

THE MICROBIOLOGICAL QUALITY OF RAW MILK AFTER INTRODUCING THE TWO DAY'S MILK COLLECTING SYSTEM

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ABSTRACT

This study was carried out to investigate the quality of raw milk after the decision of the dairies to collect the milk every two days and not daily as it has been performed till the year 2005. In wider central Slovenian production area we collected in winter and summer season 203 raw milk samples obtained from farm bulk milk tanks, collecting points and transportation tanks at the entrance to the dairy was studied. The total bacterial count, the number of coliforms, psychrotrophic micro-organisms, coagulase-positive staphylococci, yeasts and moulds were analysed using standard methods. The presence of mould species and production of aflatoxins were studied as well. The total bacterial count was higher than 100 000 cfu/ml in 48 (23.6%) out of all tested samples. Its mean value in all milk samples was 4.5 log₁₀ cfu/ml*. The mean numbers of coliform bacteria, psychrotrophic micro-organisms, yeasts and moulds together, and coagulase-positive staphylococci were 2.1 log₁₀ cfu/ml, 3.7 log₁₀ cfu/ml, 2.3 log₁₀ cfu/ml and 1.97 log₁₀ cfu/ml, respectively. The yeasts were present in 95.0% of raw milk samples with the mean concentration of 1.7 log₁₀ cfu/ml. Moulds were found in 63.3% of raw milk samples, their mean concentration was 0.6 log₁₀ cfu/ml. Isolated mould strains belonged to genera *Geotrichum* (51.5%), *Aspergillus* (33.8%), *Mucor* (5.9%), *Fusarium* (2.9%) and *Penicillium* (2.9%). None of the isolated *Aspergillus* strains with typical growth on AFPA medium produced aflatoxin M₁ on YES or YGC medium supplemented with Methyl-β-cyclodextrin.

Key words: raw milk / microbiological quality / total bacterial count / moulds / aflatoxin M₁

MIKROBIOLOŠKA KAKOVOST SUROVEGA MLEKA PO UVEDBI SISTEMA DVODNEVNEGA ZBIRANJA

IZVLEČEK

Preučevali smo mikrobiološko kakovost odkupljenega surovega mleka po odločitvi mlekarn, da bodo mleko zbirale vsake dva dni in ne več dnevno, kot so to izvajale do konca leta 2005. V osrednjem proizvodnem področju Slovenije smo v zimskem in letnem obdobju odvzeli 203 vzorcev mleka iz hladilnih bazenov posameznih proizvajalcev, zbiralnic ter transportnih cistern na sprejemu mlekarne. S standardnimi mikrobiološkimi metodami smo ugotavljali skupno število aerobnih mezofilnih mikroorganizmov, koliformnih in psihrotrofnih mikroorganizmov, število kvasovk in plesni ter koagulaza-pozitivnih stafilokokov. Ugotavljali smo tudi prisotnost posameznih vrst plesni in njihovo sposobnost tvorbe aflatoksinov. Skupno število mikroorganizmov je presegalo 100 000 ke/ml[†] v 48 (23,6 %) od vseh preiskanih vzorcev.

* Abbreviations: cfu/ml = the number of colony forming units per millilitre of the sample.

† Kratice: ke/ml = število kolonijjskih enot v ml vzorca.

Njihovo povprečno število v vseh vzorcih je bilo 4,5 log₁₀ ke/ml. Povprečno število koliformnih mikroorganizmov je znašalo 2,1 log₁₀ ke/ml, število psihrotrofnih mikroorganizmov 3,7 log₁₀ ke/ml, skupno število kvasovk in plesni 2,3 log₁₀ ke/ml in število koagulaza-pozitivnih stafilokokov 1,97 log₁₀ ke/ml. Kvasovke s povprečno koncentracijo 1,7 log₁₀ ke/ml smo ugotovili v 95 % vzorcev, plesni s povprečno koncentracijo 0,6 log₁₀ ke/ml pa v 63,3 % vzorcev. Najpogosteje smo izolirali plesni iz rodov *Geotrichum* (51,5%), *Aspergillus* (33,8 %), *Mucor* (5,9%), *Fusarium* (2,9%) in *Penicillium* (2,9%). Nobeden izmed izoliranih sevov iz rodu *Aspergillus*, ki je kazal značilno rast na gojišču AFPA, ni na gojiščih YES in YGC z metil-β-ciklodekstrinom tvoril aflatoksina M₁.

Ključne besede: surovo mleko/ mikrobiološka kakovost / skupno število mikroorganizmov / plesni / aflatoksin M₁

INTRODUCTION

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 micro-organisms therefore the microbial content of milk is a major feature in determining its quality (Rogelj, 2003). Bacterial contamination of raw milk can originate from different sources: air, milking equipment, feed, soil, faeces and grass (Coorevits *et al.*, 2008). The number and types of micro-organisms in milk immediately after milking are affected by factors such as animal and equipment cleanliness, season, feed and animal health (Rogelj, 2003). It is hypothesized that differences in feeding and housing strategies of cows may influence the microbial quality of milk (Coorevits *et al.*, 2008). Rinsing water for milking machine and milking equipment washing also involve some of the reasons for the presence of a higher number of micro-organisms including pathogens in raw milk (Bramley, 1990).

After milking, milk is cooled, which additionally influence the dynamic of microbial process (Rogelj, 2003).

The conditions during storage and transport in refrigerated tanks cause the raw milk microbiota to change from predominantly Gram-positive to predominantly Gram-negative organisms as they grow. Gram-negative bacteria usually account for more than 90% of the microbial population in cold raw milk that has been stored. The Gram-negative flora is composed mainly of psychrotrophic species of *Pseudomonas*, *Achromobacter*, *Aeromonas*, *Serratia*, *Alcaligenes*, *Chromobacterium*, *Flavobacterium* and *Enterobacter* (Garcia-Armesto *et al.*, 1997; Sørhaug *et al.*, 1997; Ryser, 1999; Martins *et al.*, 2006).

Frank (1997) also mentioned the presence of other genera: *Enterococcus*, *Proteus*, *Lactococcus*, *Streptococcus*, *Leuconostoc*, *Lactobacillus*, *Microbacterium*, *Propionibacterium*, *Micrococcus*, *Bacillus* and coliforms. Organisms unable to grow at refrigeration temperatures remain at low numbers, implying that temperature is an important factor contributing to the prevalence and proliferation of specific organisms in the milk (Jay, 1996; Frank, 1997).

Pasteurization of raw milk is effective in eliminating all but the thermotolerant microorganisms of the genera *Microbacterium*, *Micrococcus*, *Streptococcus*, *Lactobacillus*, *Bacillus*, *Clostridium*, the coryneforms, and occasionally some Gram-negative rods (Jay, 1996). Psychrotrophs can grow at refrigeration temperatures below 7 °C, produce enzymes toxins and other metabolites (Jay, 1996) and contribute to high standard plate counts in both raw and pasteurised milk. Since milk is currently handled and stored at low temperatures, these organisms hinder efforts to increase the shelf life of pasteurised milk (Frank, 1997). Most of these bacteria produce extracellular proteolytic and lipolytic enzymes that are secreted into the milk. Many of these enzymes are not inactivated by pasteurizing at 72 °C for 15 s or by Ultra-High Temperature (UHT) treatment (Griffiths *et al.*, 1981). The residual activities of these enzymes can reduce the organoleptic quality and shelf life of processed milk products (Fairbairn *et al.*, 1986). Pasteurization cannot guarantee the absence of microorganisms, when they are

present in large numbers in raw milk or due to post-pasteurization contamination. (Salmeron *et al.*, 2002).

Examination for the presence and number of specific micro-organisms is, therefore, an integral part of any quality control or quality assurance plan and it may be applied to a number of areas: raw materials, intermediate samples, finished products, or environmental/equipment sites. Milk-borne and milk-product borne outbreaks represent 2–6% of bacterial food-borne outbreaks reported by surveillance systems from several countries (De Buyser *et al.*, 2001).

Yeasts and moulds in some cheese types can periodically cause problems, both economic and sensory. They usually present in raw milk do not survive pasteurization; their presence in pasteurized milk and other milk products is caused by re-infection during manufacturing (Nelson, 1981; Jodral *et al.*, 1993). The contamination of milk products, particularly cheeses is caused by yeasts and moulds present in the environment of cheese factories, like walls and shelves of ripening rooms, air, equipment, water, milk, brine, etc. (Chapman and Sharpe, 1990; Jay, 1992). Of course, the presence of yeasts and moulds in raw milk is undesired, when in manufacturing unpasteurized milk is used.

Yeasts themselves are not commonly the cause of defect in dairy products unless they ferment lactose. In this case, they can grow rapidly and produce a characteristic yeasty or fruity flavour and obvious gas (Davis and Wilbey, 1990). They also produce metabolites, e.g. short-chain fatty acids and other compounds, with known toxic effects against undesired micro-organisms in the intestinal tract (Jakobsen and Narvhus, 1996).

Moulds are important in milk, which is used for the manufacture of cheese and other dairy products. The presence of wild types of moulds is undesirable as they may influence the organoleptic characteristics of the cheeses, they can produce mycotoxins and represent a potential health risk (Jodral *et al.*, 1993; Wouters, *et al.*, 2002). Mycotoxins are toxic metabolites produced by various fungi growing in a wide range of food and animal feedstuffs. The main mycotoxins that occur frequently are aflatoxins, ochratoxins, patulins, trichothecenes and zearalenones (Gilbert, 2002). The major toxigenic species of fungi belong to genera *Aspergillus*, *Fusarium*, *Acremonium* and *Phomopsis* (D'Mello and Macdonald, 1997). Aflatoxins are mycotoxins produced as carcinogenic, teratogenic and mutagenic secondary metabolites by some species of genus *Aspergillus* (Frisvad *et al.*, 2005) Aflatoxin B₁ represents the highest degree of toxicity for animals, followed by aflatoxin M₁, G₁, B₂ and G₂ (Gourama and Bullerman, 1995). AFM₁ may be found in the milk of animals that have been fed with feed containing AFB₁.

The objective of this work was to evaluate the level of microbiological contamination of raw milk samples taken from farm cooling milk tanks, collecting points and from bulk raw milk from transportation tanks at the entrance to a dairy. This study was carried out to investigate the quality of raw milk after the decision of the dairies to collect the milk every two days and not daily as it has been performed till the year 2005.

We wished to found out the differences in number of micro-organisms between milk samples taken in winter and summer season and at different collecting stages.

Our interest was also the identification of moulds isolated from samples and the detection of their aflatoxin production.

MATERIALS AND METHODS

Sampling

The collection of the raw milk samples took place in January to February and May to June 2006 at the central part of Slovenia. A total of 203 raw milk samples were analysed, of which 100 samples were taken in winter and 103 of them in summer season. In both seasons 60 of

samples were obtained from collecting points and farm bulk milk tanks, while 40 and 43 of them were collected from transportation tanks at the entrance to the dairy in winter and in summer, respectively. The milk samples were taken in accordance with the instructions given in ISO/DIS 707 (1995). Samples were collected in sterile bottles and transported to the laboratory in cold chain under temperature 6 °C and analysed within 2 hours of sampling.

Media, reagents and reference strains

The samples were serially decimal diluted with Quarter strength Ringer's solution (Merck, Germany) and appropriate dilutions plated on media using the pour plate method (ISO/FDIS 8261 (E), 2001).

For the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) the Baird Parker with RPF supplement agar (Biokar Diagnostics, France) was used (SIST EN ISO 6888-2, 1999).

The enumeration of coliform micro-organisms was carried out on VRBL agar (Merck, Germany) according to the standard ISO 4832 (2006).

The presence and number of total bacterial count and psychrotrophic micro-organisms were evaluated on PCA agar (Merck, Germany) with the addition of 0.1% w/v (1 g per 1 l of medium) of skimmed milk powder. The plates were incubated at 30 °C for 72 hours for aerobic mesophilic counts (EN ISO 4833, 2003) and at 6.5 °C for 10 days for psychrotrophic micro-organisms (ISO 6730, 2005).

For the enumeration of yeasts and moulds the YGC agar (Merck, Germany) was used according to the standard ISO 6611(E) (2004).

Aspergillus flavus / *parasiticus* agar (AFPA base) (Oxoid, England), supplemented with chloramphenicol selective supplement (Oxoid, England) was used for isolation and partial identification of moulds. Yeast extract sucrose agar (YES) (Samson and Hoekstra, 2000) and also yeast glucose chloramphenicol agar (YGC), both supplemented with 0.3% methyl- β -cyclodextrin (Sigma, Germany) and 0.6% sodium deoxycholate (Sigma, Germany) were used for determination of aflatoxin production (Fente *et al.*, 2001; Ordaz *et al.*, 2003).

Quarter strength Ringer's solution (Merck, Germany) was used for decimal dilution of samples.

Reference strains: *A. flavus* EXF 523 and *A. flavus* EXF 438 were kindly given as a gift from University of Ljubljana, Biotechnical Faculty, Department for Biology, Slovenia.

Inoculation and enumeration of micro-organisms

The milk samples were serially decimal diluted with Quarter strength Ringer's solution and appropriate dilutions were inoculated by pouring the plates with chosen medium (EN ISO 8261 (E), 2001).

Moulds isolation and identification

Each morphologically different mould colony from the plates with YGC medium was picked up, transferred to YGC and AFPA mediums and incubated for 5 days at 25 °C and for 42 hours at 30 °C, respectively.

Primary classification of colonies from solid mediums YGC and AFPA was based on colony characteristics (pigmentation, shape, background colour) and on microscopic examination of moulds using immersion objective magnifications 100/1.30 and 160/0.17, according to Samson and Hoekstra (2000). The identification of strains *A. flavus* and *A. parasiticus* was confirmed by reverse, yellow to orange pigmentation on AFPA medium (Pitt *et al.*, 1983).

Aflatoxin production

For the examination of aflatoxin production at isolated moulds from the genus *Aspergillus*, the YES and YGC medium, supplemented with 0.3% methyl- β -cyclodextrin and 0.6% sodium deoxycholate were used (Fente *et al.*, 2001; Ordaz *et al.*, 2003). The plates with inoculated strains were incubated for 3 days at 28 °C and then observed under UV light (365 nm).

Statistical analyses

For statistical analyses, the SAS/STAT (2000) was used. Descriptive statistics including average, standard deviation, variability coefficient, minimum and maximum was done. The Pearson's correlation coefficients between variables log number of different groups of tested micro-organisms in milk samples were calculated.

RESULTS AND DISCUSSION

According the Regulatives EU (Regulation 853, 2004) the rolling geometric average of total number of micro-organisms should not exceed 100 000 per ml of raw cow's milk from primary production. The rolling geometric average should be calculated over a two month period, with at least two samples per month. In Slovenia, the microbiological quality of raw milk is very good and is comparable with milk quality in dairy developed countries. In the year 1994 only about 60% of milk, delivered to dairies, contained up to 100 000 micro-organisms per millilitre, while in the year 2005 already 99.5% out of all 448.5 millions litres of raw milk, delivered to dairies, contained less than 100 000 m.o./ml. Up to 50 000 micro-organisms per millilitre contained 93.6% of milk (Valjavec, 2006).

Since the year 2005 some Slovenian dairies have collected the milk from the farmers every two days and not daily any more. Such milk stays in the cooling pools or tanks two days and is mixed with warm milk after every new milking. The microbiological quality and the correlation between different groups of micro-organisms in such milk are different in comparison to the fresh milked milk, which is cooled immediately and take to the dairy daily.

In this study we aimed to investigate the microbiological quality of 203 raw milk samples, collecting in winter (100 samples) and summer season (103 samples). We determined the total bacterial count higher than 100 000 cfu/ml in 48 (23.6%) out of all tested samples.

The mean total aerobic mesophilic micro-organisms, coliform bacteria, psychrotrophic micro-organisms, coagulase-positive bacteria, and yeasts and moulds counts were determined as 4.5 log₁₀ cfu/ml, 2.0 log₁₀ cfu/ml, 3.7 log₁₀ cfu/ml, 1.9 log₁₀ cfu/ml and 2.3 log₁₀ cfu/ml, respectively. The basic statistic parameters were determined in the Table 1. The highest mean values as well as minimal and maximal values were found for the number of total mesophilic aerobic count. The lowest number between all tested groups of micro-organisms was evaluated for coagulase-positive staphylococci (Table 1).

Aaku *et al.* (2004) and Arenas *et al.* (2004) reported on $5.5 \cdot 10^6$ cfu/ml and 10^6 to 10^7 cfu/ml of the total number of micro-organisms in pooled raw milk, respectively, which is higher than in our experiment ($3.2 \cdot 10^4$ cfu/ml).

The average number of coliforms, yeasts and moulds, and coagulase-positive staphylococci represented only 0.37%, 0.62% and 0.27% of the mean number of total bacterial count, respectively.

The mean number of almost all groups of micro-organisms was higher in summer season, but the correlations between their numbers and the total bacterial count stayed very similar in winter and summer season. The total bacterial count exceeded the 10^5 cfu/ml in 15% of winter samples and in 32% of summer samples. It decreased from summer to winter for 46.5%, the number of

coliforms was lower for 48.2%, the number of yeasts and moulds for 45.0% and the number of coagulase-positive staphylococci for 5.0%.

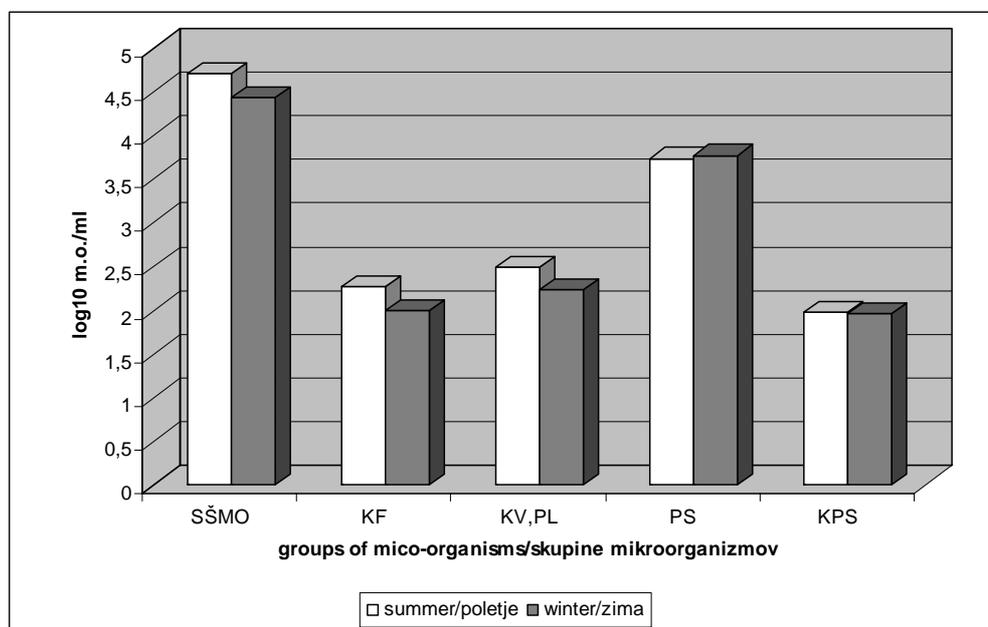
Table1. The basic statistical parameters for the log values of the number of individual groups of micro-organisms per millilitre of 203 tested samples

Preglednica 1. Osnovni statistični parametri za logaritemske vrednosti števila posameznih skupin mikroorganizmov v mililitru 203 preskušanih vzorcev

Groups of m.o. Skupine m.o.	Number of micro-organisms per ml of the sample, in log ₁₀ Število mikroorganizmov v ml vzorca, v log ₁₀					
	Average Povprečje	Median Mediana	Sd	KV	Min	Max
SŠMO	4.53	4.55	0.55	12.15	2.60	6.04
KF	2.09	2.17	1.07	51.69	0.00	4.53
KV-PL	2.32	2.44	0,71	30.95	0.30	4.18
PS	3.75	3.93	0.89	23.75	2.00	5.50
KPS	1.97	2.17	0.63	31.90	1.00	3.17

m.o.: micro-organisms / mikroorganizmi; Sd: standard deviation / standardni odklon; KV: coefficient of variation / koeficient variabilnosti; Min: minimum value / najmanjša vrednost; Max: maximum value / največja vrednost; SŠMO: total bacterial count / skupno število aerobnih mezofilnih mikroorganizmov; KF: coliform microorganisms / koliformni mikroorganizmi; KV, PL: yeasts and moulds / kvasovke in plesni; PS: psychrotrophic microorganisms / psihrotrofni mikroorganizmi; KPS: coagulase-positive staphylococci / koagulaza-pozitivni stafilokoki

The number of psychrotrophic micro-organisms was the exception in a few views. The mean of the psychrotrophs was 3.7 log₁₀ cfu/ml and represented even 17.1% of the mean of total bacterial count. Their number was in winter higher than in summer for 6.9% (Fig. 1).

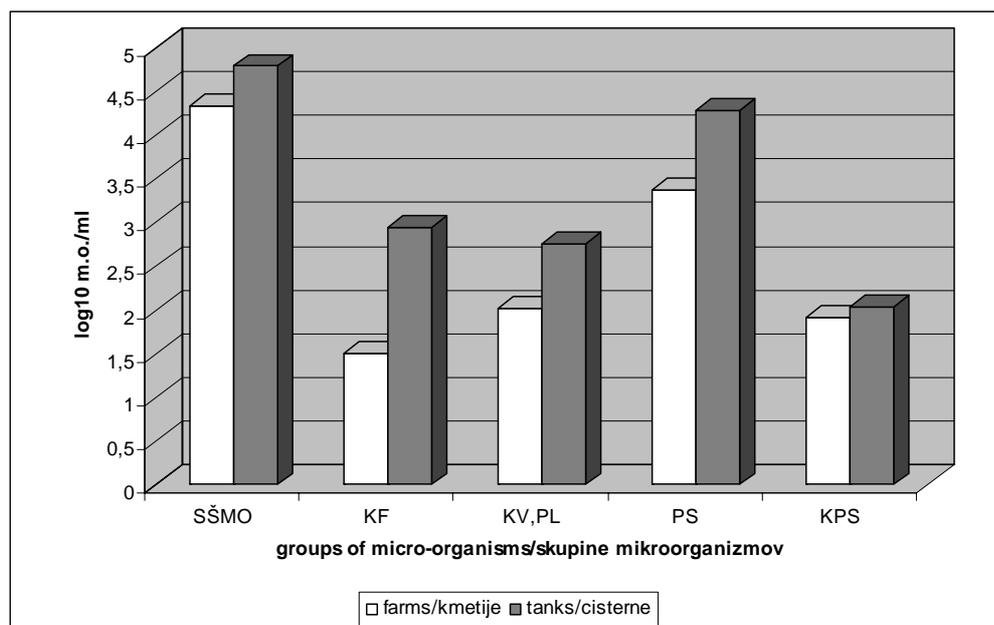


KF: coliform micro-organisms / koliformni mikroorganizmi; KV, PL: yeasts and moulds / kvasovke in plesni; PS: psychrotrophic micro-organisms / psihrotrofni mikroorganizmi; KPS: the coagulase-positive staphylococci / koagulaza-pozitivni stafilokoki

Figure 1. Average log numbers of the individual groups of micro-organisms per millilitre of milk samples in both seasons.

Slika 1. Povprečne logaritemske vrednosti posameznih skupin mikroorganizmov v mililitru vzorcev mleka v obeh sezonah.

The number of all tested groups of micro-organisms was higher in samples of raw bulk milk, collected from transportation tanks at the entrance of the dairy. The highest differences between samples taken from individual farms and from transportation tanks were in number of coliforms (about 1.44 log₁₀ cfu/ml). As it is represented in the Fig. 2, the mean total bacterial count, number of coliforms, yeasts and moulds, psychrotrophic micro-organisms and coagulase-positive staphylococci were in milk from transportation tanks higher than in milk from individual farm tanks for 10.2%, 96.3%, 36.1%, 26.9% and 6.4%, respectively. The total bacterial count exceeded 100 000 cfu/ml in 21.7% of samples from farm bulk milk tanks and in 27.5% of samples from transportation tanks at the entrance of the dairy.



KF: coliform micro-organisms / koliformni mikroorganizmi; KV, PL: yeasts and moulds / kvasovke in plesni; PS: psychrotrophic micro-organisms / psihrotrofni mikroorganizmi; KPS: the coagulase-positive staphylococci / koagulaza-pozitivni stafilokoki

Figure 2. Average values of the log number of the individual groups of micro-organisms per millilitre of milk samples from farm bulk tanks and transportation tanks at the entrance of the dairy.

Slika 2. Povprečne logaritemske vrednosti posameznih skupin mikroorganizmov v mililitru vzorcev mleka iz hladilnih bazenov pri posameznih proizvajalcih in iz transportnih cistern na sprejemu mlekarne.

Almost all correlations between individually groups of micro-organisms were in both seasons highly statistically significant ($P \leq 0.0001$), except for coagulase-positive staphylococci there was no statistically significant correlation with any other tested group (Table 2).

The correlation of the number of coliforms with total bacterial count was understandable, because the coliforms represented a part of the total bacterial count. From the same reason there was also a correlation between the number of coliform and psychrotrophic micro-organisms, because a lot of coliform bacteria are capable to growth at low temperatures. Bramley (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 micro-organisms, the majority of these coliforms are *Aerobacter* spp. In our experiment the number of coliforms represented 2.1% of the number of psychrotrophs.

The incidence of coliforms in raw milk has received considerable attention, partly due to their association with contamination of faecal origin and the consequent risk of more pathogenic

faecal organisms being present, partly because of the spoilage their growth in milk at ambient temperatures can produce, and not least due to the availability of sensitive and rapid tests for detecting and enumerating coliforms. Coliform counts regularly in excess of 100 cfu/ml are considered by some authorities as evidence of unsatisfactory production hygiene. Sporadic high coliform counts may also be a consequence of unrecognised coliform mastitis, mostly caused by *E. coli*. The coliform micro-organisms are found also on the surface of the underwashed or moisture milking equipment, (Bramley, 1990, Rogelj, 2003).

Table 2. The influence of the season and the place of sampling (farm bulk milk tanks and transportation tanks at the entrance of the dairy) on the log number of tested microorganisms from individual groups

Preglednica 2. Vpliv sezone in mesta odvzema vzorca (hladilni bazeni pri posameznih proizvajalcih ali transportne cisterne na sprejemu mlekarne) na logaritmsko število mikroorganizmov iz posameznih skupin

Groups of m.o. Skupine m.o.	Season / sezona		Sampling place / mesto vzorčenja	
	F	P	F	P
SŠMO	19.71	< 0.0001	59.42	< 0.0001
KF	7.04	0.0084	234.13	< 0.0001
KV-PL	10.91	0.0011	102.64	< 0.0001
PS	0.18	0.6679	68.07	< 0.0001
KPS	0.05	0.81	1.87	0.17

m.o.: micro-organisms / mikroorganizmi; SŠMO: total bacterial count / skupno število aerobnih mezofilnih mikroorganizmov; KF: coliform microorganisms / koliformni mikroorganizmi; KV, PL: yeasts and moulds / kvasovke in plesni; PS: psychrotrophic microorganisms / psihrotrofni mikroorganizmi; KPS: coagulase-positive staphylococci / koagulaza-pozitivni stafilokoki; F: variance ratio / vrednost variance; $P \leq 0.05$: statistically significant influence / statistično značilen vpliv; $P \leq 0.001$: highly statistically significant influence / visoko statistično značilen vpliv; $P > 0.05$: no statistically significant influence / ni statistično značilnega vpliva

In our case the average number of coliforms was about 120 cfu/ml. In about 10% of winter samples taken exclusively from farm tanks we did not detect any coliforms with standard plate count method.

Bramley (1990) also reported, that the number of psychrotrophs should represent about 10–50% of total bacterial count, which is in the agreement with our results, because the number of psychrotrophic micro-organisms represented 10.9% of total bacterial count in summer and 21.9% of them in winter. We suppose, that the cooling of raw milk in cooling tankers at individual milk producers or farms was very intensive and the environmental temperatures did not influence on the microbiological quality of milk, even if it was kept there two days. Villar *et al.* (1996) also reported about good correlation between the total bacterial count and psychrotrophic count ($r = 0.82$). The correlation coefficient between these two groups in our experiment was $r = 0.71$.

Leitner *et al.* (2008) established that refrigerated storage of good-quality milk from a single cow resulted in moderate deterioration of its quality, low level of bacterial growth (standard plate and psychrotroph counts), and low small losses of curd yield. When milk was collected from farm bulk milk tanks and from dairy silos, its quality deteriorated faster than that of single-cow milk resulting in high bacteria counts and high loss of curd yield, most of which was already apparent for the farm bulk milk tank. Statistical analyses in his study did not reveal any significant interaction between bacterial growth, milk composition, somatic cell count, and curd yield loss, indicating that other mechanisms such as enzymatic activity might be responsible. From the comparison between the high-quality milk from an uninfected cow's udder and the

commingled milk on the farm and in the dairy silos, it appears that introduction of milk coming from infected udders might cause curd yield loss (Leitner *et al.*, 2008).

Our results agreed with the Leitner's comments (2008), because we established the statistically significant influence ($P \leq 0.05$) of the season and the place of sampling (individual farms, transportation tanks) on the total bacterial count, number of coliforms and yeasts and moulds. The influence of the place of sampling was determined also on the number of psychrotrophic micro-organisms (Table 2).

To avoid the increase of the number of micro-organisms the European Regulative 853 (2004) recommends that immediately after milking, milk must be held in a clean place designed and equipped to avoid contamination. It must be cooled immediately to not more than 8 °C in the case of daily collection, or not more than 6 °C if collection is not daily. During transport the cold chain must be maintained and on arrival at the establishment of destination, the temperature of the milk must not be more than 10 °C (Regulation EC 853, 2004).

The standard deviations and variability coefficients were in most cases high, because there were rather large differences in number of micro-organisms between samples (Table 1).

The number of coagulase-positive staphylococci was not in statistically significant correlation with other groups of micro-organisms and was not dependent on the season (Table 2).

Coagulase-positive staphylococci may cause human disease through the production of toxins. The formation of effective levels of toxin requires a high number of micro-organisms (approximately 10^5 – 10^6 micro-organisms per gram of food) at a pH value greater than 5 and so the presence of coagulase-positive staphylococci at a low level does not necessarily constitute a hazard. Dairy-related outbreaks of staphylococcal intoxication have been attributed to raw milk, dried milk, cheese and ice cream. Coagulase-positive staphylococci may be present in raw milk from the surfaces of animals, udder and teat canals of a cow, particularly if lesions are present. Also, the nasal area and hands of humans are recognised sites of contamination: poor personal hygiene can result in the contamination of milk and dairy products. Essential to the production of toxin is the growth of micro-organisms. In general, *Staphylococcus aureus* and the related species *Staphylococcus intermedius* and *Staphylococcus hyicus* do not multiply at temperatures below 8 °C, and 10 °C is the minimum for toxin production. These micro-organisms are, however, resistant to salt. Pasteurisation will be effective against them but, if toxins are present, the toxins will not be inactivated. The higher counts of *Staphylococcus* recorded in spring, when milk yields are at their peak, are a cause for concern and mammary infections (Anonim., 1994; Bramley, 1990). De Buyser *et al.* (2001) reported that *Staphylococcus aureus* was by far the most frequent pathogen associated with food pathogen outbreaks (85.5% of the outbreaks) in France, followed by *Salmonella* (10.1%), *E. coli* (3%), *L. monocytogenes* (3%) and *C. jejuni* (1.5%) outbreaks.

In our experiment coagulase-positive staphylococci were found in all tested samples, but their number did not exceed 1300 bacteria per ml.

It is documented that yeasts occur in raw milk at insignificant numbers (Fleet, 1990) probably due to competitive utilization for the growth substrates by psychrotrophic bacteria of milk or owing to inhibition by metabolites excreted by bacteria (Viljoen, 2001).

The mean number of yeasts and moulds found in raw milk samples of our study was 2.3 log₁₀ cfu/ml, which is comparable to the mean yeast count (2.64 log₁₀ cfu/ml) in raw milk from farms located in different areas of Sardinia (Fadda *et al.*, 2004). The maximum values of yeasts in raw milk samples from our producers were 4.1 log₁₀ cfu/ml.

In all milk samples, two or more mould species were found, 210 of them were successfully isolated and classified to genera *Geotrichum* (51.5% of strains), *Aspergillus* (33.8% of strains), *Mucor* (5.9% of strains), *Fusarium* (2.9% of strains) and *Penicillium* (2.9% of strains). The results are close to results of Jodral *et al.* (1993) who reported that the genera most frequently isolated in the raw milk were *Geotrichum* (76.5%), *Fusarium* (45.3%) and *Aspergillus* (31,2%).

O'Brien *et al.* (2005) established about 91% of baled grass silage contaminated with moulds, which mostly belonged to genera *Penicillium*, *Geotrichum*, *Fusarium* and mucoraceous species. These genera were found in our raw milk samples too, so it could be expected that the feed was one of the possible sources of contamination of raw milk in spite of Finne Kure *et al.* (2004) adduced proofs that there are many possible sources of contamination of raw milk, beside the feed also the air and the environment.

We also expected the higher number of yeasts and moulds in milk sampled in winter, when the pasture or the hay was replaced by conserved or ensiled feed. Many authors namely reported on higher number of yeasts, moulds and consecutively the higher concentration of mycotoxins in ensiled feed, which was used mostly in winter season. These micro-organisms were very often transferred from feed to milk (Blanco *et al.*, 1988; Lopez *et al.*, 2003; Kamkar, 2005). Surprisingly, the average number of yeasts and moulds in raw milk samples was a little higher in summer (2.49 log₁₀ cfu/ml) than in winter (2.23 log₁₀ cfu/ml), while the minimal and maximal values were higher in winter, as we expected.

All 210 mould strains isolated from milk samples as well as standard strains *A. flavus* were inoculated on *A. flavus/parasiticus* agar (AFPA). *Aspergillus* strains grew on the medium, but except reference strains *A. flavus* EXF 523 and *A. flavus* EXF 438 only one strain, previously classified as genus *Aspergillus* produced a distinctive bright orange yellow reverse colour on AFPA medium as described by Frädberg *et al.* (2003) and thus identified as *A. flavus* or *A. parasiticus* species.

Only about 70% out of 24 *Aspergillus* strains, growing on YGC medium, were able to form the colonies on YES medium supplemented with methyl- β -cyclodextrin and 0.6% sodium deoxycholate. None of isolated strains could cause a typical white fluorescent zone around the colonies, visible under UV light. We could conclude, that tested mould strains did not produce aflatoxins on these two media.

In our study it was found out that only one strain belonged to species *A. flavus / parasiticus* under typical growth on AFPA medium. *A. flavus* is not a common species on cheese. Most studies showed that aflatoxins could only be produced in milk and on cheese at temperatures higher than 10 °C and a limiting a_w of 0.79 was found for growth of *A. flavus* and aflatoxin production (Scott, 1989).

CONCLUSIONS

- In our study 76.4% out of total 203 tested raw milk samples contained less than 100 000 cfu/ml. There were statistical significant differences in total bacterial count between winter and summer samples and between samples taken from individual farm bulk milk tanks and transportation tanks at the entrance of the dairy.
- The total bacterial count exceeded 100 000 cfu/ml in 21.7% of samples from farm bulk milk tanks and in 27.5% of samples from transportation tanks at the entrance of the dairy.
- The number of all tested groups of micro-organisms was higher in samples of raw bulk milk, collected from transportation tanks at the entrance of the dairy. The highest differences between these samples were in number of coliforms.
- The average of the psychrotrophs represented even 17.1% of the average total bacterial count. Their number was in winter higher than in summer.
- All samples contained coagulase-positive staphylococci which may be an indicator of mastitical diseases of milking cows. The health care of milking cows should be more intensive.
- Isolated mould strains belonged to genera *Geotrichum*, *Aspergillus*, *Mucor*, *Fusarium* and *Penicillium*. None of the isolated *Aspergillus* strains produced aflatoxin M₁.

- The microbiological quality of milk samples mostly suited the requirements of the dairies, although that there was the percent of raw bulk milk from transportation tanks, contained less than 100 000 m.o./ml lower (72.5%) in comparison with the data for total bacterial count of raw milk in Slovenia in the year 2005.
- More attention should be focused on the cleaning of transportation tanks, appropriate handling with milk and its transportation at low temperatures from farms and collecting points to the dairies, particularly in summer season.

POVZETEK

Na osnovi uredbe EU (Regulation 853/2004) geometrijsko povprečje skupnega števila aerobnih mezofilnih mikroorganizmov v enem mililitru svežega kravjega mleka ne sme presežati 100 000. V Sloveniji je mikrobiološka kakovost surovega mleka dobra in je primerljiva s kakovostjo mleka mlekarsko razvitih držav. Ob koncu leta 2005 se je večina slovenskih mlekarn odločilo, da bodo mleko odvažale iz zbirnih hladilnih bazenov posameznih proizvajalcev ter zbiralnic vsake dva dni in ne več dnevno. Tako mleko stoji v hladilnih bazenih, ob vsaki molži pa se v bazen dolije k ohlajenemu še sveže pomolzeno toplo mleko. Mikrobiološka kakovost in razmerje med različnimi skupinami mikroorganizmov v takem mleku se razlikuje od sestave mikroflore v mleku, ki se takoj ohladi in zbira vsakodnevno.

V našem poskusu smo želeli preučiti mikrobiološko kakovost 203 vzorcev surovega mleka, od tega jih je bilo 100 odvzetih v zimskem in 103 vzorci v letnem obdobju. Vzorčili smo mleko iz zbirnih hladilnih bazenov pri posameznih proizvajalcih mleka, zbiralnicah in v transportnih cisternah ob sprejemu mleka v mlekarni. Ugotavljali smo skupno število mikroorganizmov, število koliformnih in psihrotrofnih mikroorganizmov, kvasovk in plesni ter število koagulaza-pozitivnih stafilkokokov. Želeli smo ugotoviti prisotnost posameznih rodov plesni in tvorbo aflatoksinov pri vrstah rodu *Aspergillus*.

Skupno število mikroorganizmov je presežalo 100 000 ke/ml v 48 (23,6 %) od vseh preiskanih vzorcev. Njihova povprečna vrednost v vseh vzorcih mleka je bila 4,5 log₁₀ ke/ml (3.2·10⁴ ke/ml). Povprečno število koliformnih mikroorganizmov je znašalo 2.1 log₁₀ ke/ml, število psihrotrofnih mikroorganizmov 3.7 log₁₀ ke/ml, skupno število kvasovk in plesni 2.3 log₁₀ ke/ml in število koagulaza-pozitivnih stafilkokokov 1.97 log₁₀ ke/ml. Število vseh skupin mikroorganizmov, z izjemo psihrotrofov, je bilo višje v poletnem času odvzema vzorcev, čeprav je njihovo razmerje s skupnim številom mikroorganizmov ostalo podobno v obeh sezonah.

Število vseh skupin mikroorganizmov, posebno še koliformnih, je bilo višje v mleku iz transportnih cistern na sprejemu mlekarne.

Kvasovke smo ugotovili v 95 % vzorcev, plesni pa v 63,3 % vzorcev. Najpogosteje smo izolirali plesni iz rodov *Geotrichum* (51,5 %), *Aspergillus* (33,8 %), *Mucor* (5,9 %), *Fusarium* (2,9 %) in *Penicillium* (2,9 %). Nobeden izmed izoliranih sevov iz rodu *Aspergillus*, ki je kazal značilno rast na gojišču AFPA, ni na gojiščih YES in YGC z metil-β-ciklodekstrinom tvoril aflatoksin M₁.

Ugotovili smo, da je mikrobiološka kakovost surovega mleka kljub dvodnevni odvozu, še vedno v večini primerov ustrezna, čeprav je bil odstotek vzorcev v mlekarni sprejetega mleka, ki so vsebovali manj kot 100 000 mikroorganizmov/ml, v primerjavi z mikrobiološko kakovostjo mleka v Sloveniji v letu 2005, nekoliko nižji (72,5 %).

Iz rezultatov lahko povzamemo, da samo skupno število mikroorganizmov ni vedno realni pokazatelj mikrobiološke kakovosti mleka. Občasno je potrebno kontrolirati tudi prisotnost in število drugih skupin mikroorganizmov. Število koliformnih, psihrotrofnih in koagulaza-pozitivni mikroorganizmov je bilo v našem poskusu občasno visoko. Koliformni mikroorganizmi so namreč pokazatelj slabe higiene in možnega fekalnega onesnaženja, psihrotrofni

mikroorganizmi se množijo tudi pri nizkih temperaturah hlajenja in so pogosti kvarljivci mleka zaradi tvorbe termostabilnih proteolitičnih in lipolitičnih encimov, koagulaza-pozitivni stafilokoki pa so lahko prisotni v mleku mastitčnih krav. Tudi prisotnost gliv, kontaminentov iz okolja, je bila v vzorcih pogosta.

Mikrobiološka kakovost vzorcev mleka iz transportnih cistern na sprejemu mlekarne je bila, zlasti v letnem obdobju, opazno slabša v primerjavi z vzorci iz hladilnih bazenov posameznih proizvajalcev ali zbiralnic. Zato je večjo pozornost potrebno posvetiti čiščenju transportnih cistern, pravilnemu ravnanju z zbranim mlekom in njegovemu prevozu v mlekarne ob ustrezno nizkih temperaturah.

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