MICROBIOLOGICAL CONTENT OF COW MILK DEPENDING ON SEASON AND HERD SIZE IN LATVIAN ORGANIC FARMS

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Abstract

The objective of the study was to investigate the microbiological content of cow (*Bos primigenius*) milk in Latvian organic farms according to season and herd size with a purpose to detect their impact on the distribution of mastitis causing pathogens in milk. Samples were collected in 14 organic dairy farms of Latvia, 4 times through 2012: in winter, autumn, spring and summer period. Raw milk samples (n=564) obtained from cow composite milk were studied. The samples were divided into three groups in accordance with the number of cows in the cow-shed: A (3-30), B (31-60) and C (61-124). The total colony count (TTC) and the isolation of mastitis causing bacteria were analysed using standard methods. Bacterial growth occurred in 90.4% of samples. Isolated microorganisms belonged to 35 species, and the following bacteria were the most prevalent agent, including Coagulase negative staphylococci in 29.4%, *Staphylococcus aureus* in 24.1%, *Kocuria kristinae* in 12.9%, and *Enterobacteriaceae spp.* in 10.3% out of 564 milk samples. Depending on the season, the average number of TCC was the lowest in summer (4.66 ± 4.01 $\log_{10}$ CFU mL^{-1}), moderately higher in spring and winter (4.72 ± 4.18 and 4.82 ± 3.54 $\log_{10}$ CFU mL^{-1}, respectively), but significantly higher in autumn (5.43 ± 4.80 $\log_{10}$ CFU mL^{-1}). Coliforms were not isolated from group A herds instead of B (3.1%) and C (4.6%). Occurrence of *Staphylococcus aureus* was noticeably higher in B (19.1%) and C (20.8%) than in A (12.6%) herds. Most of group A herds met the milk quality requirements, while group C herds produced more contaminated milk.

Key words: raw milk, microbiological quality, organic farming.

Introduction

Over the past years, in the European Union, including Latvia, and also worldwide, the demand for organic agricultural products has noticeably increased. The organic sector in the EU has been rapidly developing during the past years. During the last decade, organic area in the EU enlarged by about 500,000 hectares per year. The whole organic area represents 5.4% of the total utilised agricultural area in Europe. In Latvia the organic dairy cows (*Bos primigenius*) make up 9.6% of the total dairy cow herd and their produced milk comprises 7% of the total produced milk amount in Latvia (Facts and figures on organic agriculture…, 2013). Based on the current growth rate of organic food consumption, it is predicted that the demand for organically produced dairy products will continue to increase (Batte et al., 2007).

Due to the high nutritional value of milk, together with the neutral pH and high water activity, the raw milk serves as an excellent growth medium for different microorganisms including many spoilage and pathogenic bacteria (Frank, 2007). Researchers have observed that numerous factors contribute to the bacterial variation, such as geographical location, season, farm size, number of animals on the farm, hygiene and farm management practices. However, in spite of the variation, all of these surveys demonstrated quite clearly that milk can be a significant source of foodborne pathogens of human health significance (Oliver et al., 2005).

The objective of the study was to investigate the microbiological content of cow milk in Latvian organic farms depending on season and herd size with a purpose to detect their impact on the distribution of mastitis causing pathogens in milk.

Materials and Methods

Sampling

Five hundred and sixty four raw milk samples from 14 organic dairy farms located in various parts of Latvia - regions of Kurzeme (3 herds), Latgale (3 herds), Vidzeme (2 herds) and Zemgale (6 herds) - were collected in winter (December, 2011), when the dairy cows were housed indoors, in autumn (October, November, 2012), in spring (April, May, 2012) and in summer period (August, 2012) when the cows were kept outdoors grazing on pastures. The organic dairy farms have been registered by the state control institutions. Dairy cows in these farms were housed either in tie stall or free stall cow-sheds. 148 samples were collected in winter, 142 in spring, 168 in summer and 106 raw milk samples in autumn. Herd size varied from 3 to 124 animals in a cow-shed including 4 herds with 3-30 cows (group A herds, n=86 samples), five - with 31-60 cows (group B herds, n=262 samples) and five herds with 61-124 cows (group C herds, n=216 samples) in a cow-shed. Herds of A, B and C group have milk production 4461 ± 257, 4372 ± 517 and 5428 ± 575 kg per cow per year, respectively. Fifteen lactating cows from each herd were chosen for sampling. Milk samples from herds of fewer than

15 cows were collected from 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 ninth lactation.

Milk samples were collected by trained farm personnel from a cow level (cow composite milk) during sampling procedure of monitoring milk quality according to the standard LVS 175:1999 ‘Sampling of raw milk’. Samples were collected in sterile, 7 mL vacutainers (Vacutest Kima, Italy) and immediately transported to the Laboratory of Microbiology of the Research Institute of Biotechnology and Veterinary Medicine ‘Sigra’ (Sigulda, Latvia) maintaining cold chain under the temperature of 10 °C, then frozen at -20 °C for 2-6 weeks until the examination was done.

Microbiological examination

The samples were defrosted at a room temperature and serially decimally diluted with Maximum Recovery Diluent (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.

Microbiological examination has been described in detail previously (Gulbe and Valdovska, 2012). Samples presenting more than three sizes of microbial pathogens were considered contaminated (Laboratory and field handbook on bovine mastitis, 1999).

Statistical analysis

For statistical analysis, the IBM SPSS Statistics version 21 was used. Descriptive statistics for the total colony count and occurrence of several microorganisms including average standard error and frequencies was done. To determine whether the effect of season and herd size was significant in explaining the variations in the total colony count and isolated bacteria, the data were analysed by bivariate correlation. Data are presented as mean ± standard error, and a probability value $p<0.05$ was considered statistically significant.

Results and Discussion

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).

In our results, bacterial growth had occurred in 90.4% of the examined milk samples. Isolated microorganisms belonged to 35 species, - we divided them into 16 groups and included one group of mixed culture (this means that the sample contains 2 and more varieties of bacteria) (Fig. 1a, b).

Coagulase negative staphylococci (CoNS), *Staphylococcus aureus, Kocuria kristinae* and *Enterobacteriaceae spp.* were the most prevalent agents and were isolated in 166 (29.4%), 136 (24.1%), 73 (12.9%) and 58 (10.3%) out of 564 raw milk samples, respectively. Above mentioned bacteria, except for *Enterobacteriaceae spp.*, were distributed in milk samples almost of each herd throughout the year. Bacteria from genus *Enterobacteriaceae* were present mostly in samples from group C herds all year round, more rarely in samples from group B herds (only in spring and autumn), but these microorganisms were not present in samples of group A herds. Isolated *Enterobacteriaceae spp.* includes *E. coli, Klebsiella oxytoca, Serratia marcescens, Kluyvera ascorbata, Pantoea agglomerans* and other undifferentiated species. CoNS group includes *S. equorum, S. kloosii, S. saprophyticus, S. simulans, S. haemolyticus, S. vitulus* and other undifferentiated CoNS species.

More rarely distributed bacteria belongs to genus of *Micrococcus spp.* in 35 (6.2%), *Corynebacterium spp.* in 30 (5.3%), *Bacillus spp.* in 23 (4.1%), a group of other Gram-positive bacteria in 20 (3.5%), and *Streptococcus spp.* in 17 (3.0%) out of 564 raw milk samples. *Micrococcus spp.* includes *M. sedentarius* and other undifferentiated species. *Corynebacterium spp.* includes *C. freundii*, *C. aquaticum* and *C. kutscheri*. Other Gram-positive bacteria consist of *Lactococcus lactis ssp. lactis, Lactococcus lactis ssp. cremoris, Pediococcus pentosaceus, Gemella haemolysans* and other undifferentiated species. *Streptococcus spp.* includes *S. uberis* and other undifferentiated streptococci except for *S. agalactiae*.

We isolated *Aerococcus spp.* with occurrence 3.7% of examined raw milk samples. Since the *Aerococcus spp.* was isolated only from two herds during one season, this occurrence cannot be applied to all investigated herds.

The frequency of other isolated bacteria does not exceed 3% of all examined samples, and this category includes other Gram-negative bacteria in 14 (2.5%), coagulase positive staphylococci (CoPS) in 10 (1.8%), *Enterococcus spp.* in 10 (1.8%) and *S. agalactiae* in 7 (1.2%) out of 564 milk samples. Other Gram-negative bacteria contains *Acinetobacter baumanii, Arcanobacterium pyogenes* (formerly *Actinomyces pyogenes*), *Pseudomonas fluorescens* and other undifferentiated species. *Bacillus spp.* includes *B. brevis, B. cereus, B. licheniformis, B. subtilis* and other undifferentiated species. *Enterococcus spp.* includes *E. faecalis* and other undifferentiated species, but CoPS contains *S. intermedius* and other

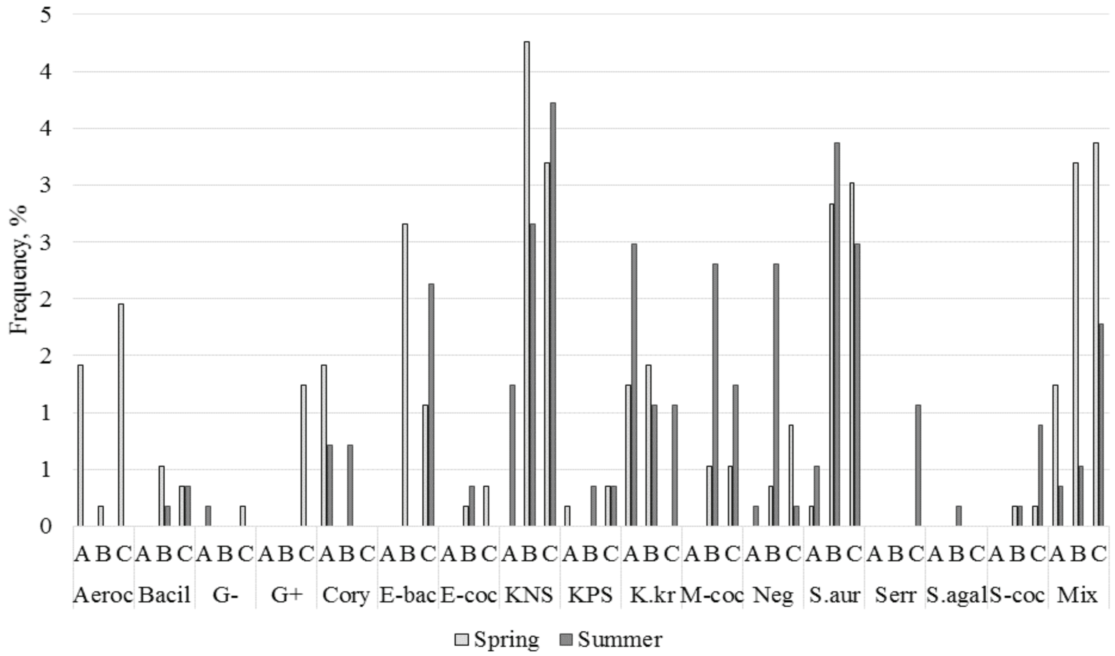

Figure 1a. The occurrence of isolated bacteria in raw milk samples depending on season and herd size, % of samples in each group.

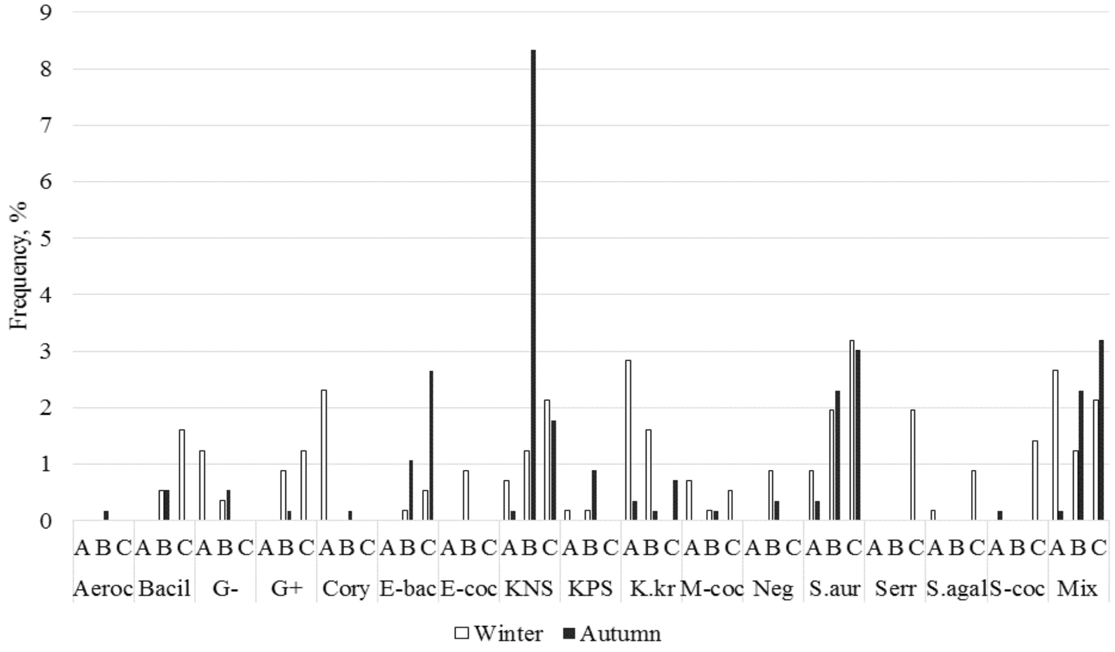

Figure 1b. The occurrence of isolated bacteria in raw milk samples depending on season and herd size, % of samples in each group: □ Winter; ■ Spring; ■ Summer; ■ Autumn; Aeroc – *Aerococcus spp.*; Bacil – *Bacillus spp.*; G- - other Gram-negative microorganisms; G+ - other Gram-positive microorganisms; Cory – *Corynebacterium spp.*; E-bac – *Enterobacteriaceae spp.*; E-coc – *Enterococcus spp.*; KNS – coagulase negative staphylococci; KPS – coagulase positive staphylococci; K.kr – *Kocuria kristinae*; M-coc – *Micrococcus spp.;* Neg – negative culture; S.aur – *Staphylococcus aureus*; Serr – *Serratia spp.*; S.agal - *S. agalactiae*; S-coc – *Streptococcus spp.*; Mix – mixed culture.

undifferentiated coagulase-positive staphylococci except for *S. aureus*.

SPSS Bivariate correlation analysis revealed that the effect of herd size, season and isolated bacteria on TTC was significant ($p<0.01$); the impact of herd size and season on isolated bacteria was significant too ($p<0.05$) while the seasonal effect was not significant on the isolated bacteria groups.

All isolated bacteria groups depending on season and herd size are presented in Figure 1a, b.

According to the Directives of European Union (Regulation 853/2004), the geometric average of the total number of microorganisms should not exceed 100,000 CFU per mL of raw cow milk from primary production. We determined the TCC higher than 100,000 CFU mL^{-1} in 14.4% and 85.6% of samples with TCC lower than 100,000 CFU mL^{-1} with mean TCC 5.7 ± 4.9 and 4.1 ± 3.0 log_{10} mL^{-1} out of all tested samples, respectively. There are quite large variations by TCC in different milk worldwide. K.G. Torkar and S.G. Teger (2008) and D.N. Prabhavathy and D. Sowmya (2012), reported on TCC in 24% raw milk samples in quantity of 4.46 log_{10} CFU mL^{-1} and 4.5 log_{10} CFU mL^{-1}, of the total number of microorganisms in raw milk, respectively, and this is similar to our experiment (4.9 ± 4.1 log_{10} CFU mL^{-1}). Other researchers report significantly higher TCC, for example, E.N. Aaku et al. (2004) and R. Arenas et al. (2004) - 6.7 log_{10} CFU mL^{-1} and 7-8 log_{10} CFU mL^{-1}, respectively.

In the literature an opinion prevails that the seasonal climate variations have an effect on microbial content of raw milk (Osteras et al., 2006; Riekerink et al., 2007; Nobrega and Langoni, 2011; Zucali et al., 2011).

K.G. Torkar and S.G. Teger report a decrease of TCC from summer to winter (Torkar and Teger, 2008). In our study the average number of the total colony count (TCC) was the lowest in summer (4.66 ± 4.01 log_{10} CFU mL^{-1}), moderately higher - in spring and winter (4.72 ± 4.18 and 4.82 ± 3.54 log_{10} CFU mL^{-1}, respectively), but significantly higher - in milk samples taken in autumn (5.43 ± 4.80 log_{10} CFU mL^{-1}). The average increase of TCC in autumn samples largely is because of samples from group C herds, as we can see it in Figure 2 that displays distribution of TCC in accordance with season and herd size.

K.G. Torkar and S.G. Teger (2008) explains that there exists a correlation between TCC and certain individual groups of microorganisms because they represent a part of the total bacterial amount in milk. There is an especially positive correlation between the number of coliform and psychrotrophic microorganisms because a lot of coliform bacteria are capable to grow at low temperatures (Torkar and Teger, 2008). A.J. Bramley and C.H. McKinnon (1990) reported that some species of the genera making up the coliform group of bacteria are psychrotrophic and constitute 10 – 30% of the whole group of microorganisms, the majority of these are *Aerobacter spp.*

Figure 3 displays distribution of TCC in accordance with five most frequent, isolated group of microorganisms. It also shows the average TCC

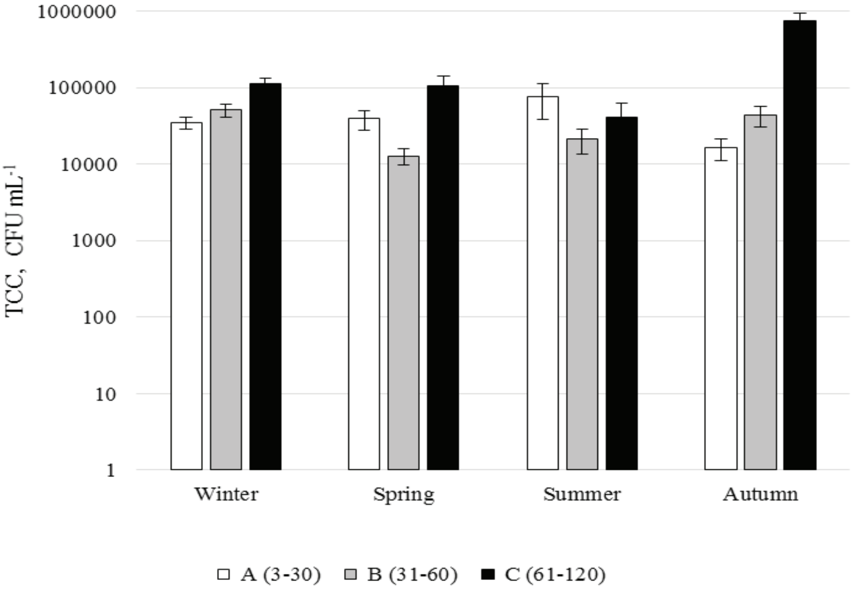

Figure 2. The mean number of total colony count in raw milk samples depending on season and herd size: cows in shed ☐ 3-30 ▨ 31-60 ■ 61-124.

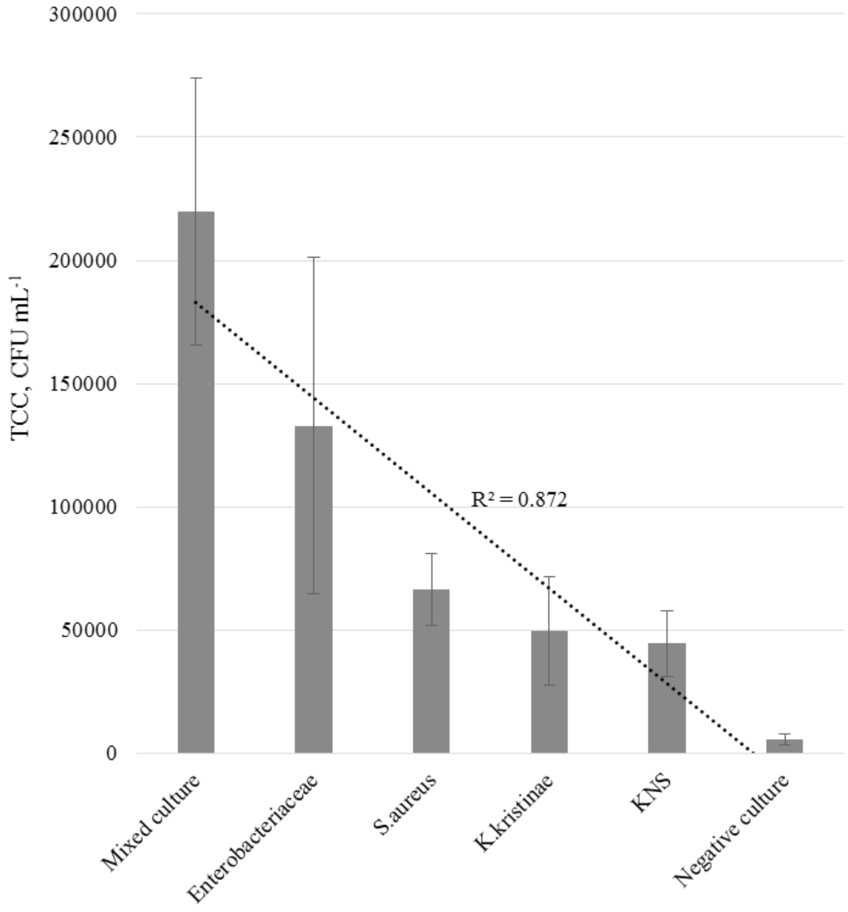

Figure 3. The mean number of total colony count in raw milk samples depending on bacterial agent.

in milk samples with negative bacterial culture when culturing was performed on blood agar and selective mediums.

The highest TCC is associated with mixed culture, substantially lower - with *Enterobacteriaceae spp*., but the major mastitis pathogens - *S. aureus* and *K. kristinae* together with CoNS - creates further reduced increase of TCC in raw milk.

In literature there is also data on the seasonal impact on the prevalence of certain bacteria species in cow milk. O. Osteras with colleagues refer to the highest prevalence of *Streptococcus dysgalactiae* and CoNS during April and May (late indoor season), and the highest prevalence of *S. aureus* and *Streptococcus uberis* - during June and July (the outdoor season) (Osteras et al., 2006). M. Zucali et al. (2011) report that occurrence of CoPS is higher in cold season in comparison with the other staphylococcus. Occurrence of *E. coli* is higher in summer than in winter (Olde Riekerink, 2007).

In this study, analysing the most widely spread bacterial groups we found that *Aerococcus spp.* occurrence is high in spring (n=20), but it was almost absent in samples of other seasons (n=1 in autumn). *Bacillus spp.* is most distributed in winter (n=12) milk samples, less so in other seasons (n=5 in spring, n=3 in summer and winter) and this observation is confirmed by other investigations – A.D. Sutherland and R. Murdoch, who report that the occurrence of mesophilic *Bacillus spp.* was the highest in the winter and lowest in the summer/autumn while psychrotrophic baccili occurrence was conversely lowest in the winter and highest in the late summer/autumn (Sutherland and Murdoch, 1994). The most of our isolated *Bacillus spp.* belong to mesophilic species (*B. brevis, B. licheniformis, B. subtilis)* while *B. cereus* is psychrotrophic one. Psychrotrophic microorganisms, from a food spoilage perspective, are the most important organisms present in dairy products because they cause unpasteurized milk spoilage during its storage in the refrigerator (Champagne et al., 1994). *Enterobacteriaceae spp.* prevalence is the highest in spring and autumn (n=21 and n=12), less frequent in summer (n=12) and winter (n=4). CoNS are less distributed in raw milk in winter (n=23) than following seasons (n=42 in spring, n=43 in summer, n=58 in autumn), but prevalence of CoPS is higher in cold season (n=9 in cold season toward n=5 in hot season) – these observations are in line with the investigations of the O. Osteras group (Osteras et al., 2006). *Serratia spp.* are detected only in winter (n=11) and summer (n=6). Prevalence of *K. kristinae* is lower in autumn (n=7) than in the following seasons (n=25 in winter, n=15 in spring, n=26 in summer).

The quality of milk can be estimated by the count of somatic cells (SCC), the total colony count of mesophilic aerobic and facultative anaerobic microorganisms, coliforms and *S. aureus* (Nikolajeva, 2011). In literature there is some data about the impact of the herd size on SCC, but it is hard to find out any findings how the herd size affects the bacterial diversity in milk. Therefore, in our study we made a short comparison of milk quality with other published investigations based on the total colony count toward SCC: several researchers have found out that the herd size has a significant impact on the quality of milk (Allore et al., 1997; Khaitsa et al., 1999; Ely et al., 2003). H.G. Allore et al. observed twice as frequent, elevated (SCC > 500,000 mL^{-1}) somatic cell count in herds with fewer than 27 lactating cows, compared to herds with > 62 cows (Allore et al., 1997), whereas L.O. Ely et al. report that herds with more than 449 cows had lower SCC than other smaller herds (Ely et al., 2003).

In this study, the average number of TTC was the lowest in B (4.50 ± 3.94 $\log_{10}$ CFU mL^{-1}), moderately higher in A (4.62 ± 4.17 $\log_{10}$ CFU mL^{-1}) and the highest in group C herds (5.40 ± 4.84 $\log_{10}$ CFU mL^{-1}). Significantly higher ($p<0.05$) TCC was in the milk samples taken in autumn (5.43 ± 4.80 $\log_{10}$ CFU mL^{-1}).

Coliforms from *Enterobacteriaceae spp.* were not isolated from group A herds instead of B (3.1%) and C (4.6%). Prevalence of *S.aureus* was noticeably higher in B (19.1%) and C (20.8%) than in group A (12.6%) herds. Percentage is calculated for occurrence of bacteria in each herd group during the year.

Conclusions

1. Bacterial growth occurred in 90.4% of examined milk samples. Isolated microorganisms belong to 35 species. Coagulase negative staphylococci, *Staphylococcus aureus, Kocuria kristinae* and *Enterobacteriaceae spp.* were the most prevalent agents and were isolated in 166 (29.4%), 136 (24.1%), 73 (12.9%), and 58 (10.3%) out of 564 raw milk samples, respectively.
2. Depending on season, the average number of total colony count was the lowest in summer (4.66 ± 4.01 $\log_{10}$ CFU mL^{-1}), moderately higher in spring and winter (4.72 ± 4.18 and 4.82 ± 3.54 $\log_{10}$ CFU mL^{-1}, respectively). Significantly higher the total colony count was in milk samples taken in autumn (5.43 ± 4.80 $\log_{10}$ CFU mL^{-1}) from large (C) herds.
3. Depending on the herd size, the average number of total colony count was the lowest in medium (B) (4.50 ± 3.94 $\log_{10}$ CFU mL^{-1}), moderately higher in small (A) (4.62 ± 4.17 $\log_{10}$ CFU mL^{-1}) and the highest in large (C) herds (5.40 ± 4.84 $\log_{10}$ CFU mL^{-1}).
4. Coliforms were not isolated from small herds instead of medium (3.1%) and large (4.6%) herds. Occurrence of *S.aureus* was noticeably higher in medium size (19.1%) and large size herds (20.8%) than in small herds (12.6%).
5. Milk which is produced in small organic herds, meets the milk quality requirements more than milk from large herds, taking into account the following milk quality indicators: the total colony count, presence of coliforms and presence of *S. aureus.*

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