

THE PRESENCE OF SOME PATHOGEN MICRO ORGANISMS, YEASTS AND MOULDS IN CHEESE SAMPLES PRODUCED AT SMALL DAIRY-PROCESSING PLANTS

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ABSTRACT

The presence of pathogenic and some indicator micro organisms was studied in 40 samples of cheese comprising 14 curd samples, 13 samples of soft ripened salted or non-salted cheese and 13 samples of semi-hard cheese manufactured at five small dairy-processing plants. The mean number of coagulase-positive staphylococci in all tested samples was 2.5×10^4 cfu g⁻¹, while the number of *E. coli* bacteria was 1.4×10^6 cfu g⁻¹. In 20.0% out of 40 samples tested, the number of coagulase-positive staphylococci exceeded the prescribed regulations, particularly in soft cheese (12.5%) and curd (7.5%). About 17.5% of samples were contaminated with *E. coli* in higher concentrations than national valid regulations allowed. The number of *E. coli* was mostly exceeded in soft cheese and curd in 12.5% and 5.0% of all examined samples, respectively. One sample of semi hard cheese was contaminated with sulphite-reducing clostridia. *Proteus* was detected in 3 samples (7.5%) and *L. grayi* in 1 (2.5%) sample. *Salmonella* and *L. monocytogenes* were not detected. According to the valid regulations 9 (22.5%) samples in our investigation did not reach the adequate microbiological quality. Both, yeasts and moulds were isolated from 60% of tested cheese samples with average concentrations of 5.8×10^4 cfu g⁻¹ and 2.0×10^4 cfu g⁻¹, respectively. The genera *Geotrichum* (91.9%), *Moniliella* (5.4%) and *Aspergillus* (2.7%) were the most frequently isolated strains from examined cheese samples. The *Aspergillus* strains did not belong to the species *A. flavus* or *A. parasiticus* and did not produce aflatoxins.

Key words: milk products / cheese / microbiology / pathogen micro-organisms / yeasts / moulds / aflatoxins

PRISOTNOST NEKATERIH PATOGENIH MIKROORGANIZMOV, KVASOVK IN PLESNI V VZORCIH SIRA, PROIZVEDENIH V MALIH MLEKARSKO-PREDELOVALNIH OBRATIH

IZVLEČEK

Proučevali smo prisotnost patogenih in nekaterih indikatorskih mikroorganizmov v 40 vzorcih sira, od tega v 14 vzorcih skute, 13 vzorcih soljenega ali nesoljenega svežega (mehkega) sira in 13 vzorcih poltrdega tipa sira, proizvedenih v petih malih mlekarско-predelovalnih obratih. Povprečno število koagulaza-pozitivnih stafilokokov v vseh vzorcih je znašalo $2,5 \times 10^4$ KE[†] g⁻¹, medtem ko je bilo število bakterij vrste *E. coli* $1,4 \times 10^6$ KE g⁻¹. Izmed 40 vzorcev je v 20.0 % število koagulaza-pozitivnih stafilokokov presevalo slovenske veljavne normative, posebno v vzorcih mehkega sira (12,5 %) in skute (7,5 %). Okrog 17,5 % vzorcev je bilo okuženo z

* Cfu: colony forming units

† KE: kolonijske enote

bakterijami *E. coli* v višjih koncentracijah, kot to dovoljujejo slovenski veljavni predpisi. Število bakterij *E. coli* je bilo previsoko v vzorcih mehkega sira (12,5 %) in skute (5,0 %). En vzorec poltrdega sira je vseboval sulfid-reducirajoče klostridije. Vrste rodu *Proteus* smo ugotovili v 3 (7,5 %) in *L. grayi* v 1 (2,5 %) vzorcih. *Salmonella* in *L. monocytogenes* nista bili ugotovljeni v nobenem izmed preiskanih vzorcev. Izmed 40 preiskanih vzorcev jih 9 (22,5 %) ni ustrezalo veljavnim slovenskim normativom glede njihove mikrobiološke kakovosti. Tako kvasovke kot plesni so bile izolirane iz 60 % preiskanih vzorcev. Povprečno število kvasovk je bilo $5,8 \times 10^4$ KE g⁻¹ in plesni $2,0 \times 10^4$ KE g⁻¹ vzorca. Najpogosteje so bile izolirane plesni iz rodov *Geotrichum* (91,9 %), *Moniliella* (5,4 %) in *Aspergillus* (2,7 %). Sev iz rodu *Aspergillus* ni pripadal vrstama *A. flavus* /*parasiticus* in tudi ni tvoril aflatoksinov.

Ključne besede: mlečni izdelki / siri / mikrobiologija / patogeni mikroorganizmi / kvasovke / plesni / aflatoksini

INTRODUCTION

The number and types of micro-organisms present in milk and dairy products depends on the microbial quality of milk used, heat treatment of milk, the conditions in which the products are manufactured, the temperatures and duration of storage, feeding of the animals, season, area, general sanitation in the plant, quality of starter cultures, occurrence of phages, quality of rinsing water, etc. (Bramley, 1990; Anonim., 1994; Harkye and Ebenezer, 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. The most common spoilage micro-organisms in milk and dairy products are *Pseudomonas*, 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 bacterial food-borne outbreaks reported by surveillance systems from several countries. The cheeses represent 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*, etc.) (De Buysier *et al.*, 2001).

L. monocytogenes is a food-borne pathogen that can contaminate dairy products (Menendez *et al.*, 2001). It is a psychrotroph and can grow on contaminated cheese at low temperatures. This bacteria is fairly heat-tolerant, widely distributed in dairy farms, and frequently found in raw milk. Some strains can survive pasteurization, and adapt to acidic environments. The most commonly occurring species in food are *L. innocua* and *L. monocytogenes*. Although *Listeria* is mostly inactivated under normal conditions of pasteurization (Farkye and Vedamuthu, 2002), problems can arise from post-pasteurisation contamination. 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 which are very likely to be due to environmental contamination (Meyer Broseta *et al.*, 2003).

The contamination of raw milk with *Salmonella* usually occurs as a result of transfer of faeces from an animal to the milk via unclean teats and udders. Such contamination can pass into milk during milking and, once present on milking parlour equipment, it 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).

Sulphite-reducing clostridia (mostly *Cl. perfringens*) are spore-forms that are present in sediment of various types and in the intestinal tracts of man 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).

Examination of the presence of *E. coli* as an indicator of faecal contamination and/or poor hygienic practices has traditionally been done in dairy plants. It is well known that some strains might be enteropathogenic or enterotoxigenic. Both of these groups have been responsible for outbreaks of diseases involving cheese and milk (Anonim., 1994).

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 while 10 °C is the minimum for toxin production. These micro-organisms are, however, resistant to salt. Pasteurization will be effective against them but if toxins are present, they 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).

Pasteurization cannot guarantee the absence of pathogenic micro-organisms when they are present in large numbers in raw milk or due to post-pasteurization contamination (Salmeron *et al.*, 2002).

The production of milk products should be in accordance with legal regulations for good sanitary practice on removal from the processing establishment. The microbiological criteria for cheese are *L. monocytogenes* and *Salmonella* (absent in 25 g or 1 g of sample), *S. aureus* (m=1 000, M=10 000, n=5, c=2, for fresh cheese: m=10, M=100, n=5, c=2), *E. coli* (m=10 000, M=100 000, n=5, c=2, for soft cheese m=100, M=1000, n=5, c=2) and coliforms at 30 °C for soft cheese (m=10 000, M=100 000, n=5, C=2) (Off. J. of the European Communities, 1992, consolidated in 2004; Pravilnik..., Ur. l. RS, 2004). The microbiological criteria for foodstuffs according the Regulation EU no. 2073, (Off. J. of the European Communities, 2005) are more strict for *E. coli* in cheese (m=100, M=1000, n=5, c=2). The allowed number of *S. aureus* depends on the type of examined cheese and on the thermization of the milk, used for cheese production. These criteria are designed for the food products during or at the end of the production process, except for the presence of *Salmonella*, which should be estimated in milk products on the market.

Yeasts and moulds in some cheese types can periodically cause problems, both economic and sensory. Usually present in raw milk, they do not survive pasteurization; their presence in pasteurized milk and other milk products is caused by re-infection during manufacturing (Nelson, 1987; Jodral *et al.*, 1993). The contamination of milk products, particularly cheeses is due to yeasts and moulds presence in environment of cheese factories, like walls and shelves of ripening rooms, air, equipment, water, milk, brine, etc. (Chapman and Sharpe, 1990; Jay, 1992).

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 typical yeasty or fruity flavour and obvious gas (Davis and Wilbey, 1990).

On the other hand, some specific strains in a starter cultures for soft cheese production or in the maturation and aroma formation play an important role in many cheese varieties, contributing with their metabolic properties to the ripening process. They metabolize lactic acid in the cheese, and raise the pH of the microenvironment in the area adjacent to the surface, and allow good growth and metabolic activities of the bacteria in the smear. Additionally, the lysis of yeast cells liberates vitamins and amino acids, which stimulate the bacteria, and provide flavour precursors. 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).

Although moulds have little practical importance in raw milk, they are important in pasteurized milk, particularly when it is used for the manufacture of cheese and other dairy products. The characteristic feature of some mould-ripened cheese types is extensive proteolysis and lipolysis. 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). The major toxigenic species of fungi belong to genera *Aspergillus*, *Fusarium*, *Acremonium* and *Phomopsis* (D'Mello and Macdonald, 1997).

For this purpose we wanted to find out the presence of pathogens and indicator micro-organisms including yeasts and moulds in 40 samples of curd, soft and semi-hard cheese from individual small dairy-processing plants that sold their products on the market. The objective of this study was also the identification of isolated mould strains and detection of their aflatoxin production.

MATERIAL AND METHODS

Sampling

A total of 40 samples of cheese comprising 14 curd samples, 13 samples of soft salted or non-salted cheese and 13 samples of semi-hard cheese were collected from November 2004 to January 2005. The cheeses were manufactured at five individual small dairy-processing plants that sold their products on the market.

The samples were taken in accordance with the instructions given in ISO/DIS 707, (1995).

Methods

Media

For the detection of *L. monocytogenes* according to EN ISO 11290-1 (1996), *Listeria* Enrichment Broth (Biokar Diagnostics, France) as pre-enrichment (inc. 30 °C/24–48 h) and 1 Fraser broth (Merck, Germany) as enrichment broth (inc. 37 °C/24–48 h) were used. 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 (Biomérieux, France) were used for confirmation and identification.

For the detection of *Salmonella* in cheese samples Buffered peptone water (Biokar Diagnostics, France) as a non-selective pre-enrichment medium and Selenite cystein buffer (Biokar Diagnostics, France) as an enrichment medium (ISO 6579, 2002) were used. 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 (Biomérieux, France) were used for confirmation and identification.

Detection of *Proteus* spp. was carried out with inoculation of the sample into Nutrient broth (Biokar Diagnostics, France (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 enumeration of bacteria *Escherichia coli* the chromogenic medium COLI ID (Biomerieux, France) (inc. 37 °C/24 h) and identification of the selected colonies with API 10 S strips (Biomerieux, France) were used.

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 Petrifilm™ Staph Express Count System (3 M™, USA) was used for confirmation.

The presence of sulphite-reducing clostridia spores 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 number of yeasts and moulds in samples the yeast-extract-glucose-chloramphenicol agar (YGC) (Merck, Germany) was used. Yeast and mould colonies growing on the plates were counted after 5 days incubation at 25 °C. (ISO 6611/IDF 94, 2004).

Inoculation

For the enumeration of yeasts, moulds, coagulase-positive staphylococci and *E. coli* the primary suspensions of cheese samples were prepared with 3–5 min homogenization of 10 g of the sample in 90 ml of sterile 20% di-potassium hydrogen phosphate (Merck, Germany) using Bagmixer® 400 (Interscience, France). The primary suspensions of cheese samples were prepared with quarter-strength Ringer's solution and inoculated by pouring the plates with chosen medium (EN ISO 8261 (E), 2001). For detection of *Listeria* spp., *Salmonella* spp. and *Proteus* spp., after the previous pre-enrichment, the culture has been spread out onto the solid selective media and later the identification took place.

Moulds isolation and identification

Each morphologically different mould colony from the plates was picked up, transferred on to the YGC and AFPA (Oxoid, England) mediums and incubated at 25 °C/5 days and 30 °C/42 hours, 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 magnified 100/1.30 (160/0.17), according to Malloch and 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, 1983). The reference strains *A. flavus* EXF 523 and *A. flavus* EXF438 were kindly given as a gift from the University of Ljubljana, Biotechnical Faculty, Department for Biology.

Statistical analyses

For statistical analyses the SAS/STAT (SAS/STAT User's Guide, 2000) and Excel XP were used.

Descriptive statistics including average, standard deviation, minimum and maximum were calculated.

The CORR Procedure was taken for calculating the correlation coefficients between variables log number of yeasts, moulds, coagulase-positive staphylococci and *E. coli* in different types of products and in products offered by different producers.

RESULTS

Pathogen micro-organisms in cheese samples

The mean number of coagulase-positive staphylococci in all tested samples was 2.5×10^4 cfu g^{-1} , while the number of bacteria *E. coli* was 1.4×10^6 cfu g^{-1} . Because the samples were collected in one instead of in five units ($n=1$ instead $n=5$), we estimated that the product was unsuitable according regulations, when the number of micro-organisms exceeded the maximum value M. In 20% out of 40 samples tested the number of coagulase-positive staphylococci exceeded the prescribed regulations (Pravilnik..., 2004), particularly in soft cheese (12.5%) and curd (7.5%). About 17.5% of samples were contaminated with *E. coli* in higher concentrations than regulations according Pravilnik..., (2004) allowed. The number of *E. coli* was mostly exceeded in soft cheese (12.5%) and in curd (5.0% of samples) (Tables 1 and 2, Figure 1). According the regulations ES 2073/2005, the number of *E. coli* and *S. aureus* exceeded in 37.5% and 20% of samples, respectively. One sample of semi-hard cheese was contaminated with sulphite-reducing clostridia. *Proteus* sp. was detected in 3 samples (7.5%) and *L. grayi* in 1 (2.5%) sample. *Salmonella* sp. and *L. monocytogenes* were not detected in any sample.

Table 1. The number of coagulase-positive staphylococci in cheese samples (in cfu g^{-1})
Preglednica 1. Število koagulaza-pozitivnih stafilokokov v vzorcih sira (v KE g^{-1})

Statistical parameters Statistični parametri	Samples Vzorci			Mean (all samples) Povprečje (vsi vzorci)
	Curd Skuta	Soft cheese Mehki sir	Semi-hard cheese Poltrdi sir	
Mean / povprečje	1.4×10^2	7.2×10^4	5.0×10^1	2.5×10^4
Min	≤ 10	≤ 10	≤ 10	≤ 10
Max	1.3×10^3	1.0×10^6	3.0×10^1	1.0×10^6
Sd	3.6×10^2	2.7×10^5	9.6	1.6×10^5

Sd – standard deviation / standardni odklon; Min – minimal values / minimalne vrednosti; Max – maximal values / maksimalne vrednosti;

Table 2. The number of *E. coli* in cheese samples (in cfu g^{-1})
Preglednica 2. Število *E. coli* v vzorcih sira (v KE g^{-1})

Statistical parameters Statistični parametri	Samples Vzorci			Mean (all samples) Povprečje (vsi vzorci)
	Curd Skuta	Soft cheese Mehki sir	Semi-hard cheese Poltrdi sir	
Mean / povprečje	1.6×10^6	2.2×10^6	1.0×10^3	1.4×10^6
Min	≤ 10	≤ 10	≤ 10	≤ 10
Max	2.3×10^7	1.5×10^7	9.1×10^3	2.3×10^7
Sd	6.1×10^6	5.2×10^6	2.6×10^3	4.7×10^6

Sd – standard deviation / standardni odklon, Min – minimal values / minimalne vrednosti, Max– maximal values / maksimalne vrednosti

The correlation coefficient between the number of coagulase-positive staphylococci and the number of *E. coli* in curd is $r=0.93$ ($P<0.01^{**}$ significant) in soft cheese $r=0.64$ ($P<0.01^{**}$ significant) and in hard cheese $r=-0.16$ ($P>0.05$, not significant).

The correlation between the number of coagulase-positive staphylococci in all three types of cheese samples is negative as well as between the number of *E. coli*, except between the number of *E. coli* in soft and semi-hard cheese ($r=0.59$, $P<0.05$ * significant).

Yeasts and moulds in cheese samples

The yeasts in concentration above 10 cells per gram of the sample were determined in 24 (60%) out of 40 samples. In this concentration yeasts were present in 9 (64.3%) out of 14 of curd samples, in 11 (84.6%) out of 13 soft cheese samples and in 4 (21.4%) out of 13 semi-hard cheese samples. The statistical parameters are represented in Table 3.

Moulds were present in the number up to 10 per gram of the sample in 24 (60%) out of 40 tested samples. In this concentration moulds were found in 10 (71.4%) out of 14 of curd samples, in 9 (69.2%) out of 13 soft cheese samples and in 5 (38.4%) out of 13 semi-hard cheese samples. The statistical parameters are presented in Table 4.

Table 3. The number of yeasts in cheese samples (in cfu g⁻¹)
Preglednica 3. Število kvasovk v vzorcih sira (v KE g⁻¹)

Statistical parameters Statistični parametri	Samples Vzorci			Mean (all samples) Povprečje (vsi vzorci)
	Curd Skuta	Soft cheese Mehki sir	Semi-hard cheese Poltrdi sir	
Mean / povprečje	1.4×10^5	1.5×10^4	1.3×10^4	5.8×10^4
Min	≤ 10	≤ 10	≤ 10	≤ 10
Max	7.8×10^5	1.5×10^5	1.6×10^5	7.8×10^5
Sd	2.8×10^5	3.9×10^4	4.4×10^4	1.8×10^5

Sd – standard deviation / standardni odklon, Min – minimal values / minimalne vrednosti, Max – maximal values / maksimalne vrednosti

Table 4. The number of moulds in cheese samples (in cfu g⁻¹)
Preglednica 4. Število plesni v vzorcih sira (v KE g⁻¹)

Statistical parameters Statistični parametri	Samples Vzorci			Mean (all samples) Povprečje (vsi vzorci)
	Curd Skuta	Soft cheese Mehki sir	Semi-hard cheese Poltrdi sir	
Mean / povprečje	2.1×10^4	4.0×10^4	5.8×10^1	2.0×10^4
Min	≤ 10	≤ 10	≤ 10	≤ 10
Max	1.2×10^5	4.7×10^5	3.8×10^2	4.7×10^5
Sd	4.2×10^4	1.2×10^5	1.1×10^2	7.8×10^4

Sd – standard deviation / standardni odklon, Min – minimal values / minimalne vrednosti, Max – maximal values / maksimalne vrednosti

The correlation coefficient between number of yeasts and moulds in curd is $r=0.55$ ($P<0.05^*$ statistically significant) in soft cheese $r=0.10$ ($P>0.05$ statistically not significant) and in hard cheese $r=-0.24$ ($P>0.05$, statistically not significant).

The comparison between the numbers of micro-organisms in different cheese samples is represented in Figure 1.

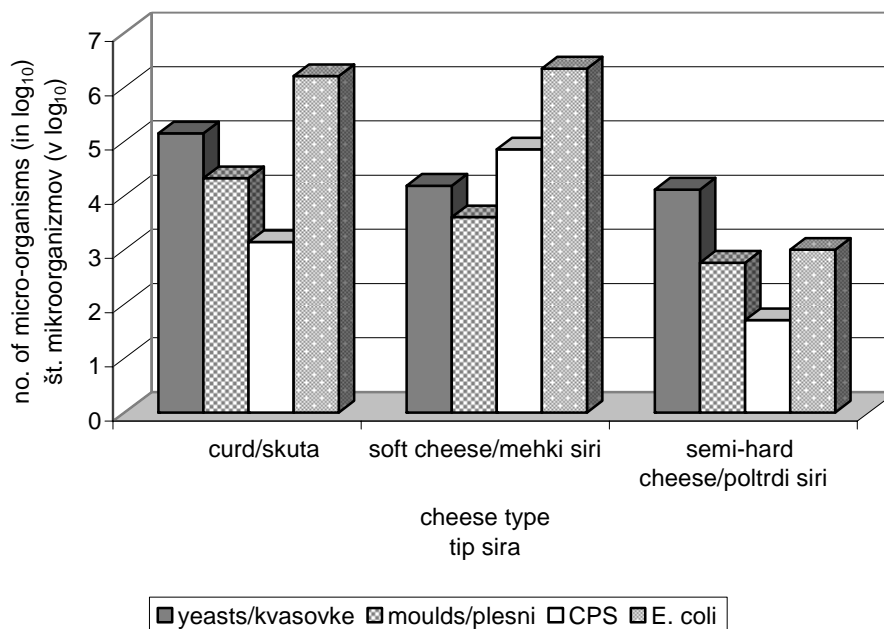


Figure 1. The \log_{10} values of the mean number of yeasts, moulds, coagulase-positive staphylococci (CPS) and *E. coli* per g (g^{-1}) of different types of cheese samples.

Slika 1. \log_{10} vrednosti povprečnega števila kvasovk, plesni, koagulaza-pozitivnih stafilokokov (CPS) in *E. coli* v gramu (g^{-1}) vzorca različnih tipov sira.

The microbiological quality of samples from different small food-processing plants

The samples of cheese were collected from five small food-processing plants that offered their milk products on the market. At sampling we tried to take approximately the same number and type of product at every plant. But some of them are specialized on specific types of cheeses. In spite of this we compare the microbiological quality of samples between plants. The results of the comparison are expressed in the Figure 2.

The samples collected from plants A and B were particularly most often highly contaminated (in 43% of cases), while plant C offered faultless products. Only one sample of the plant D and plant E, respectively did not correspond to valid regulations, because they contained very high number of *E. coli*. The samples collected at plant E contained also *Proteus* sp. and *L. grayi*, while the number of other micro-organisms was very low.

From 24 cheese samples we isolated 37 mould strains that mostly belonged to genus *Geotrichum* (91.9%) as found out after morphological and microscopic examinations. Only 2 (5.4%) isolates were classified in genus *Moniliella* and 1 strain (2.7%) in genus *Aspergillus*.

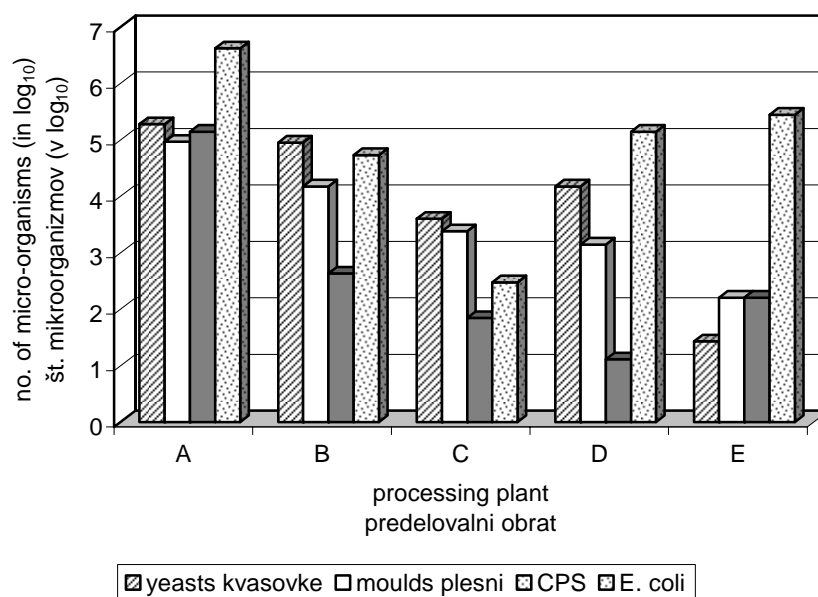


Figure 2. The log₁₀ values of the mean number of yeasts, moulds, coagulase-positive staphylococci (CPS) and *E. coli* per g (cfu g⁻¹) of cheese samples produced by small dairy-processing plants (signed from A to E).

Slika 2. Log₁₀ vrednosti povprečnega števila kvasovk, plesni, koagulaza-pozitivnih stafilokokov (CPS) in *E. coli* na gram (KE g⁻¹) vzorcev sirov, proizvedenih v malih mlekarско-proizvodnih obratih (označenih od A do E).

The growth on AFPA medium and aflatoxin production

All isolated mould strains including standard strains *A. flavus* were inoculated on *A. flavus* and *A. parasiticus* agar (AFPA). Isolated *Aspergillus* strain grew on the medium, but it did not produce a distinctive bright orange yellow reverse colour on AFPA medium after 42–48 h incubation at 30 °C (Frädberg, *et al.*, 2003) as it was detected at standard strains.

Only about 70% out of 24 *Aspergillus* sp. strains, growing on YGC medium, were able to form the colonies on YES medium supplemented with methyl-β-cyclodextrin and 0.6% sodium deoxycholate. On these mediums none of isolated strains could produce colonies with typical white under UV light fluorescent zone.

DISCUSSION

About 40% of all cow milk produced in Slovenia each year is processed into different sorts of cheese (Valjavec, 2000). There is not so many data about the microbiological quality of cheeses which are not processed in larger dairies. Therefore we evaluated the presence of pathogen micro-organisms, yeasts and moulds in 40 samples of cheese comprising 14 curd samples, 13 samples of soft salted or non-salted cheese and 13 samples of semi-hard cheese, which were manufactured at individual small dairy-processing plants selling their artisan products on the market.

Pathogen micro-organisms in cheese samples

Coagulase-positive staphylococci (*S. aureus*) and *E. coli* in cheese are frequently used as indicators of hygienic quality and show lack of microbiological safety (IFST, 1998). In our study

the number of coagulase positive staphylococci and *E. coli* was rather high (2.5×10^4 cfu g⁻¹ and 1.4×10^6 cfu g⁻¹, respectively). The samples of curd and particularly soft cheese were contaminated most often in the range from 10^2 to 10^4 cfu g⁻¹ for staphylococci and in the range of about 10^6 cfu

g⁻¹ for *E. coli*. The samples of semi-hard cheese were less contaminated with *E. coli* (10^3 cfu g⁻¹) and staphylococci (10^1 cfu g⁻¹) and fulfilled the criteria of valid Slovenian regulations (Pravilnik...Ur.l., 2004), but one sample was contaminated with clostridia and one with *Proteus*. *Salmonella* and *L. monocytogenes* were not detected in any of cheese samples tested.

According to Pravilnik., Ur.l. RS (2004) nine (22.5%) samples in our investigation did not reach the adequate microbiological quality, while the normatives EU (ES 1073, 2005) of the number of coagulase-positive staphylococci and *E. coli* were exceeded in 37.5% of samples.

Sk *et al.*, (2004) reported about 24% of white pickled cheese samples where the number of *S. aureus* (coagulase-positive staphylococci) was unacceptable according to Turkish microbial standards. These numbers are higher than our results.

Higher results were reported also by Borges *et al.* (2003) who evaluated the hygienic-sanitary quality of 43 Coalho cheese samples produced in different regions of Brazil. *E. coli* was confirmed in 93.1% of samples. Coagulase-positive staphylococci were observed in 93.1% of samples ranging from 1.0×10^1 to 2.0×10^9 cfu g⁻¹. The presence of *Salmonella* was confirmed in 34.9% and *Listeria* sp. in 6.9% of cheese samples (Borges *et al.*, 2003).

Very close to our results is the number of coagulase positive staphylococci according to the study of Brindani *et al.* (2001) where only one out of 50 samples of Ricotta cheese was contaminated with coagulase-positive staphylococci (4.0×10^4 cfu g⁻¹), but *Salmonella* was present in one sample and *L. monocytogenes* in two samples.

On the other hand, Menendez *et al.* (2001) established low average numbers of *S. aureus* (<61 per g of cheese) and *E. coli* (<52 per g of cheese) in 24 Tetilla cheese samples. *L. monocytogenes* was detected in two of the 24 samples. None of the samples yielded *Salmonella* spp.

The reasons for higher contamination of soft cheeses lay in their relatively high moisture and low acid content, which makes them easily susceptible to microbiological spoilage. The high salt content of brine and its dehydrating effect provide a few more days of shelf life, but the coagulase-positive staphylococci are, however, resistant to salt. (Anonim., 1994; Farkye and Vedamuthu, 2002; Tekinşen and Özdemir, 2005).

There were differences in microbiological quality of cheese samples offered by different processing plants. These differences were not only the consequence of the offering the different types of cheese with different quality, but also of the hygienic conditions at milking process and cheese production.

The best way to ensure good shelf life of soft cheeses is to observe strict sanitary practices throughout the milking, manufacture steps, and post-manufacture handling. All the equipment used should be properly cleaned and sanitized. The quality and the pH of water used for washing the curd are very important. The water should be of potable quality and pH preferably slightly acidic or neutral (Farkye and Vedamuthu, 2002).

Yeasts and moulds in cheese samples

The samples of cheese were obtained in autumn and winter season, when the pasture or the hay was replaced by conserved or ensiled feed. Many authors reported on the higher number of yeasts, moulds and consecutively the higher concentration of mycotoxins in ensiled feed which is used mostly in winter season (Blanco, *et al.*, 1988; Lopez, *et al.*, 2003; Kamkar, 2005). For the needs of Slovenian cattle-breeding, about a half of the whole required voluminous forage should be conserved (Babnik and Verbič, 2003), but data on its contamination with yeasts and moulds are rare. The contamination of the feed also varies according to the area, moisture, climatic

differences and temperature (Jay, 1992; Sassahara, 2005). Some moulds like *A. flavus* and *A. parasiticus* can easily grow in feed having moisture between 13% and 18% and environmental moisture between 50% and 60%, furthermore they can produce toxin (Jay, 1992).

Beside the feed, the main sources of contamination of milk and milk products with yeasts and moulds are also environment and, particularly, the air (Finne Kure, *et.al.*, 2004)

It is documented that yeasts occur in raw milk at insignificant numbers (Fleet, 1996) 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).

Yeasts were found in our study in 60% of tested cheese samples in the mean number of 5.8×10^4 ($4.7 \log_{10}$) cfu g⁻¹. More contaminated cheese samples were offered by plants A and B. Producers A and B processed mostly more contaminated curd and soft cheese, while the plant E more low contaminated semi-hard cheese (Figure 2). The soft cheese samples were contaminated more frequently (84.6%), while the semi-hard cheese samples only in 21.4%. The mean values of yeasts were in samples of curd a bit higher ($5.1 \log_{10}$) than in samples of soft cheese and semi hard-cheese ($4.1 \log_{10}$). The higher contamination was probably the consequence of improper hygienic conditions during processing. Robinson and Tamime (2002) explicitly reported that yeasts as spoilage organisms generally enter in milk and cheese as contaminants from the air, or from improperly stored containers used for packaging the product. Brines are a potent source of yeasts too. The manufacturing process, the pH and a_w values, ripening time, etc., might also be the reasons of differences in number of yeasts in various types of cheese. Var *et al.* (2006) reported on the increase of yeast number between the 15th to the 30th day of ripening of the Kashar cheese and the decrease was observed after 30 to 60 day of ripening, depending on the antimicrobial agent or packaging material used. In the further ripening the number of yeasts was not changed drastically. Our results are in range or even lower than data obtained in other studies. For example, the number of yeasts in Spanish Armanda cheese samples after 1 month-ripening counts 10^6 cfu g⁻¹ (Tornadijo *et al.*, 1998). Mean yeast counts of artisan Portuguese ewes' cheese ranged from 2.7 to 6.4 \log_{10} cfu g⁻¹ (Pereira-Dias *et al.*, 2000) and yeast counts from 10^3 in curd and 10^5 cfu g⁻¹ in one month old cheese were shown by Hatzikamari *et al.* (1999). Borges *et al.* (2003) reported that all (43) tested Coalho cheese samples from Brazil were contaminated with yeasts and moulds in from 1.7×10^4 to 1.6×10^9 cfu g⁻¹.

About 60% of cheese samples were contaminated with moulds in concentration of about 2.0×10^4 cfu g⁻¹, which is probably the consequence of contamination from the environment. Higher number of yeasts and moulds in cheese samples were also obtained in one of our previous studies (Godič Torkar and Golc Teger, S., 2004). The contamination with moulds in samples of curd and soft cheese was in the range of 10^4 cfu g⁻¹ and was much higher than the contamination level of semi-hard cheese samples (mean value 58 cfu g⁻¹). For this reason the number of moulds in the samples collected from plant E that offered mostly semi-hard cheese was lower than in samples offered by other producers. The non-contaminated samples were available mostly at plants D and E. The samples from plant C were of the worst quality and were highly contaminated with yeasts and moulds.

We advised the producers that a lot of attention should be focused on the hygiene during cheese processing. Finne Kure *et al.*, (2004) also think that it is important to have focus on the air to reduce mould growth on the cheese. He recommended treating the mouldy cheese carefully. The level of mould spores that are able to grow on cheese will increase dramatically in the air if the mouldy cheese is not handled with care. The workers handling mouldy cheese should not enter the production rooms after this operation. The ventilation system may also contain a lot of mould spores and it is, therefore, necessary to have maintenance of this system in order to reduce spreading of spores from the ventilation systems (Finne Kure *et al.*, 2004).

It was not surprising that 91.9% of strains isolated from cheese samples in our investigation were classified as *Geotrichum* spp., because Chapman and Sharpe (1990) reported that the high

- The samples from the plant C were of the worst quality and they were highly contaminated with yeasts and moulds.
- The samples collected from the plants A and B were particularly most often highly contaminated (in 43% of cases).
- 91.9% of mould strains isolated from cheese samples in our investigation were classified as *Geotrichum* spp.
- We advised the producers that a lot of attention should be focused on the hygienic conditions during milking and cheese processing.

POVZETEK

V Sloveniji je kar nekaj individualnih majhnih živilsko–predelovalnih obratov, kjer proizvajalci mleko predelajo v mlečne izdelke, zlasti v različne tipe sirov in skuto. Te izdelke prodajajo na tržnicah v večjih mestih. Higienska kakovost in zdravstvena ustreznost proizvedenih sirov in skute je odvisna od ustrezne mikrobiološke kakovosti namolzenega mleka kot surovine, kakovosti molže, razmer, v katerih se mleko predeluje v sire, temperature hranjenja mleka in sirov, krme, sezone, uporabe različnih starterskih kultur, higiene mlekarskega pribora, okolja, ne nazadnje tudi vode, ki se uporablja pri predelovalnem procesu.

Preverjanje prisotnosti in števila specifičnih, posebno patogenih in potencialno patogenih mikroorganizmov, je pomemben dejavnik pri kontroli in sistemu zagotavljanja kakovosti proizvodnje in samih izdelkov.

Namen našega dela je bil ugotoviti prisotnost nekaterih patogenih in potencialno patogenih mikroorganizmov v 40 vzorcih skute in sirov, vzorčenih pri petih malih mlekarsko–predelovalnih obratih, ki ponujajo svoje izdelke na eni izmed slovenskih tržnic. Ugotavljali smo tudi število in prisotnost kvasovk in plesni, saj zlasti plesni rodu *Aspergillus*, ki kot proizvajalec aflatoksinov povzročajo tudi zdravstveno rizičnost živila.

Največji problem je predstavljalo povišano število koagulaza–pozitivnih stafilokokov (*S. aureus*) in *E. coli*. Najpogosteje so bili kontaminirani vzorci skute in zlasti mehkih sirov v območju od 10^2 do 10^4 KE g^{-1} stafilokokov in okrog 10^6 KE g^{-1} za *E. coli*. Vzorci poltrdega sira so bili manj pogosto kontaminirani in so vsi izpolnjevali zahteve veljavnih predpisov. *Salmonella* in *L. monocytogenes* nista bili ugotovljeni v nobenem izmed preiskanih vzorcev. Po veljavnih mikrobioloških kriterijih (Pravilnik..., Ur.l., 2004) je bilo 9 (22.5 %) preiskanih vzorcev neustreznih. Okrog 60 % vzorcev je vsebovalo kvasovke in plesni. Koncentracija plesni v skuti in mehkih sirih se je gibala v območju 10^4 KE g^{-1} in je bila veliko višja kot koncentracija v poltrdih sirih (povprečje 58 KE g^{-1}), medtem ko se je število kvasovk v vseh vzorcih gibalo v območju od 10^4 do 10^5 KE g^{-1} . Ugotovili smo razlike v mikrobiološki kakovosti vzorcev sira pri različnih proizvajalcih.

Kar 91,9 % izoliranih sevov plesni je pripadalo rodu *Geotrichum* spp. Samo en sev je pripadal rodu *Aspergillus*, ki pa ga nismo mogli uvrstiti v vrsti *A. flavus/parasiticus* in ni tvoril aflatoksinov.

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moisture soft cheeses, cottage cheese and cream cheese were subject to spoilage by species *Geotrichum*. A member of the genus *Geotrichum*, *G. candidum*, also called *Oospora lactis*, is the most important species in foods (Jay, 1992). It is recognised as real milk mould, which is very often isolated from milk and, therefore, it is not surprising that this fungus plays also a role with *Penicillium* and *Mucor* in the manufacturing of some dairy products (Wouters *et al.*, 2002). *G. candidum* inhibits the growth of *Listeria monocytogenes*, produces several enzymes for the breakdown of protein and fat and plays a key role in the ripening of camember cheese (Wouters *et al.*, 2002). It also plays an important role in competition with undesirable contaminants in the cheese (Nielsen *et al.*, 1998). Its lipases and proteases release fatty acids and peptides that contribute to the development of distinctive flavour and aroma in cheese (Tornadijo *et al.*, 1998).

Only 2 (5.4%) isolates were classified in genus *Moniliella* and 1 strain (2.7%) in genus *Aspergillus*. Surprising, *Penicillium* spp. was not isolated from samples in spite of Scott (1989), and Fine Kure and Skaar (2000) reported that genus *Penicillium* is most frequent mould in cheese, followed by *Aspergillus*, *Cladosporium*, *Geotrichum* and *Mucor*. In our samples of cheese the genus *Aspergillus* was rare too, probably owing to low temperatures of milk chilling and cool season. Equally Bullerman (1981) suggests that *Aspergillus* species, in contrast to *Penicillium* species, cannot grow at low temperatures.

In cheese samples there are only a few different sorts of moulds because the pasteurisation reduces the presence of contaminants from the group of yeasts and moulds. At the same time the environmental factors in winter are not advantageous for secondary contamination during cheese production (Scott, 1989, Fine Kure and Skaar, 2000).

A. flavus is not a common species on cheese too. Most studies showed that aflatoxins could only be produced 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).

In our study we found out that only one strain of the genus *Aspergillus*, but it was not identified as *A. flavus* or *A. parasiticus* after typical growth on AFPA medium. This strain did not produce the aflatoxin detected on YGC or YES medium supplemented with methyl- β -cyclodextrin.

CONCLUSIONS

- In our study the coagulase-positive staphylococci (*S. aureus*) and *E. coli* present in samples of cheese were most often the problem of hygienic quality and show lack of microbiological safety.
- The samples of curd and particularly soft cheese were contaminated most often in the range from 10^2 to 10^4 cfu g⁻¹ for staphylococci and in the range of about 10^6 cfu g⁻¹ for *E. coli*. The samples of semi-hard cheese were less contaminated with *E. coli* and staphylococci and fulfilled the criteria of valid regulations.
- *Salmonella* and *L. monocytogenes* were not detected in any of the cheese samples examined.
- According to the valid regulations 9 (22.5%) samples in our investigation did not reach the adequate microbiological quality.
- About 60% of samples were contaminated with yeasts and moulds, which is probably the consequence of the contamination from the environment.
- The contamination with moulds in samples of curd and soft cheese was in the range of 10^4 cfu g⁻¹ and was much higher than the contamination level of semi-hard cheese samples (mean value 58 cfu g⁻¹).
- There were differences in microbiological quality of cheese samples offered by different processing plants.

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