection with *Serratia spp.,* some strains of *E.coli* and *Klebsiella spp.,* less frequently *Pseudomonae spp.* may result in a severe clinical mastitis in cows. All of isolated bacteria can be causative agents for mastitis.

Isolated bacteria

1) In association with categories of somatic cell count

Bacterial growth occurred in 97% of samples. *S. aureus, Micrococcus kristinae* (*M. kristinae)* and coagulase negative staphylococci (CoNS) were the most prevalent agents with very high SCC and were isolated 29.6%, 11.1%, 11.1% milk samples, respectively. *S.aureus, M. kristinae* and microorganisms of genus *Enterobacteriaceae* were the most prevalent agents with high SCC and were isolated 20.5%, 13.6% and 13.6% milk samples, respectively. The most prevalent agents isolated from samples with low SCC were *M. kristinae, CoNS* and *S. aureus* with incidence 15.3%, 15.3% and 10.6% milk samples, respectively. All isolated bacteria groups are showed in Table 2.

Table 2

Incidence of bacteria in cows' composite milk samples

Isolated bacteria	Low SCC <200,000 mL^{-1}		High SCC 200,000-500,000 mL^{-1}		Very high SCC >500,000 mL^{-1}	
	n=85	%	n=44	%	n=54	%
S. aureus	9	10.6	9	20.5	16	29.6
M. kristinae	13	15.3	6	13.6	6	11.1
CoNS[1]	13	15.3	4	9.1	6	11.1
Bacillus cereus	2	2.4	3	6.8	5	9.3
C. aquatica	3	3.5	4	9.1	3	5.6
Enterobacteriaceae[2]	7	8.2	6	13.6	4	7.4
Lactic acid bacteria[3]	3	3.5	3	6.8	5	9.3
CoPS[4]	1	1.2	1	2.3	0	0.0
Other Gram-positive	15	17.6	1	2.3	2	3.7
Other Gram-negative	3	3.5	2	4.5	0	0.0
Culture negative	4	4.7	1	2.3	0	0.0
Other microorganisms[5]	12	14.1	4	9.1	7	13.0

CoNS[1] includes *S. saprophyticus*, *S. kloosi, S. equorum* and other undifferentiated; *Enterobacteriaceae*[2] include*Serratia spp*., *Pseudomonae fluorescens*, *E. coli*, *Klebsiella oxytoca* and other undifferentiated; Lactic acid bacteria[3] include *Lactococcus lactis ssp. lactis, Lactococcus lactis ssp. cremoris* and *Pediococcus pentosaceus*; CoPS[4] (Coagulase positive staphylococci) includes *S. intermedius* and other undifferentiated except for *S. aureus*; Other microorganisms[5] include *Micrococcus spp*., *Bacillus spp., Enterococcus faecalis*, *Gemella haemolysans, Acinetobacter baumanii, Actinomyces pyogenes* and *S. agalactiae*.

Isolated microorganisms can be divided into several groups depending on their significance in milk quality and effect on udder health: Group of **lactic acid bacteria** *(Lactococcus lactis ssp. lactis, Lactococcus lactis ssp. cremoris, Pediococcus pentosaceus, Enterococcus faecalis)* is able to ferment lactose to lactic acid. They are normally present in the milk and are also used as starter cultures in the production of cultured dairy products. **Spoilage bacteria** are involved in spoilage of milk, if they are psychrotrophic organisms *(P. fluorescens, Bacillus spp., Micrococcus spp., Corynebacterium spp.)*. Most psychrotrophs are destroyed by pasteurization temperatures; however, some like *P. fluorescens* can produce proteolytic and lipolytic extracellular enzymes which are heat resistant and capable of causing spoilage. Some species and strains of *Bacillus, Corynebacterium, Lactobacillus, Micrococcus*, and *Streptococcus* can survive pasteurization and grow at refrigeration temperatures which can cause spoilage problems (Lin, 1997). **Coliform bacteria** *(E. coli, K. oxytoca)* are indicator organisms; they are closely associated with the presence of pathogens but not necessarily pathogenic themselves. They also can cause rapid spoilage of milk, because they are able to ferment lactose with the production of acid and gas, and are able to degrade milk proteins. The main bacterium of this group is *E. coli*. Some serotypes of *E. coli* can cause food poisonings and alimentary intoxications in human, the most dangerous among them are enterohemorrhagic *E. coli* strains, especially serotype O157:H7 (Usajewicz and Nalepa, 2006). **Pathogenic bacteria** can be divided into contagious *(S. aureus, S. agalactiae)* and environmental *(Staphylococcus spp., Bacillus cereus (B. cereus), M. kristinae, Acinetobacter baumanii, Actinomyces pyogenes)* pathogens. There have been a number of foodborne illnesses resulting from the ingestion of raw milk from a mastitic cow, or dairy products made from properly pasteurized milk (Nikolajeva, 2011; Douglas Goff, 1995; Guidelines, 2002). *S.aureus* is the bacterium with the largest interest in food toxicoinfections, because of some *S. aureus* strains are able to produce staphylococcal enterotoxins that cause gastroenteritis (Bennett and Hait, 2011).

2) In association with categories of milk secretion

According to categorization of milk secretion, the most distributed mastitis pathogens are *S. aureus, M. kristinae, B. cereus* and CoNS in cases of subclinical mastitis and latent mammary infection. (see Figure 2).

In this study ***S. aureus*** was the most frequent isolated pathogen - 35% of subclinical mastitis and 24% of latent udder infection cases. *S. aureus* is a common cause of bovine mastitis and its incidence is still high. A. Jemeljanovs et al. (2008) referred to 27%, J.M.B. Haguingan et al. (2010) - 31% and I. Klimiene et al. (2011) refered to 19% incidence of *S. aureus* in cases of subclinical mastitis. ***M. kristinae*** incidence in our study was 17% of subclinical mastitis and 34% of latent udder infection cases. Literature contains little information about *M. kristinae*. Most of these microorganisms are commensals to the human skin flora and cause infection in some cases. J.M.B. Haguingan et al. (2010) referred to 0.5% incidence of *M. kristinae* in cases of bovine subclinical mastitis. We isolated ***B. cereus*** with incidence of 11% of cases of subclinical mastitis. Since the *B. cereus* was isolated only from two herds, this incidence cannot be

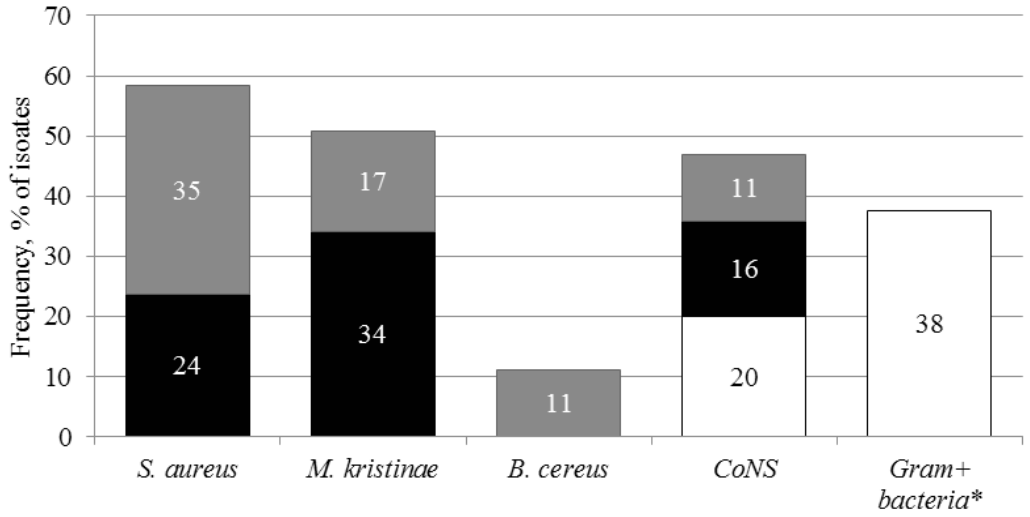

Figure 2. The incidence of the most distributed bacteria in cows' composite milk samples according to udder health categories: □ Normal secretion, ■ Latent infection, ▩ Subclinical infection, * other Gram-positive bacteria, including undifferentiated environmental non-pathogenic bacteria.

applied to all investigated herds and further data are required. *Bacillus* spp., including *B. cereus* are widely distributed in nature, and it is a frequent contaminant in raw milk and dairy products. In order to claim that *B. cereus* is a cause of intramammary infection, pure culture and association with high SCC or clinical signs of udder disease must be identified (Gonzales, 1996). There are authors that refer to fairly high incidence (30%) of contamination of raw milk by *B. cereus* (Hassan et al., 2010). In the context with subclinical mastitis, J.M.B. Haguingan et al. (2010) referred to presence of *B. cereus* in 7.6% of milk samples. Incidence of **coagulase negative staphylococci** in our study was 11%, 16% and 20% in cases of subclinical mastitis, latent udder infection and normal secretion, respectively. The most frequently diagnosed CoNS was *S. saprophyticus* - 5% of all cases of mastitis and latent udder infection. CoNS have traditionally been considered to be minor mastitis pathogens that can cause mastitis as opportunistic bacteria. The main reason for this is that mastitis caused by CoNS is mild, and usually remains subclinical (Taponen et al., 2006). The significance of CoNS, however, needs to be reconsidered as in many countries they have become the most common mastitis-causing agents (Pitkala et al., 2004). A. Jemeljanovs et al. (2008) admitted that CoNS incidence was 27% from subclinical mastitis secretion. Researchers have isolated more than ten different CoNS species from milk obtained from mastitis affected bovine udders. Most commonly reported species are *S. chromogenes, S. epidermidis, S. simulans* and *S. hyicus* (Thorberg et al., 2006; Pyörälä and Taponen, 2009; Klimiene et al., 2011). Some CoNS isolated from mastitis may be opportunists from the environment, but in S. Pyörälä and S. Taponen (2009) opinion, it is very likely that at least the main species infecting the bovine mammary gland are specifically adapted to the udder environment.

Further investigations are necessary to determine the source of microorganisms detected in milk and find out which ones are involved in mastitis aetiology.

Conclusions

1. The mean value of total mesophilic aerobic and facultative anaerobic microorganisms' count in the samples with low, high and very high SCC was 4.7, 5.0 and 5.0 log10 cfu mL^{-1}, respectively.
2. Bacteria from genus *Enterobacteriaceae* were found in 11.6% of milk samples, including coliforms of 2.6%.
3. The yeasts were present in 57% of milk samples with the mean concentration of 3.1 log10 cfu mL^{-1}.
4. Moulds were found in 27% of all milk samples and mean concentration was 4.4 log10 cfu mL^{-1}. Identified mould strains belonged to genera *Absidia*, *Aspergillus*, *Geotrichum*, *Mucor* and *Penicillium*.
5. In cases of subclinical mastitis and latent mammary infection, the most distributed pathogens were *Staphylcoccus aureus*, *Micrococcus kristinae*, *Bacillus cereus* and coagulase negative staphylococci. In cases of normal secretion the most isolated bacteria were gram-positive undifferentiated environmental bacteria and coagulase negative staphylococci.

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4. Family orientation to traditional relationship with professionals in rural area where families are information receivers but not coordinators, social isolation is determined as a value that protects from social intolerance, comprehension that could make a risk to family isolation in the rural area where children are with special needs.
5. Technological opportunities of knowledge society can develop new solutions in family educations that could be economically suitable and attainable with infrastructure improvements, using existent professionals and activating family resources. Information obtained in e-environment, e-communication with professionals and online consultations, family education organisations as e-courses can be future opportunity potentials.

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