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THE MICROBIOLOGICAL QUALITY OF SOME CRITICAL CONTROL POINTS IN THE CHEESE PRODUCTION OF INDIVIDUAL SLOVENIAN CHEESE-MAKERS

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ABSTRACT

The microbiological quality of 98 samples taken at some critical control points during the milking and processing of 14 semi-hard cheese made from raw cow milk by individual Slovenian producers was studied. The sampling points were: swabs from cows' udders, milking machines inner surfaces before and after milking, fresh raw and mixed milk from vats, whey immediately after curdling, brine, cheese after one month of ripening and after the following month of being kept vacuum packed at 6 °C. The high number of micro-organisms on the inner surfaces of washed milking machines before milking revealed ineffective cleaning (washing) by about 60% of cheese producers. There were no seasonal differences in the number of micro-organisms, except that the number of coliforms was higher in spring. The average of total number of micro-organisms was $4.9 \cdot 10^5$ cfu/ml in raw milk and $5.5 \cdot 10^6$ cfu/ml in mixed milk from a vat (raw fresh milk mixed with milk kept for about 18–24 hours at room temperature), which did not grow significantly during cheese-processing. The number of coliforms in raw and mixed milk was in the range of $3.4 \cdot 10^5$ cfu/ml and fell to $5.4 \cdot 10^4$ cfu/ml in whey. The average number of enterococci, aerobic spore-forming micro-organisms, yeasts and moulds, lactobacilli, lactococci, proteolytic and lipolytic micro-organisms in milk and in whey were in the same logarithmic range of about $2.2 \cdot 10^4$, 310, 3.5, $31.2 \cdot 10^4$, $2.1 \cdot 10^6$, $6.2 \cdot 10^3$ and $1.7 \cdot 10^4$ cfu/ml of the sample, respectively. *Listeria* spp. was isolated from 5.3% (cows' udders, milking machine, milk and whey), while none of the examined samples were positive to the presence of *Salmonella* spp. and *Campylobacter* spp. *Proteus* was present in 7 (7%) cases of milk and whey. Clostridia were detected in 10 (10%) samples (swabs, raw milk, whey). *E. coli* was isolated from 12 (12%) samples of swabs, raw and mixed milk, whey and brine. After one month of ripening the average total bacterial count was $9.2 \cdot 10^7$ cfu g^{-1} of cheese, of these $6.8 \cdot 10^7$ represented lactic-acid producers and $2.2 \cdot 10^7$ represented non-lactic acid producers. The average number of coliforms, enterococci, aerobic spore-forming micro-organisms, yeasts and moulds, lactococci, lactobacilli, proteolytic and lipolytic micro-organisms were $2.0 \cdot 10^5$, $6.3 \cdot 10^6$, 280, 960, $2.5 \cdot 10^7$, $9.8 \cdot 10^7$, 450 and $9.8 \cdot 10^4$ cfu g^{-1} of cheese, respectively. *Salmonella* spp., *Listeria* spp., *Proteus*, sulphite-reducing clostridia and *Campylobacter* spp. were not detected in cheese samples. *E. coli* was found in 4 (30%) of samples while coagulase positive staphylococci were present in 9 (64%) of cheese samples. A high number of enterococci (from a min. 3.10^3 to a max. 15.10^7 cfu g^{-1}) and coliforms (from a min. 10 to a max. 19.10^5 cfu g^{-1}) were detected as well. After one month of keeping vacuum-packed ripened cheeses at 6 °C, the number of micro-organisms did not rise significantly, except for the number of yeasts and moulds which grew to $3.6 \cdot 10^4$ cfu g^{-1} of cheese. Because of improper milking and processing hygiene conditions, three (21%) of the tested cheese samples did not correspond to the microbiological criteria according to the applicable regulations.

Key words: cheese-making / critical control points / microbiological quality / Slovenia

MIKROBIOLOŠKA KAKOVOST NA NEKATERIH KRITIČNIH KONTROLNIH TOČKAH PROIZVODNJE SIRA PRI POSAMEZNIH SLOVENSKIH SIRARJIH

IZVLEČEK

Proučevali smo mikrobiološko kakovost 98 vzorcev, odvzetih na nekaterih kritičnih kontrolnih točkah molže in proizvodnje 14-poltrdih sirov iz surovega mleka pri posameznih slovenskih sirarjih. Jemali smo vzorce brisov površine vimena krav molznic, notranjih površin molznih strojev pred in po molži, surovega mleka takoj po molži, mešanega mleka iz sirarskega kotla pred sirjenjem, sirotke takoj po koagulaciji, slanice, sirov po enomesečnem zorenju in vakumsko pakiranih sirov po nadaljnjem enomesečnem skladiščenju pri 6 °C. Visoko število mikroorganizmov na površini opranih molznih strojev pred molžo kaže na neučinkovito čiščenje (pranje) pri okrog 60 % sirarjev. Sezonskih razlik v številu mikroorganizmov nismo zasledili, razen nekoliko povišanega števila koliformnih mikroorganizmov v spomladanskem obdobju. Skupno število mikroorganizmov je bilo $4{,}9 \cdot 10^5$ kolonijskih enot ke/ml v surovem mleku in $5{,}5 \cdot 10^6$ ke/ml v mešanem mleku iz sirarskega kotla (sveže pomolženo surovo mleko primešano mleku, hranjenem 18–24 ur pri sobni temperaturi) in ni statistično značilno naraščalo med sirjenjem. Število koliformnih mikroorganizmov v surovem in mešanem mleku se je gibalo v območju okrog $3{,}4 \cdot 10^5$ ke/ml in se znižalo do vrednosti $5{,}4 \cdot 10^4$ ke/ml v sirotki. Povprečno število enterokokov, aerobnih sporotvornih mikroorganizmov, kvasovk in plesni, lactobacilov, laktokokov, proteolitičnih in lipolitičnih mikroorganizmov je bilo v mleku in sirotki v enakem logaritemskem območju $2{,}2 \cdot 10^4$, 310, 3,5, $31{,}2 \cdot 10^4$, $2{,}1 \cdot 10^6$, $6{,}2 \cdot 10^3$ in $1{,}7 \cdot 10^4$ ke/ml vzorca za vsako skupino mikroorganizmov. *Listeria* sp. je bila izolirana v 5,3 % vzorcev (vimena, molzni stroji, mleko, sirotka), medtem ko v nobenem od preiskanih vzorcev nismo zasledili bakterij vrst *Salmonella* spp. in *Campylobacter* spp. *Proteus* je bil prisoten v 7 (7 %) vzorcih mleka in sirotke. Sulfid-reducirajoči klostridiji so bili ugotovljeni v 10 (10 %) vzorcih (brisi, surovo mleko, sirotka). *E. coli* je bila izolirana iz 12 (12 %) vzorcev brisov, surovega in mešanega mleka, sirotke in slanice. Po enomesečnem zorenju je bilo povprečno število aerobnih mezofilnih mikroorganizmov okrog $9{,}2 \cdot 10^7$ ke g^{-1} sira, od teh je bilo $6{,}8 \cdot 10^7$ kislinotvornih in $2{,}2 \cdot 10^7$ nekislinotvornih mikroorganizmov. Povprečno število koliformnih mikroorganizmov, enterokokov, aerobnih sporoformnih mikroorganizmov, kvasovk in plesni, laktokokov, laktobacilov, proteolitičnih in lipolitičnih mikroorganizmov je bilo $2{,}0 \cdot 10^5$, $6{,}3 \cdot 10^6$, 280, 960, $2{,}5 \cdot 10^7$, $9{,}8 \cdot 10^7$, 450 in $9{,}8 \cdot 10^4$ ke g^{-1} sira. Bakterij vrst *Salmonella* spp., *Listeria* spp., *Proteus* in *Campylobacter* spp. nismo zasledili v nobenem od vzorcev sirov. *E. coli* smo našli v 4 (30 %) vzorcih, medtem ko so bili koagulaza pozitivni stafilokoki prisotni v 9 (64 %) vzorcih sirov. Ugotovili smo tudi visoko število enterokokov (od najmanj 3.10^3 do največ 15.10^7 ke g^{-1}) in koliformnih mikroorganizmov (od najmanj 10 do največ 19.10^5 ke g^{-1}). Po enomesečnem skladiščenju vakumsko pakiranih vzorcev sirov pri 6 °C se število mikroorganizmov ni statistično značilno zvišalo, le število kvasovk in plesni je poraslo do $3{,}6 \cdot 10^4$ ke g^{-1} sira. Zaradi neustrezne higiene molže in postopka sirjenja trije (21 %) vzorci sirov niso ustrezali kriterijem mikrobiološke kakovosti po veljavnih predpisih.

Ključne besede: sirarstvo / kritične kontrolne točke / mikrobiološka kakovost / Slovenija

INTRODUCTION

About 40% of cow milk produced in Slovenia each year is processed into different sorts of cheese (Valjavec, 2000; Valjavec, 2003). Some of these kinds of cheese are made from raw milk at small, artisanal cottage cheese-makers. Using raw instead of pasteurised milk keeps a larger proportion and diversity of strains belonging to endogenous lactic acid flora and secondary flora that may play an important role in the development of many desirable characteristics in cheese, particularly its specific sensory properties. The native, particularly lactic-acid flora, can also be used as protective cultures to inhibit harmful micro-organisms in milk (Salmeron *et al*., 2002).

The number and types of micro-organisms present in milk and dairy products at any particular period depended on the microbial quality of the raw materials, the conditions in which the

products were produced and the temperatures and duration of storage, feeding of the animals, season, area, using different starter cultures etc. (Anonim., 1994). Rinsing water for milking machine and cheese-making equipment washing also involve some of the reasons for the presence of a higher number of micro-organisms including pathogens in raw milk and raw milk products (Bramley, 1990).

Testing 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. The most common spoilage micro-organisms in milk and dairy products are *Pseudomonas* spp, coliforms, *Bacillus* spp, *Clostridium* spp, lactic-acid producing bacteria, yeasts and moulds, enterococci, etc. On the other hand, milk-borne and milk-product borne outbreaks, caused mostly by cheeses, represent 2–6% of the bacterial food-borne outbreaks reported by surveillance systems from several countries (De Buysier *et al*., 2001). Cheese represents a large risk of bacterial food-borne outbreaks because of pathogen micro-flora, divided into pathogens of current concern (*Salmonella* spp., *Campylobacter* spp., coagulase-positive staphylococci, *Listeria monocytogenes* etc.), and those which cause disease only occasionally (*Escherichia coli*, *Bacillus cereus*, *Clostridium perfringens*, *Clostridium botulinum*, *Streptoccoccus zoepidemicus* etc.) (Anonim., 1994).

For this reason the production of milk products should be in accordance with legal regulations for good sanitary practice. According to the standards to be met when collecting raw milk from production, holding or for the acceptance at a treatment or processing establishment, raw milk intended for direct human consumption and raw cow milk for the manufacture of products made with raw milk whose manufacturing process does not involve any heat treatment must only meet a few microbiological standards: the plate count at 30 °C should be ≤ 100 000 micro-organisms per ml (geometric average over a period of two months, with at least two samples a month), *Staphylococcus aureus* per ml n=5, m=500, M=2000 and c=2, somatic cell count ≤ 400 000 and absence of antibiotics. The microbiological criteria for cheese made from raw milk are the absence of *Listeria monocytogenes* and *Salmonella* spp. in 25 g of sample (n=5, c=0), *Staphylococcus aureus* (m=1 000, M=10 000, n=5, c=2) and *Escherichia coli* (m=10 000, M=100 000, n=5, c=2) (Off. J. of the European Communities, 1992; Pravilnik.., Ur. l. RS, 2004). For milk that does not comply with the standards, pasteurisation is the primary mean of ensuring that related cheese does not represent a health risk. Still, even industrial pasteurisation cannot guarantee the absence of pathogenic micro-organisms because they are present in large numbers in raw milk or due to post-pasteurisation contamination. Pasteurisation also reduces a large proportion of lactic acid bacteria and secondary flora that may play an important role in the development of many desirable characteristics in cheese (Salmeron *et al*., 2002).

The aim of the present study was to determine variations in the different microbial groups affecting the manufacture or sanitary quality of cheese at different critical control points of milking and cheese production.

For this purpose we wanted to find out the presence of pathogens and indicator micro-organisms in 14 semi-hard cheeses after one month of ripening made from raw cow milk by individual Slovenian producers.

The same micro-organisms were established in a total of 98 samples taken during milking and processing of cheese mentioned before: swabs from udders and milking machines, fresh raw and mixed milk from vats, whey and brine. Drinking water used for cleaning milking machines and cheese production was examined as well.

MATERIAL AND METHODS

Sampling

The milk and milk product samples were taken in accordance with the instructions given in ISO/DIS 707 (1995). The samples of water were taken in accordance with the instructions given in ISO 5667-2 (1991).

98 samples were taken from sampling points of milk producing and cheese manufacturing: swabs from cows' udders surfaces before milking, swabs from the surfaces of cleaned milking machines and milking machines after milking (liners and claws), raw fresh milk after milking, mixed milk from vats before starting cheese manufacturing (fresh milk mixed with 12–24-hour-old milk kept at room temperature), whey after curdling, brine, cheese after one month of ripening, ripened cheese after one month of being kept vacuum packed at 6 °C, drinking water used for cleaning the milking machine and cheese vat.

Detection and enumeration of micro-organisms

Preparation of test samples, initial suspensions and decimal dilutions were carried out according to ISO/FDIS 8261 (E) (2001).

Swabs, milk, whey, brine samples

For the detection of ***Listeria monocytogenes*** in swabs, milk and milk products according to EN ISO 11290-1 (1996), we used *Listeria* enrichment broth as pre-enrichment (inc. 30 °C/24–48 h) and 1 Fraser broth as enrichment broth (inc. 37 °C/24–48 h). Palcam (Biokar Diagnostics, France), Oxford (Biokar Diagnostics, France) and ChromAgar Listeria (Mast Diagnostica, Germany) were used for isolation. The immunological method Tecra Unique Listeria (Tecra, Australia) and API Listeria strips (Biomerieux, France) were used for confirmation and identification.

For the detection of *Salmonella* in swabs, milk and milk products we used Buffered peptone water as a non-selective pre-enrichment medium and Selenite cystein buffer as an enrichment medium (ISO 6579, 2002). XLD agar (Biokar Diagnostics, France), BSA agar (Biokar Diagnostics, France) and Rambach agar (Merck, Germany) were used for isolation. The immunological method Tecra Unique Salmonella (Tecra, Australia) and API 10 S strips (Biomerieux, France) were used for confirmation and identification.

Detection of *Proteus* spp. in swabs, milk and milk products was carried out with inoculation of the sample into Nutrient broth (inc. 37 °C/24 h), spreading the colonies on the Brilliant Green Agar according to Edel and Kampelmacher (Biokar Diagnostics, France), typical colonies were confirmed and identified on a Kligler iron slant agar (Merck, Germany) and with API 10 S strips (Biomerieux, France).

For the detection of thermotolerant *Campylobacter* species in swabs, milk and milk products the Preston broth, Karmali agar and Columbia blood agar (Oxoid) were used according to ISO 10272 (E) (1995). The identification was carried out by using API Campy strips (Biomerieux, France).

For the enumeration of bacteria *Escherichia coli* in swabs, milk and milk products the chromogenic medium COLI ID (Biomerieux, France) (inc. 37 °C/24 h) was used.

For the enumeration of coagulase positive staphylococci (*Staphylococcus aureus* and other species) in swabs, milk and milk products the Baird Parker with RPF supplement agar (Biokar Diagnostics, France) was used (SIST EN ISO 6888-2, 1999). The Petrifilm^{TM} Staph Express Count System (3 M^{TM}, USA) was used for confirmation.

The enumeration of coliform micro-organisms in swabs, milk and milk products was carried out on VRBL agar (Merck, Germany) according to the standard IDF 73B, (1998).

For the enumeration of faecal enterococci in swabs, milk and milk products the KF Streptococcus agar with a TTC supplement (Biokar Diagnostics, France) was used according to the standard FIL- IDF 149A (1997).

The presence of sulphite-reducing clostridia spores in swabs, milk and milk products was detected after heating the samples at the temperature of 80 °C/10 minutes, inoculating the SPS agar according to Angelotti (1962) (Merck, Germany) and incubation in anaerobic conditions (inc. 35 °C/24–48 h).

The presence of total bacterial count at 30 °C and aerobic bacteria spores in swabs, milk, whey, cheese and brine was enumerated on PCA agar (Merck, Germany) with the addition of 0.1% w/v (1 g per 1 l of medium) of skimmed milk powder, according to the standards EN ISO 4833 (2003) and Anonim. (2002).

For the enumeration and differentiation of lactic-acid- and non-lactic-acid-producing micro-organisms at 30 °C in swabs, milk, whey, cheese and brine the CLA agar with Chinablue and lactose was composed and used according to the Methodenbuch, M 7.16.2 (1985).

For the enumeration of lactococci in swabs, milk, whey, cheese and brine the M17 agar (Merck, Germany) was used according to Terzaghi and Sandine (1975).

For the enumeration of lactobacilli in swabs, milk, whey, cheese and brine the MRS agar (Merck, Germany) was used according to De Man *et al*. (1960).

For the enumeration of yeasts and moulds in milk, whey, cheese and brine the YGC agar (Merck, Germany) was used according to the standard ISO 6611(E) (1992).

The enumeration of lipolytic micro-organisms in milk, whey, cheese and brine the Tributyrin agar supplemented with Glycerintributyrat (Merck, Germany) was determined according to the Methodenbuch, M 7.6 (1985).

For the enumeration of proteolytic micro-organisms in milk, whey, cheese and brine the Milk Agar was composed and used according to the Methodenbuch, M 7.3.3 (1985).

The enumeration of lactolitic clostridia spores in milk, whey, cheese and brine after heating the samples at the temperature of 80 °C/10 minutes pH-modified RCM agar was proceeded according to the Methodenbuch, M 7.18.3.1 (1995).

For the detection of inhibitory substances in raw and mixed milk the Delvotest SP (DSM, the Netherlands) was used.

Somatic cell count in raw and mixed milk was done using the Fossomatic 5000 (Foss Electric, Denmark).

Water samples

The enumeration of viable micro-organisms was carried out with a colony count on PCA agar culture medium (Merck, Germany) after aerobic incubation at 22 °C/72 h (first set of plates) and at 37 °C/24 h (second set of plates) (SIST EN ISO 6222, 1999).

For the detection and enumeration of intestinal enterococci in water samples the membrane filtration method on a Slanetz-Bartley medium (Biokar Diagnostics, France) for isolation and Bile Esculin Azide agar (Biokar Diagnostics, France) for confirmation (SIST EN ISO 7899-2, 2000) were used.

Coliform micro-organisms and presumptive *Escherichia coli* in water were detected by the MPN method (ISO 9308-2, 1990).

The presence of sulphite-reducing anaerobes (clostridia) spores was detected after heating the samples at a temperature of 75 °C/10–15 minutes according to ISO 6461/2 (1986).

Statistical analyses

The results were analysed with SAS/STAT (1990) statistical procedure. The basic statistical parameters (mean, median, standard deviation, coefficient of variation, maximum and minimum values) and correlation procedure of log values of different groups of micro-organisms were calculated. F-test according to Scheffer for estimation of differences for log values of groups of micro-organisms depending on the area, season, type of feeding, type of cheese-making procedure, individual cheese-maker was used.

RESULTS AND DISCUSSION

The microbiological quality of milking machine surfaces, raw and mixed milk, whey, brine and cheese samples

Milking machines

Cousins *et al*. (1981) reported that inadequately disinfected milk-contact surfaces of milking equipment, including milk cans and bulk tanks, were the major sources of bacteria in milk after it left the udder until collection. The proportion of number of bacteria recovered by rinsing a milking machine during milking is known to be at least 10% or more of the number available to the milk because of the rough inside surfaces with bacterial biofilms.

In our study swabs were taken at two different critical points: liners and claw surfaces. The represented results are the averages of both measurements. It also has to be admitted that washing and in some cases disinfection immediately followed after an earlier milking.

The total number of micro-organisms on 1 cm^2 of milking machine surfaces before and after milking was $14.7 \cdot 10^3$ and $15.0 \cdot 10^3$ cfu, respectively. The number of non-lactic-acid producers was 3 times higher than lactic-acid producers, while the number of coliforms and enterococci was 230 cfu/cm^2 and 120 cfu/cm^2, respectively. It is well known that coliforms can rapidly build-up in moist, milk residues in milking equipment, which then becomes the major source of contamination of produced milk. However, relatively low coliform counts in milk do not necessarily indicate effectively cleaned and disinfected equipment (Bramley, 1990). The original source of enterococci in a milking machine is not clear because cow faeces are not considered the source of enterococci in cheese. Their natural habitats are human and animal intestinal tracts, yet they are also found in soil, on plants, and in the intestines of insects and birds. At the production of farmhouse raw-milk cheese, the enterococcus strains are found in cheese, in milk, on milking machines even after chlorination, on milking equipment surfaces, in water used on the farm and on cows' teats (Gelsomino *et al*., 2002).

The results showed very small differences between the number of micro-organisms on milking machine surfaces before and after milking. According to the applicable regulations (Pravilnik., Ur. l. SFRJ, 1989) the high number of micro-organisms on the surfaces of milking machines even after being cleaned shows improper washing (cleaning) by about 60% of cheese producers. Bramley (1990) also reported that a higher ratio of non-lactic-acid producers reveals the presence not only of coliforms and enterococci but probably also other harmful micro-organisms like micrococci, N group streptococci and mastitis streptococci, asporogenous Gram positive rods like *Microbacterium, Corynebacterium*, sporeforms, Gram negative rods like *Pseudomonas*, enterobacteria etc., which are mostly also present in raw milk (Bramley, 1990).

There were no seasonal differences in number of micro-organisms, except that the number of coliforms was higher in spring (Fig. 1).

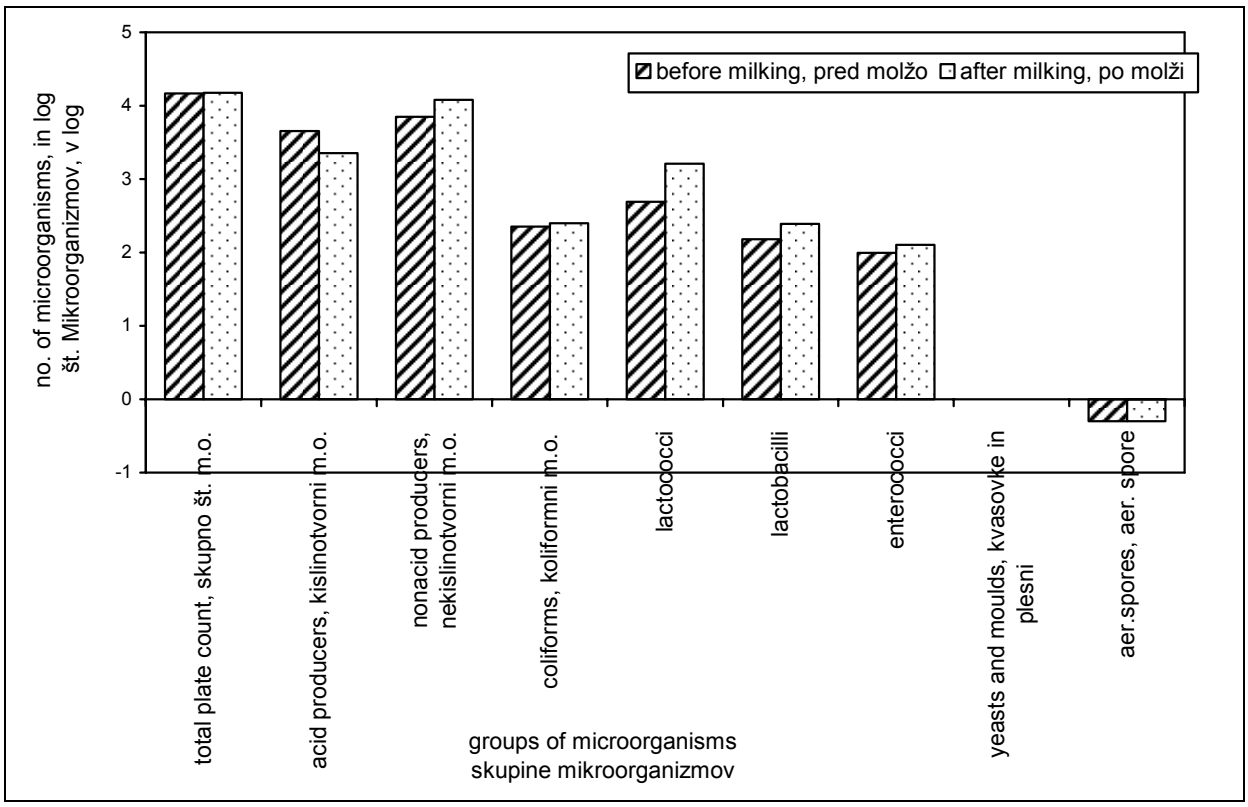

Figure 1. The average number (in log) of micro-organisms belonging to different groups expressed as the number of colony-forming units cfu per 1 cm^2 before and after milking of the inner surface of milking machines.

Slika 1. Povprečno število (v log) mikroorganizmov različnih skupin mikroorganizmov pred in po molži, izraženo kot število kolonijskih enot ke na 1 cm^2 notranje površine molznih strojev.

Raw and mixed milk and whey samples

The total number of micro-organisms was $4.9 \cdot 10^5$ cfu/ml in raw milk and $5.5 \cdot 10^6$ cfu/ml in mixed milk from a vat and did not rise significantly during cheese-processing. Arenas *et al*. (2003) reported on the same values ($5.5 \cdot 10^6$ cfu/ml) of the total number of micro-organisms in raw milk for Genestoso cheese production. The lactic-acid and non-lactic-acid producers in both types of milk samples were at a ratio of 1 to 1. The number of non-lactic-acid producers is unexpectedly high, which showed a possible contamination during cheese-processing. Particularly the number of coliforms in raw and mixed milk was high (in the range of $3.4 \cdot 10^5$) and fell to $5.4 \cdot 10^4$ in whey. 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. 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. (Bramley, 1990).

The average number of enterococci, aerobic spore-forming micro-organisms, yeasts and moulds, lactobacilli, lactococci, proteolytic and lipolytic micro-organisms in milk and in whey

was in the same logarithmic range of about $2.2 \cdot 10^4$, 310, 3.5, $31.2 \cdot 10^4$, $2.1 \cdot 10^6$, $6.2 \cdot 10^3$ and $1.7 \cdot 10^4$ cfu/ml of the sample, respectively. The number of lactobacilli and lactococci did not increase much during the whole cheese-processing (Fig. 2).

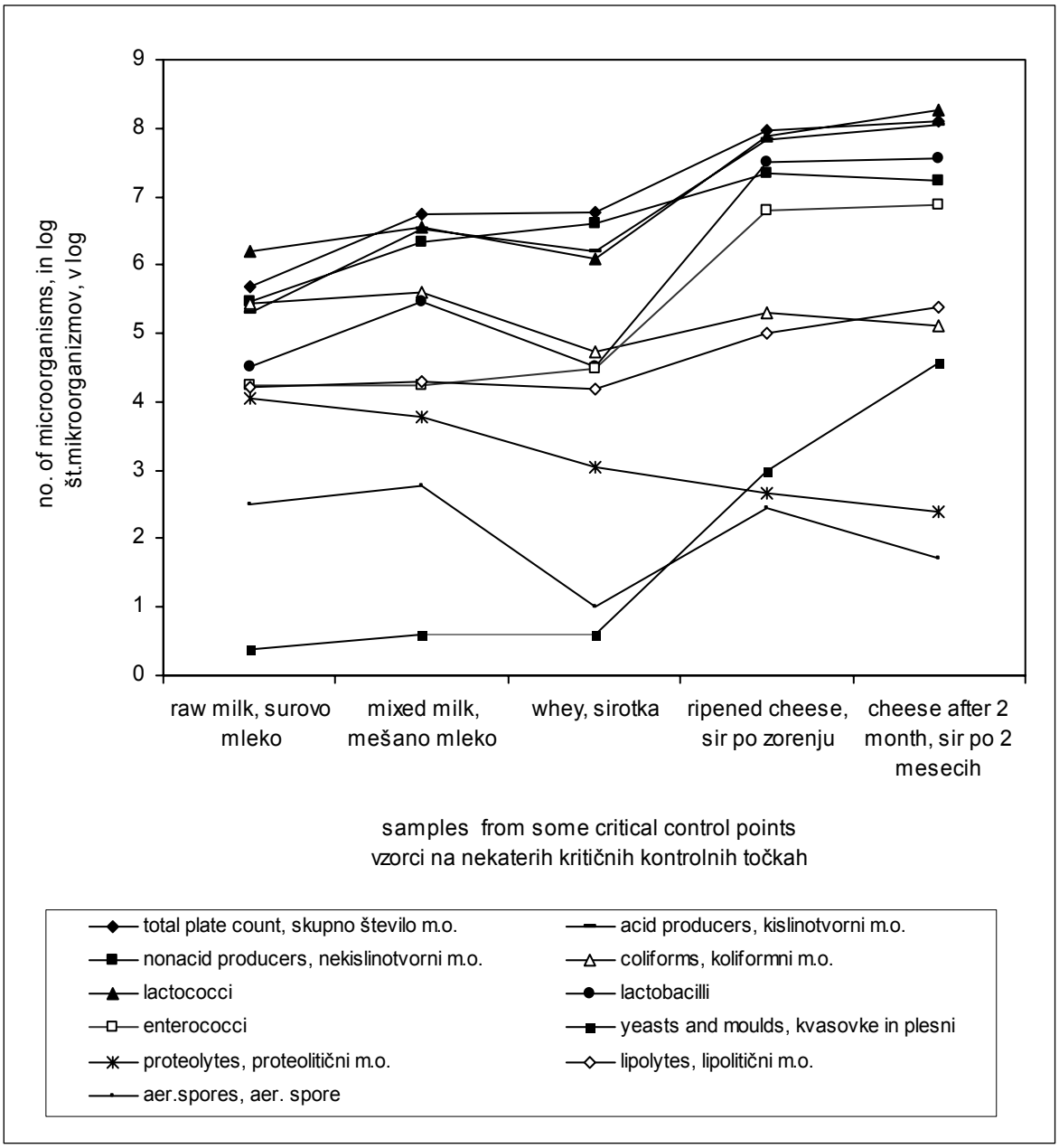

Figure 2. The average number (in log) of different groups of micro-organisms expressed as the number of colony-forming units cfu per 1 ml (g) of samples.

Slika 2. Povprečno število (v log) mikroorganizmov različnih skupin, izraženo kot število kolonijskih enot ke v 1 ml (g) vzorcev.

Arenas *et al*. (2003) found a lower number of lactococci on M17 medium ($2.6 \cdot 10^4$) and enterococci ($2.3 \cdot 10^3$) than we did in our experiment. Estepar *et al*. (1999) studied the quality of Penamellera cheese where the average of total counts on a PCA medium in starting milk was close to 10^6 cfu/ml, the number of coliforms, lactococci, lactobacilli, enterococci and yeasts and moulds was at about 10^4, 2.10^5, 5.10^3, 8.10^3, 8.10^4, respectively. Comparing the results we saw

that the number of the total count of micro-organisms, enterococci and coliforms was relatively close to our results, the numbers of lactococci and lactobacilli were lower and the number of yeasts and moulds was much higher than in our experiment.

In about 60% of tested raw milk samples the number of total plate count of micro-organisms at 30 °C was higher than 50 000 cfu/ml in (without a geometric average calculation). These samples were according to the norms (Council Directive 92/46/EEC, 1992; Pravilnik…, Ur. l. RS, 2004) not appropriate for cheese production. The number of somatic cells per ml were higher than 400 000 in 15.3% of samples. No inhibitory substances were detected in milk samples.

Cheese samples

After one month of ripening the average of total bacterial count was $9.2 \cdot 10^7$ cfu g^{-1} of cheese, of these $6.8 \cdot 10^7$ represented lactic-acid producers and $2.2 \cdot 10^7$ represented non-lactic-acid producers. The average number of coliforms, enterococci, aerobic spore-forming micro-organisms, yeasts and moulds, lactococci, lactobacilli, proteolytic and lipolytic micro-organsims were $2.0 \cdot 10^5$, $6.3 \cdot 10^6$, 280, 960, $2.5 \cdot 10^7$, $9.8 \cdot 10^7$, 450 and $9.8 \cdot 10^4$ cfu g^{-1} of cheese, respectively. After one month of keeping vacuum-packed ripened cheese samples at 6 °C, the number of micro-organisms did not rise significantly, but the number of yeasts and moulds grew to $3.6 \cdot 10^4$ cfu g^{-1} of cheese (Fig. 2).

A high number of enterococci (from a min. $3 \cdot 10^3$ to a max.$15 \cdot 10^7$ cfu g^{-1}) and coliforms (from a min. 10 to a max. $19 \cdot 10^5$ cfu g^{-1}) were detected (Fig. 3, 4). Gelsomino *et al*. (2002) reported that enterococci were widely distributed in raw milk cheese and were generally thought to positively affect the development of flavour.

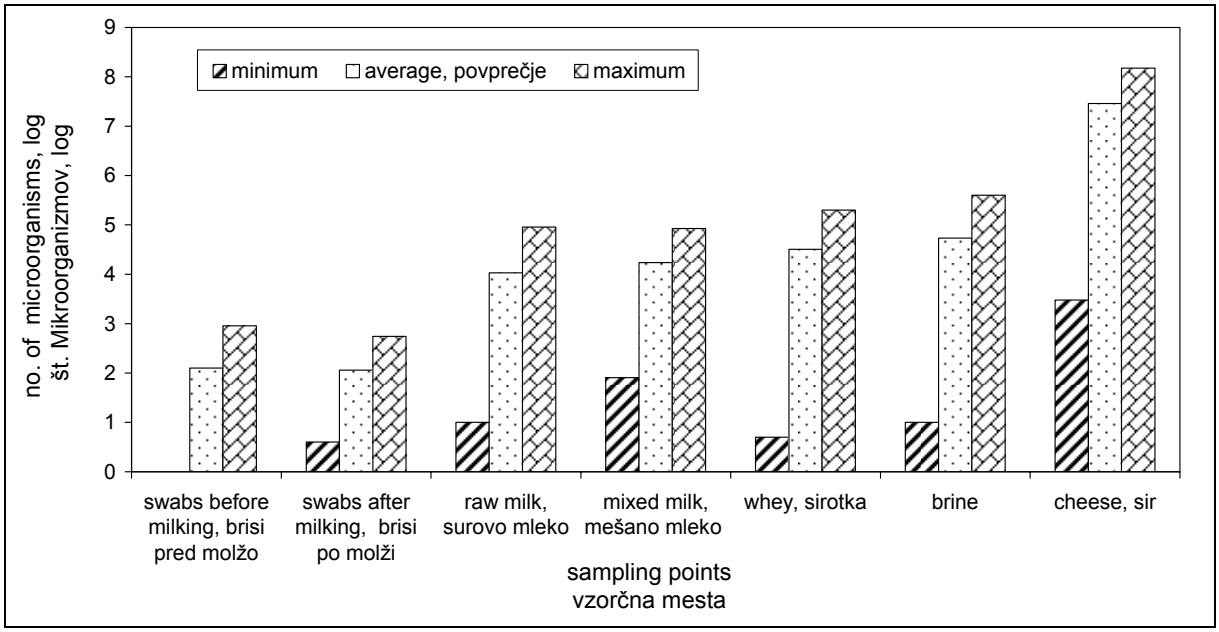

Figure 3. The average number (in log) of enterococci in 98 samples taken at different sampling points of milking and cheese manufacturing (the results of swabs before and after milking are expressed in number of colony-forming units cfu per 1 cm^2 of milking machine surfaces and in other samples as the number of cfu per 1 ml or 1 g).

Slika 3. Povprečno število (v log) enterokokov v 98 vzorcih, odvzetih na različnih kontrolnih točkah molže in sirjenja (brisi površine molznih strojev pred in po molži so izraženi kot število kolonijskih enot ke na 1 cm^2, a pri ostalih vzorcih kot število ke na 1 ml ali g).

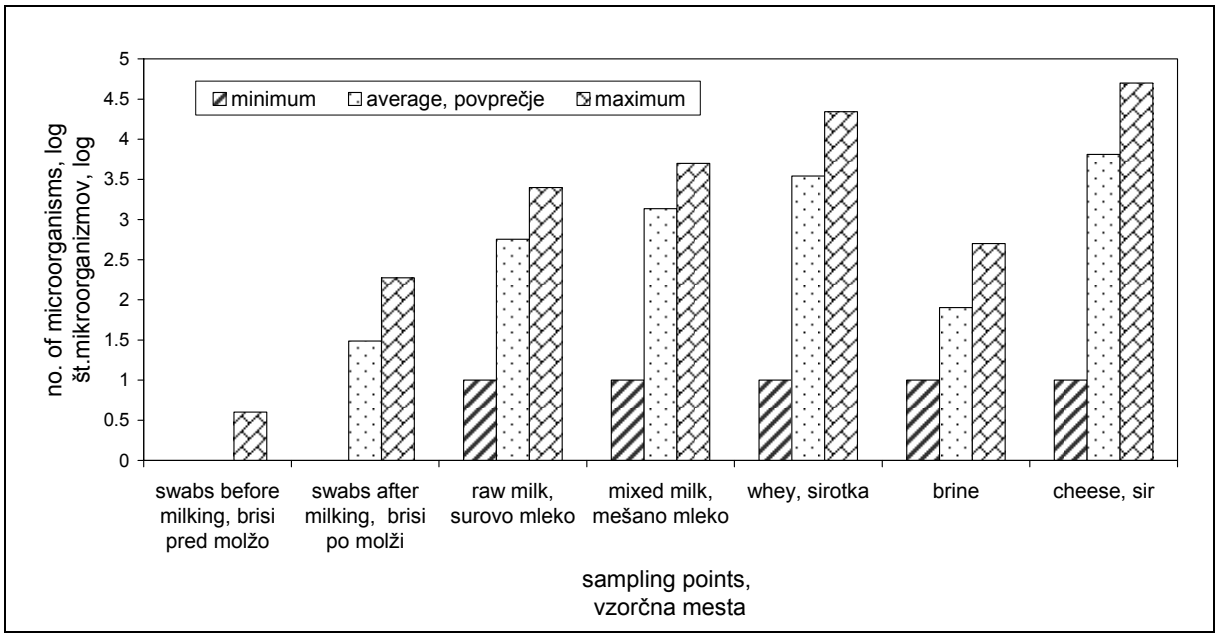

Figure 4. The average number (in log) of coliforms in 98 samples taken at different sampling points of milking and cheese manufacturing (the results of swabs before and after milking are expressed in number of colony forming units cfu per 1 cm^2 of milking machine surfaces and in other samples as the number of cfu per 1 ml or 1 g).

Slika 4. Povprečno število (v log) koliformnih mikroorganizmov v 98 vzorcih, odvzetih na različnih kontrolnih točkah molže in sirjenja (brisi površine molznih strojev pred in po molži so izraženi kot število kolonijskih enot ke na 1 cm^2, a pri ostalih vzorcih kot število ke na 1 ml ali 1 g).

The results of Estepar *et al*. (1999) showed a somewhat higher average number of total bacterial count, coliforms, enterococci and particularly yeasts and moulds, while the number of lactobacilli and lactococci was the same or a little lower than our results. Estepar also reported that the lactic acid bacteria soon became dominant after manufacturing, both on the surface and the interior of cheese. The growth of lactococci was parallel to total aerobic counts. The same data were found in our study (Fig. 2).

Arenas *et al*. (2003) reported on somewhat higher values of total count of micro-organisms ($2.6 \cdot 10^8$ cfu g^{-1} of cheese), while the number of lactococci ($3 \cdot 10^4$) and enterococci ($4.6 \cdot 10^5$) was lower than in our cheese samples.

Menendez *et al*. (2001) studied the characteristics of 24 Tetilla raw cow milk cheese, where the number of total count of micro-organisms, lactococci on M17 agar and enterococci was $3 \cdot 10^9$ cfu/ml, $2 \cdot 10^9$ cfu/ml and $2 \cdot 10^7$ cfu/ml, respectively. High mean counts of coliforms ($1.2 \cdot 10^6$ cfu/ml), and yeasts ($2.7 \cdot 10^4$ cfu/ml) were also measured.

Brine

The total number of micro-organisms in brine was about $4.0 \cdot 10^6$ cfu/ml, while a high number of lactococci (10^8 cfu/ml), lactobacilli ($2.0 \cdot 10^7$ cfu/ml), enterococci ($5.0 \cdot 10^4$ cfu/ml), and yeasts and moulds ($6.0 \cdot 10^4$ cfu/ml) was also established (Fig. 5).

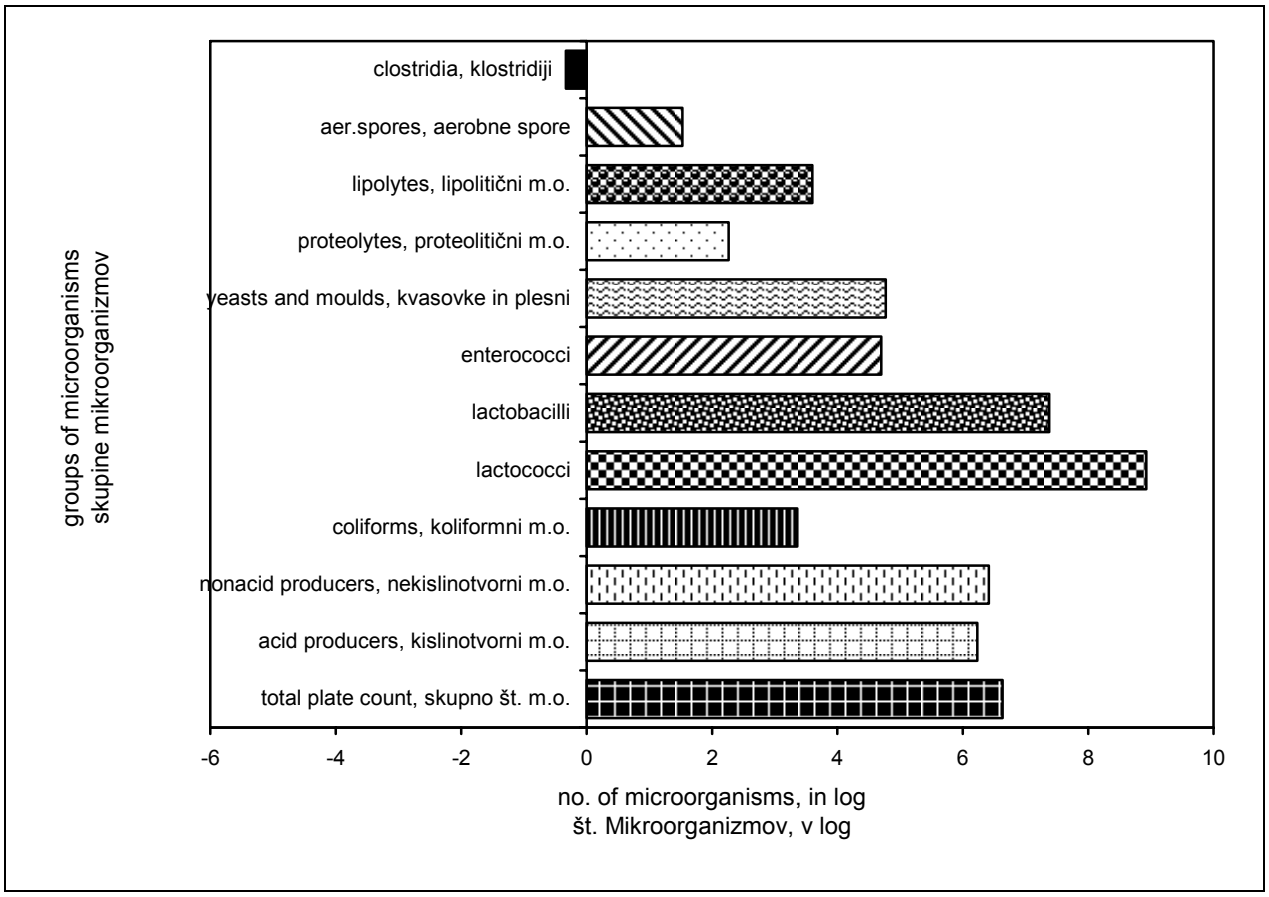

Figure 5. The average number (in log) of different groups of micro-organisms expressed as the number of colony-forming units cfu per 1 ml of brine samples.

Slika 5. Povprečno število (v log) mikroorganizmov različnih skupin, izraženo kot število kolonijskih enot ke v 1 ml vzorca slanice.

Pathogen micro-organisms in samples taken at sampling points in milking and cheese manufacturing

In this experiment the presence of pathogen micro-organisms *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli*, *Campylobacter* spp., sulphite-reducing clostridia and coagulase-positive staphylococci (*Staphylococcus aureus* and related species) was established in samples of udder surfaces, milking-machine surfaces, raw and mixed milk, whey after curdling, as well as cheese and brine.

Listeria spp. was isolated from 7% of samples of four cows' udder surfaces, one swab from milking machine inner surfaces, two milk and one whey samples, while none of the samples examined were positive to the presence of *Salmonella* spp. and *Campylobacter* spp. *L. monocytogenes* is a food-borne pathogen that can contaminate dairy products (Menendez *et al*., 2001). *Listeria monocytogenes* is in contrast to *Salmonella* a psychrotrophic microorganism and can survive at low temperatures. The growth of this organism on contaminated cheese can occur. The most commonly occurring species in food are *L. innocua* and *L. monocytogenes*. Outbreaks of listeriosis resulting from the consumption of dairy foods contaminated with *L. monocytogenes* have prompted concern about the behaviour of this organism during processing and the subsequent storage of various dairy products and about control of the hazard the bacterium poses to the dairy industry. Although *Listeria* is inactivated under normal conditions of pasteurisation (Schaack and Marth, 1988), problems can arise from post-pasteurisation contamination. Bacteria

can enter cheese at many stages during its processing. Meyer Broseta *et al*. (2003) reported that the presence of *L. monocytogenes* in farm raw milk was low (only 2.4% of samples taken monthly from milk tankers). A seasonal effect (with peaks in winter) was observed. The farm milk contamination is, most often, a sporadic event. The number of bacterial cells of *Listeria* was also very low (below 3 bacteria per millilitre with a most probable concentration of 0.1 cfu/ml). Such low levels are very likely to be due to environmental contamination.

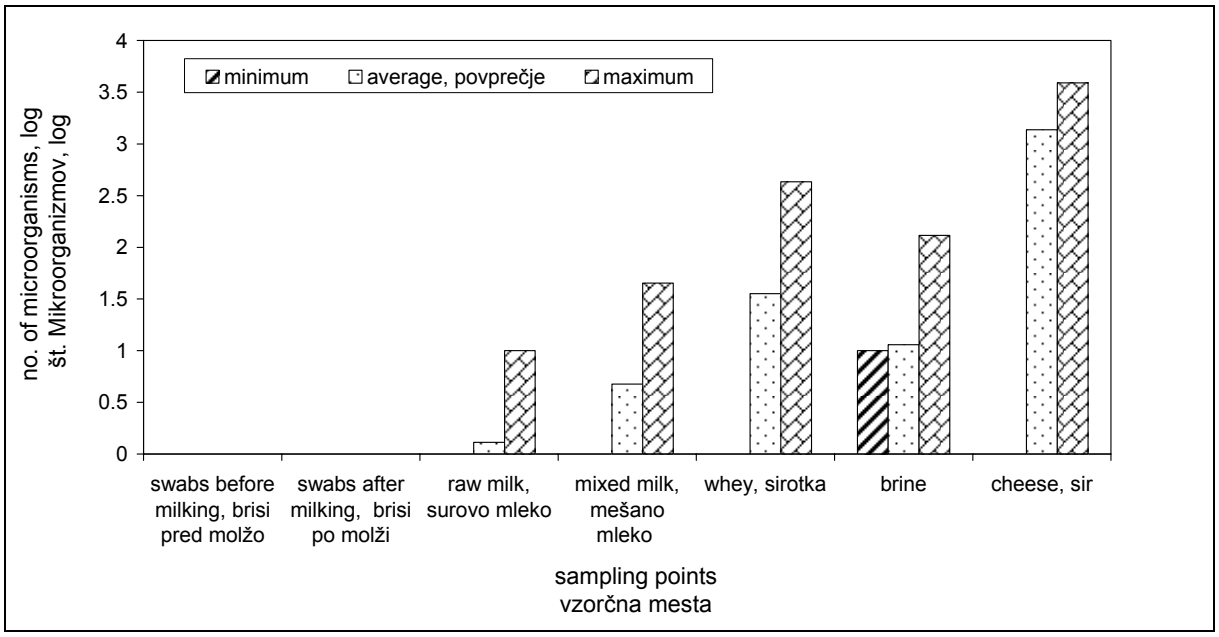

Figure 6. The average number (in log) of coagulase-positive staphylococci (*S. aureus*) in 98 samples taken at different sampling points of milking and cheese manufacturing (the results of swabs taken before and after milking are expressed in number of colony forming units cfu per 1 cm^2 of milking machine surfaces and in other samples as number of cfu per 1 ml or 1 g).

Slika 6. Povprečno število (v log) koagulaza-pozitivnih stafilokokov v 98 vzorcih, odvzetih na različnih kontrolnih točkah molže in sirjenja (brisi površine molznih strojev pred in po molži so izraženi kot število kolonijskih enot ke na 1 cm^2, a pri ostalih vzorcih kot število ke na 1 ml ali 1 g).

The contamination of raw milk with *Salmonella* usually occurs as a result of the transfer of faeces from an animal to milk via unclean teats and udders. Such contamination can pass into milk during milking and, once present, on milking parlour equipment that can then readily proliferate and spread if such equipment is not adequately cleaned and sanitised. Its growth in milk should be limited by effective refrigeration (<8 °C). Effective milking parlour hygiene (cleaning and disinfection of udders and teats), cleaning and sanitisation of milking equipment and subsequent milk storage systems are essential elements in preventing the spread of this organism (McManus and Lanier, 1987).

Campylobacter jejunii and *Campylobacter coli*, which cause *Campylobacter* enteritis, may be commonly isolated from cow faeces and this is considered to be the main source of infection of raw milk. *Campylobacter* species do not generally grow at temperatures below 30 °C and are sensitive to the conditions necessary for growth. Therefore, growth is unlikely to occur in milk and dairy products. The infectious dose for these micro-organisms is, however, low and consequently growth may not be a prerequisite of infection. In raw milk, the *Campylobacter*

number will normally be reduced during cold storage. Both *Campylobacter* species are sensitive to milk pasteurisation (Anonim., 1994).

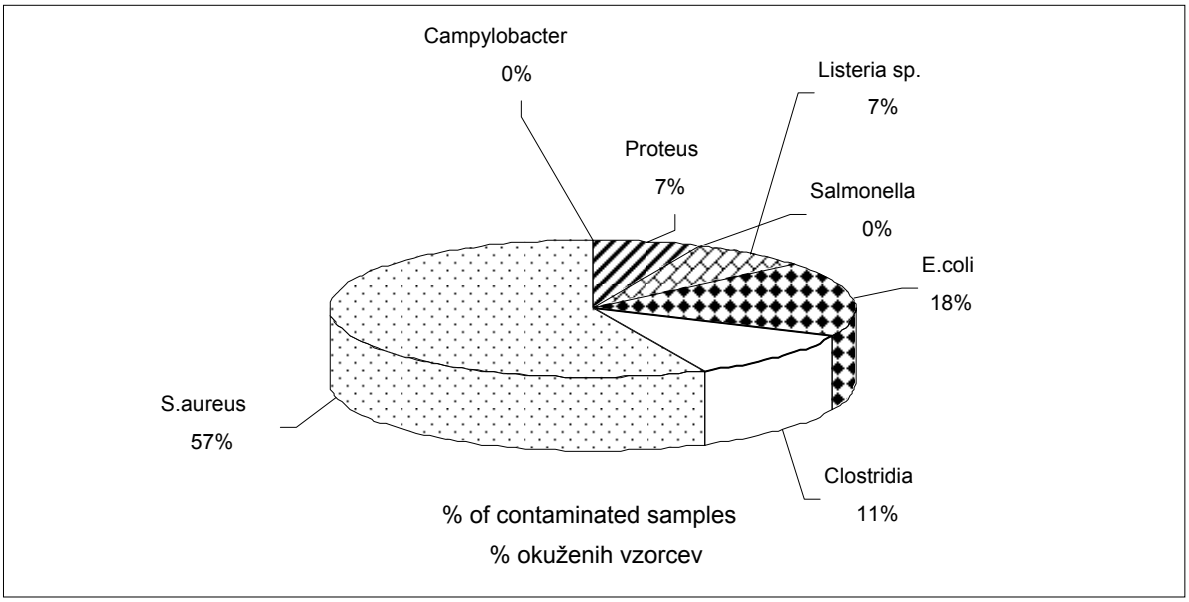

Figure 7. The percentage (%) of samples contaminated by pathogen micro-organisms of all 98 samples tested.
Slika 7. Odstotek (%) s patogenimi mikroorganizmi kontaminiranih od skupno 98 preizkušenih vzorcev.

Proteus was present in 7 (7%) cases of milk and whey. Habeš (2002) reported similar results. In his study *Proteus* spp. was present in 7.28% of 840 raw and pasteurised milk samples taken in Bosnia in a 4-year period.

Sulfite-reducing clostridia (mostly *Cl. perfringens*) were detected in 10 (10%) samples (swabs, raw milk, whey). These spore-forms are present in sediment of various types, and in the intestinal tracts of men and animals. They gain entry to milk via faeces, soil and feedstuff, especially silage. Strains may be psychrotrophic, mesophilic or thermophilic. Since most strains are strictly anaerobic, they have the greatest potential importance as spoilage organisms of cheese and canned milk products. They produce a number of soluble toxic substances (Gilmour and Rowe, 1990).

Escherichia coli was isolated in our experiment from 12 (12%) samples of swabs, raw and mixed milk, whey and brine. Testing for *E. coli* as an indicator of faecal contamination and/or poor hygienic practices has traditionally been done in dairy industry. It is well known that some strains may be enteropathogenic or enterotoxigenic. Both of these groups have been responsible for outbreaks involving cheese and milk (Anonim., 1994).

The number of coagulase-positive staphylococci (*Staphylococcus aureus* and related species) exceeded the norms of the European Communities (M=2 000 bacteria per ml of sample) in 14.4% of raw milk samples. The average number of these organisms in raw milk was $5.7 \cdot 10^2$ and increased to $3.5 \cdot 10^3$ in whey samples (Fig. 6). The presence of coagulase-positive staphylococci was examined in 57% of samples (raw milk, mixed milk, whey, brine, cheese samples) (Fig. 7).

De Buysier *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.

Coagulase-positive staphylococci (*Staphylococcus aureus* and related species) 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 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 hyius* 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. Therefore, toxins may be present in the absence of viable micro-organisms. 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) (Fig. 7).

Pathogen micro-organisms in cheese samples

Salmonella, *Listeria* spp., *Proteus*, sulphite-reducing clostridia, *Campylobacter* were not detected in cheese samples. *E. coli* was found in 4 (30%) of samples on levels from 10–3400 cfu g^{-1} of cheese, while the coagulase positive staphylococci (*S. aureus* and related species) were present in 9 (64%) of samples and ranged from 100 to 50 000 cfu g^{-1} of cheese. Their average number in cheese samples ($6.5 \cdot 10^3$) was in the same log range as in the whey samples (Fig. 6). These results showed a high contamination with these two types of micro-organisms in comparison with the results of Menendez *et al*. (2001) who established low average numbers of *Staphylococcus aureus* (<61 per g/cheese) and *Escherichia coli* (<52 per g/cheese) in 24 Tetilla cheese samples. *Listeria monocytogenes* was detected in two of 24 samples. None of the samples yielded *Salmonella* spp.

A significant correlation (correlation coefficient $r>0.80$, $P<0.0001$) between the total bacterial count, the number of lactic-acid, non-lactic-acid-producers, enterococci, lactococci and lactobacilli in swabs, milk and cheese samples was established (data not shown).

There were no significant differences in the number of micro-organisms between spring and autumn seasons, except for enterococci (P=0.0004**). Significant differences between the microbiological quality of samples from individual cheese-makers were also established (P=<0.0001***). There is no statistically significant influence on microbiological quality between different Slovenian areas where cheese production takes place, using starter cultures in cheese production or not, and between types of feeding (pasture, feeding in a cowshed, etc).

Highly statistically significant differences in number of enterococci between cheese producers (P=<0.0001***) and between seasons (P=0.0041**) were found. There were also statistically significant differences between the number of bacteria *E. coli* between seasons (P=0.041), while the differences in *E. coli* numbers between producers were not statistically significant (P=0.36).

Water samples

Water used in the process of milk production should be of bacteriologically potable quality. The purity of properly treated supplies taken direct from the mains is assured, but bacterial contamination can be introduced from storage tanks not properly protected against rodents, birds, insects and dust. Bacteria may also come from dirty wash troughs, or the carrying of buckets and hoses. Many farms rely on untreated water supplies from boreholes, wells, lakes, springs and rivers; some of these may be contaminated at source with micro-organisms of faecal origin, e.g.

coliforms, faecal streptococci and clostridia. In addition, a wide variety of saprophytic micro-organisms derived from soil or from vegetation may be present, including *Pseudomonas* spp., coliforms and other Gram-negative rods, *Bacillus* spores, coryneform bacteria and lactic acid bacteria. The numbers of these contaminants vary widely. If untreated water gains access to milk or is used for rinsing equipment and containers, any micro-organisms present in the water will contaminate the milk although the numbers of micro-organisms added may not be significant in terms of the cfu/ml of milk. However, multiplication of some of the water-borne bacteria in any residual water in the equipment will result in a more serious contamination and may lead to the establishment and development of some undesirable types of micro-organisms, e.g. psychrotrophic Gram-negative rods, in the milking equipment.

For these reasons, in areas where farm water supplies are bacteriologically unsatisfactory the chemical disinfection or sanitation of milking equipment is always delayed until just before the next milking, and the disinfectant solution is merely drained from the equipment before it is used for milking. This practice prevents recontamination resulting from rinsing with untreated water. Chlorination, by dosing with hypoclorite, is frequently recommended for water of unsatisfactory bacteriological quality used for the final rinsing of equipment, because it helps to reduce the risk of bacterial multiplication in residual water left in milking machines that are cleaned and sanitised in the one operation (Cousins *et al*., 1981).

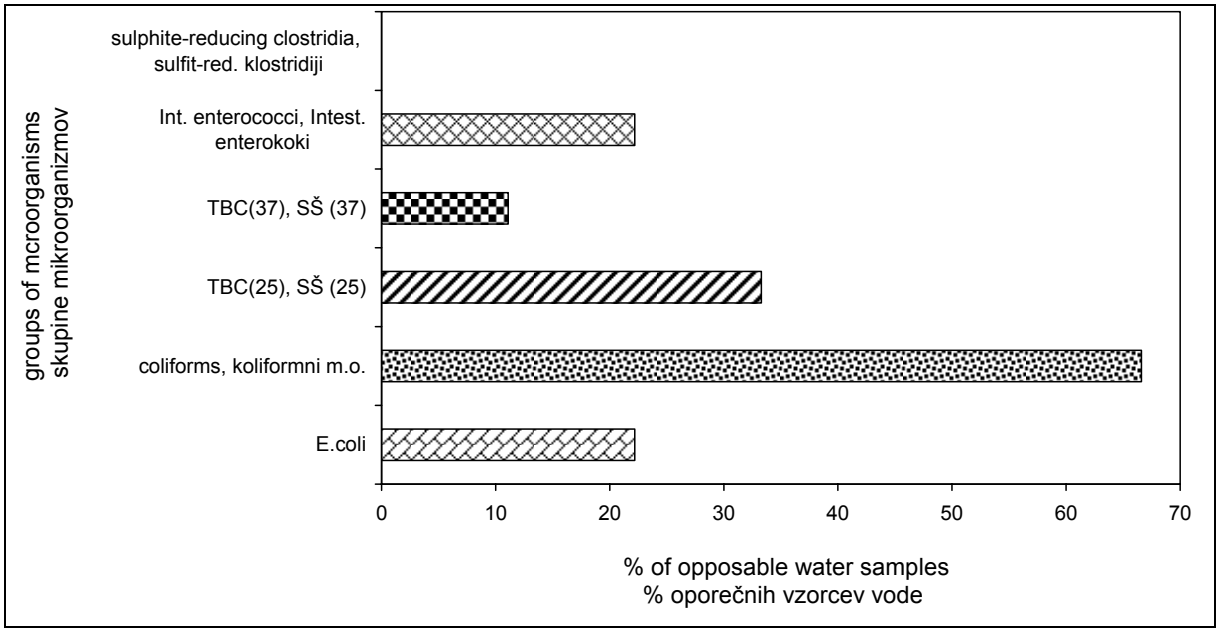

Figure 8. The percentage (%) of water samples not corresponding to the applicable regulations due to the presence or higher number of indicator micro-organisms: TBC (37): total bacterial count inc. at 37 °C; TBC (25): total bacterial count inc. at 25 °C, sulfite-reducing clostridia, coliforms, intestinal enterococci and *E. coli.*

Slika 8. Odstotek (%) vzorcev vode, ki niso ustrezali veljavnim predpisom zaradi povišanega števila indikatorskih mikroorganizmov: SŠ (37): skupno število m.o. ob inkubaciji pri 37 °C; SŠ (25): skupno število m.o. ob inkubaciji pri 25 °C, sulfit-reducirajočih klostridijev, koliformnih m.o., intestinalnih enterokokov in *E. coli*.

The number of total bacterial count and the presence of *E. coli*, coliforms and faecal streptococci in water samples taken at taps or water pipes in cheese-makers and in milking parlours were established in our study. The results showed that 78% of water samples did not correspond to the microbiological criteria according to the applicable regulations (Pravilnik, Ur.

l. RS, 2004). *E. coli* was present in 22% of samples, coliforms in 67% and faecal enterococci in 22% of the samples. The number of viable micro-organisms at 37 °C was exceeded in 11% of samples, while the number of micro-organisms at 22 °C was exceeded in 33% of samples (Fig. 8).

System of critical control points

In recent years, the hazard analysis critical control points (HACCP) concept has been proposed as the best approach to ensure food safety. The results of this study also underline the need to achieve food safety and reduce risk, to implement the hazard analysis critical control points (HACCP) concept and quality assurance from the farm to the dairy plant, and to set up and apply EU directive 92/46 on milk hygiene (Silva, 2003; De Buyser, 2001). It is very important for cheese-makers to set up the system of critical control points and to investigate the direct and cross-contamination sources in their cheese production. The milking machines, production pipelines, equipment such as vats, plastic wraps, pressing cloths, starter cultures and the hands of workers were direct contamination sources. In addition, the hands of workers and the water used in a facility played a role in direct cross-contamination. The air in the facility was a critical control point for yeast and mould contamination.

Routine microbiological monitoring of the hygienic quality of raw milk should be employed using not only the total plate count, but also indicator bacteria such as *E. coli* or coliforms and incentive payment schemes should be considered where milk is intended to be used without a bacterial destruction stage in the process, i.e. for raw milk cheese, to encourage the adoption of high hygienic standards (McManus and Lanier, 1987). The inclusion of other pathogen micro-organisms like *L. monocytogenes* on the list of organisms subject to the HACCP has recently also been called for (Silva *et al*., 2003).

CONCLUSIONS

- In our study 21% of tested cheese samples did not correspond to the microbiological criteria according to the applicable EU and Slovenian regulations.
- The high number of micro-organisms on the surfaces of washed milking machines before milking showed ineffective cleaning (washing) by about 60% of cheese producers.
- Greater contamination usually appeared during cheese-processing and not during milking.
- In 78% of drinking water samples the results exceeded the microbiological criteria according to the applicable regulations so greater attention should also be paid to water quality.
- It is suggested that more importance should be given to milking and cheese production hygiene, as well as to the determination and control of critical points in firms for improving cheese quality and preventing food-borne pathogenic outbreaks.
- The authors believe that milk and cheese producers should employ the HACCP and quality assurance practices in the production stages of milk from the farm up to and including the dairy plant, while also setting up and implementing EU Directive 92/46.

POVZETEK

V Sloveniji je posebno v odročnejših predelih kar nekaj individualnih majhnih sirarn, kjer proizvajalci mleko sami predelajo v sire, včasih tudi v skuto. Pogosto uporabljajo za sirjenje surovo, nepasterizirano mleko, kar omogoči boljši izkoristek mleka, ohrani pa se tudi naravna, za tisto področje značilna mlečnokislinska mikroflora, ki igra pomembno vlogo pri senzoričnih

značilnostih proizvedenih sirov. Higienska kakovost in zdravstvena ustreznost proizvedenih sirov zavisi od ustrezne mikrobiološke kakovosti mleka kot surovine, razmer, v katerih se mleko predeluje v sire, kakovosti molže, temperature hranjenja mleka in sirov, krme, sezone, uporabe različnih starterskih kultur, vode, uporabljene za napajanje, pranje molznega in mlekarskega pribora itd.

Preverjanje prisotnosti in števila specifičnih mikroorganizmov v različnih fazah prireje mleka in njegove predelave je pomemben dejavnik pri kontroli in sistemu zagotavljanja kakovosti proizvodnje.

Namen našega dela je bil ugotoviti mikrobiološko kakovost, oziroma prisotnost posameznih skupin mikroorganizmov na nekaterih kritičnih kontrolnih točkah prireje mleka in sirjenja. V ta namen smo ugotavljali prisotnost patogenih in indikatorskih mikroorganizmov v 14 vzorcih poltrdega sira, proizvedenih pri posameznih sirarjih na različnih področjih Slovenije ter v 98 vzorcih, odvzetih v postopku molže in predelave mleka v sir. Odvzeli smo brise površine vimena krav molznic ter notranjih površin molznih strojev, vzorce surovega mleka takoj po molži, mešanega mleka iz sirarskega kotla, sirotke po usirjanju, slanice, sirov po enomesečnem zorenju in vakuumsko pakiranih sirov po naknadnem enomesečnem skladiščenju pri temperaturi 6 °C. Odvzeli smo vzorce vode, namenjene pranju molznega in mlekarskega pribora.

Ugotovili smo, da 3 (21 %) vzorci sirov glede mikrobiološke kakovosti niso ustrezali kriterijem slovenske zakonodaje. Visoko število mikroorganizmov na notranjih površinah molznih strojev (kolektorji, sesne gume) pred molžo kažejo na neučinkovitost pranja (čiščenja, dezinfekcije) pri kar 60 % proizvajalcev. V postopku predelave mleka v sir je prišlo pogosto do večje okužbe kot v postopku molže. Kar 78 % vzorcev vode glede mikrobiološke kakovosti ni ustrezalo predpisanim kriterijem, zato je potrebno veliko pozornost usmeriti na problem zagotavljanja kakovosti vode v odročnejših predelih. Prav tako predlagamo, da posamezni sirarji posvetijo večjo pozornost izboljšanju higiene pri molži in sirjenju ter se tako izognejo slabi mikrobiološki in senzorični kakovosti ter zdravstveni oporečnosti svojih proizvodov. Vzpostavitev sistema kontrole kritičnih točk v posameznih stopnjah prireje in predelave mleka v smislu sistema HACCP in zagotavljanja kakovosti je zaželena in tudi zakonsko predpisana z direktivami EU.

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