BOVINE MASTITIS AS THE PRIMARY CONTAMINATION SOURCE OF MILK AND MILK PRODUCTS WITH *S. AUREUS* ENTEROTOXINS

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Summary. The milk of bovine mastitis has been identified as primary sources of contamination of milk and milk products with staphylococcal enterotoxins. The aim of the present study was to investigate the prevalence of enterotoxin producing *S.aureus* in milk of cows with mastitis, in raw milk and milk products in Latvia. Special emphasis was given to find out if the mastitic milk in Latvia is a primary contamination source of milk and milk products with *S.aureus* enterotoxins.

A total of 476 samples of raw milk of bovine mastitis from Latvian farms, 690 samples of raw milk, 330 samples of milk products and 313 samples of milk, and sour cream and cottage cheese, taken on Latvian markets, were analyzed.

The reverse passive latex agglutination assay *SET-RPLA Staphylococcus enterotoxin A, B, C, D detection kit* (TD 9000, Oxoid, U.K., 1996) was used to determine the enterotoxin serotypes.

The enterotoxin producing *S.aureus* in milk of bovine mastitis was present in 77.3% of samples examined, in raw milk – 29.7% of samples, in machine-made milk products – 24.1% of samples, and in sour cream and cottage cheese – 15.2% of samples were positive, respectively.

The results from this study confirmed, that milk of bovine mastitis can be identified as the primary source of contamination of milk and milk products with *S. aureus* enterotoxins in Latvia. It is likely that the contamination of raw milk and milk products with enterotoxin producing *S.aureus* in Latvia could be etiologically concerned with a high prevalence of bovine mastitis.

Key words: *Staphylococcus aureus*, enterotoxins, bovine mastitis, milk, milk products.

KARVIŲ MASTITAS – PIRMINIS PIENO IR PIENO PRODUKTŲ UŽKRĖTIMO ŠALTINIS *S. AUREUS* ENTEROTOKSINAIS

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Santrauka. Šio darbo tikslas buvo išsiaiškinti *S. aureus* gaminamo enterotoksino vyravimą karvių mastitiniame ir žaliaviniame piene bei Latvijos pieno produktuose, įrodyti, kad karvių mastitinis pienas yra pirminis ir svarbiausias pieno ir pieno produktų užkrėtimo šaltinis *S. aureus* enterotoksinais.

Buvo ištirta 476 karvių mastitinio pieno mėginiai iš Latvijos fermų, 690 žaliavinio pieno mėginiai, 330 mėginių paimtų iš perdirbamosios pramonės įrenginių, ir 313 pieno, grietinės ir kaimiško sūrio mėginių, paimtų iš Latvijos parduotuvių.

Enterotoksino serotipai nustatyti naudojant lateksinį agliutinacijos rinkinį *SET-RPLA Staphylococcus enterotoxin A, B, C, D detection kit* (TD 9000, Oxoid, U. K., 1996).

S. aureus gaminamo enteroksino karvių mastitiniame piene rasta 77,3 proc., žaliaviniame – 29,7 proc., perdirbamosios pramonės įrenginiuose – 24,1 proc. ir net 15,2 proc. *S. aureus* enterotoksino rasta pieno produktuose iš Latvijos parduotuvių.

Enterotoksinas A yra vyraujantis *S. aureus* štamas mastitiniame piene ir Latvijos pieno produktuose.

Šis paplitimas patvirtina, kad karvių mastitinis pienas yra pirminis ir svarbiausias pieno bei pieno produktų užkrėtimo šaltinis *S. aureus* enterotoksinais.

Raktažodžiai: *Staphylococcus aureus*, enterotoksinai, karvių mastitas, pienas, pieno produktai.

Introduction. It is widely known fact that *Staphylococcus aureus* is the pathogenic agent of the bovine mastitis that causes substantial losses to the agricultural production (Bulletin of the International Dairy Federation 394, 2005). The largest part of the strains is enterotoxigenic. (Olson 1970; Heeschen 1995; Holeckova et.al, 2002). Independently from the clinical form of the mastitis an etiology of mastitis is connected with *S.aureus* in 30% of cases in Nordic countries and 40% in England (Pyörälä, 1995; Pitkälä, et al., 2004). In relation to toxicological infections of food products it should be noted that a part of it may be connected with

occurrence in the nutrition of the sub-clinical mastitis milk and milk products. The results of researches show high occurrence of enterotoxin-forming staphylococcus in milk of mastitis bovines. Typical prevalence of enterotoxin-forming *S.aureus* strains has been proved in Germany - 58,7% (Zschock, et al., 2005), Trinidad - 53,6 % (Adesiyun, 1995) and Japan - 67,8% (Katsuda, 2005). Relatively smaller ratio of enterotoxigenic *S.aureus* strains has been isolated in Korea – 22.3% (Lim et al., 2004) and USA - 13,8% (Cenci-Goga et al., 2003) and 28,6% (Kenny, 1993). In publications of different authors the milk of mastitis bovine has been identified as primary sources of contamination of milk and milk products with staphylococcal enterotoxins (Cenci-Goga B.T. et.al, 2003; Loncarevic S. et.al., 2005).

The information about occurrence of enterotoxin-forming *S.aureus* in milk of mastitis bovine is quite controversial. The information about situation in Latvia is not available so the **purpose of this work** is to identify the proportion of enterotoxin-forming *S.aureus* strains in milk of mastitis bovine and to determine a primary contamination source of milk and milk products with *S.aureus* enterotoxins.

Materials and method. In frame of this work the 476 samples of non-pasteurised milk obtained from Latvian farms from bovine with clinical and sub-clinical mastitis were investigated. Analysis were performed also for 690 samples of aggregate milk, 330 samples of individual milk taken at milk processing factories and 313 samples of milk, sour cream and curds taken at different Latvian markets and commercial places. *S.aureus* was determined using internationally recognized method *LVS EN ISO 6888-1 : 1999* with application of selective culture media (Baird-Parker agar) and confirmation with rabbit plasma coagulase. In order to perform an identification of isolated plasma coagulating staphylococcus the biochemical test system API-STAPH (20500, BioMérieux, France) has been used. This test allows the precise determination of *S.aureus* strain. For confirmation of enterotoxin-forming *S.aureus* strains the enzyme linked fluorescence assay (ELFA) has been applied (Pimbley, Patel, 1998). The presence of staphylococcus enterotoxins in samples was confirmed using *BioMérieux* analyser *miniVIDAS* and special enterotoxin determination tests *VIDAS Staph enterotoxin SET* (30701, *BioMérieux*, France).

The reason of application of enzyme linked fluorescence assay for identification of enterotoxin-forming S.aureus strains is connected with high sensitivity and selectivity of the test. The application of this testing system allows determining the presence of enterotoxins serotypes A, B, C and D) (Pimbley, Patel, 1998). After the determination of strains of isolated enterotoxin-forming *S.aureus* the task of our work was to identify the enterotoxin serotypes of the specific *S.aureus* strains.

For serotyping of enterotoxins the reverse passive latex agglutination test with *SET-RPLA Staphylococcus enterotoxin A, B, C, D detection kit* (TD 9000, Oxoid, U.K., 1996) has been used. The application of this test allowed to identify the type of enterotoxins formed by isolated *S.aureus* strains and to describe population of enterotoxin serotypes forming *S.aureus* in Latvia.

Results. The overview of the isolated *Staphylococcus aureus* and enterotoxin-forming strains in Latvia is given in Table 1.

Table 1. **The prevalence of *Staphylococcus aureus* and enterotoxins producing strains isolated from mastitis milk and milk products in Latvia**

Samples	No. of samples analysed	No. of samples, where the *S.aureus* detected	No. of enterotoxin producing *S.aureus* detected
Mastitis bovine milk	476	119 (25.0%)	92 (77.3%)
Raw milk	690	155 (22.5%)	46 (29.7%)
Machine-made milk products	330	29 (8.8%)	7 (24.1%)
Milk and milk products on market	313	138 (44.1%)	21 (15.2%)

The results show that coagulase positive *S.aureus* more frequently occurs in milk and milk products taken at Latvian markets - 44.1% of the cases. The occurrence of S.aureus in commercially produced milk products was identified in 8.8% of the cases. The difference in number of samples with isolated S.aureus between mastitis bovine milk and raw milk is not significant (25.0% and 22.5% of the cases respectively). The largest ratio of enterotoxin-forming *S.aureus* strains has been isolated from milk of mastitis bovine.

The results of analysis of milk and milk products are summarized in the Table 2.

Taking into account the prevalence of small farms in Latvia the significant number of milk samples produced in such condition was taken for the analysis in frame of this research. The inclusion of different milk products sources allows better identification of the occurrence of enterotoxin-forming *S.aureus* in all significant milk distribution chains. The presence of *S.aureus* was established in 8.8% of the commercially produced milk samples. The occurrence of *S.aureus* in milk produced in small farms is significantly higher (44.1%). 24.1% of *St.aureus* strains isolated from commercially produced milk are able to produce enterotoxins. The production of enterotoxins is specific for 15.2% of *S.aureus* strains isolated from milk samples taken at markets and open commercial places.

The information about staphylococcal enterotoxins in analysed samples is presented in Table 3 and Figure 1. The *S.aureus* producing enterotoxin A (SEA) was isolated

as the dominant strain in all samples. The formation of enterotoxins B (SEB) and C (SEC) is less typical. The simultaneous formation of different serotypes of enterotoxins is not specific since only one strain produced both SEA and SEB enterotoxins.

Table 2. **The prevalence of *Staphylococcus aureus* and enterotoxins producing strains isolated from milk and milk products in Latvia**

Name	*No of analysed samples*	*No. of samples, where S.aureus detected*	*No. of samples the enterotoxin producing S.aureus strains were found*	
		No.	*No.*	%
Butter	6	1 (16.7%)	0	0
Cheese	92	4 (4.3%)	1	25.0
Fermented milk products	52	0	0	0
*Other milk products**	180	24 (13.3%)	6	25.0
Machine-made milk products (total)	**330**	**29 (8.8%)**	**7**	**24.1**
Milk	116	51 (43.9%)	5	9.8
Cottage cheese	100	34 (34.0%)	10	29.4
Sour cream	97	53 (54.6%)	6	11.3
Milk and milk products on market (total)	**313**	**138 (44.1%)**	**21**	**15.2**

* Other milk products - curd, sour cream, deserts on milk base, curd snack

Table 3. **The prevalence of *Staphylococcus aureus* enterotoxin serotypes in Latvia**

Samples	SEA	SEB	SEC	SEA+SEB	Unidentified SE*
Mastitis bovine milk[1)]	54	13	25	0	0
Raw milk[2)]	40	2	3	1	0
Machine-made milk products [3)]	7	0	0	0	0
Milk products on market [4)]	9	3	7	0	2
TOTAL	110	18	35	1	2

* SE – Staphylococcus enterotoxin. [1)] Joffe, Birģele, 2006. [2)] Joffe, 2002. [3)] Joffe, 2002. [4)] Joffe, Birģele, 2006.

Discussions. The ratio of the enterotoxin-forming *S.aureus* strains in milk of bovine mastitis in Latvia is very high – 77.3%. Different scientists have published the results with indication on high occurrence of enterotoxin-forming strains in farms with mastitis infected cows. (Olson C.J. et.al, 1970; Ng K.L.D., Tay L., 1993; Cenci-Goga et. al, 2003). The results of the work demonstrates that ratio of enterotoxin-forming *S.aureus* in mastitis bovine milk is not connected with occurrence of *S.aureus* since major part of isolated strains forms enterotoxins. Our results indicate that enterotoxins forming *S.aureus* strains are distributed throughout all areas of Latvia. Thus the potential risk of occurrence of these toxins-forming strains in food products is relatively high. The role of *S.aureus* enterotoxins in pathogenesis of bovine mastitis is not yet fully clarified although the high ratio of enterotoxin-forming strains as the main factor of pathogenesis of *S.aureus* bacteria indicates the significant role of enterotoxins in pathogenesis of mastitis (Katsuda, 2005).

Assuming that the major source of contamination of non-pasteurised milk is the milk coming from the farms with mastitis infected cows (Holeckova et.al, 2002) the aggregate milk samples were also used in this work. The comparison with results obtained from milk of mastitis bovine shows that occurrence of the enterotoxin-forming strains is lower. Although our assumption was not directly confirmed since enterotoxin-forming *S.aureus* strains in samples of aggregate milk are found only in 29.7% cases, some authors (Loncarevic S. et.al., 2005; Cenci-Goga B.T. et.al, 2003) support this correlation. An aggregate sample was formed from milk coming from farms with different occurrence of *S.aureus* and different clinical form of mastitis. During the combination of the milk the staphylococcus are mixed and the concentration of cell diminishes. The occurrence of enterotoxin-forming *S.aureus* strains should be taken into account. This fact indicates the possibility of presence of enterotoxins in milk products ready for consumption. Furthermore the results of analysis of mastitis infected cows show the connection between contamination of milk and occurrence rate of mastitis in Latvia.

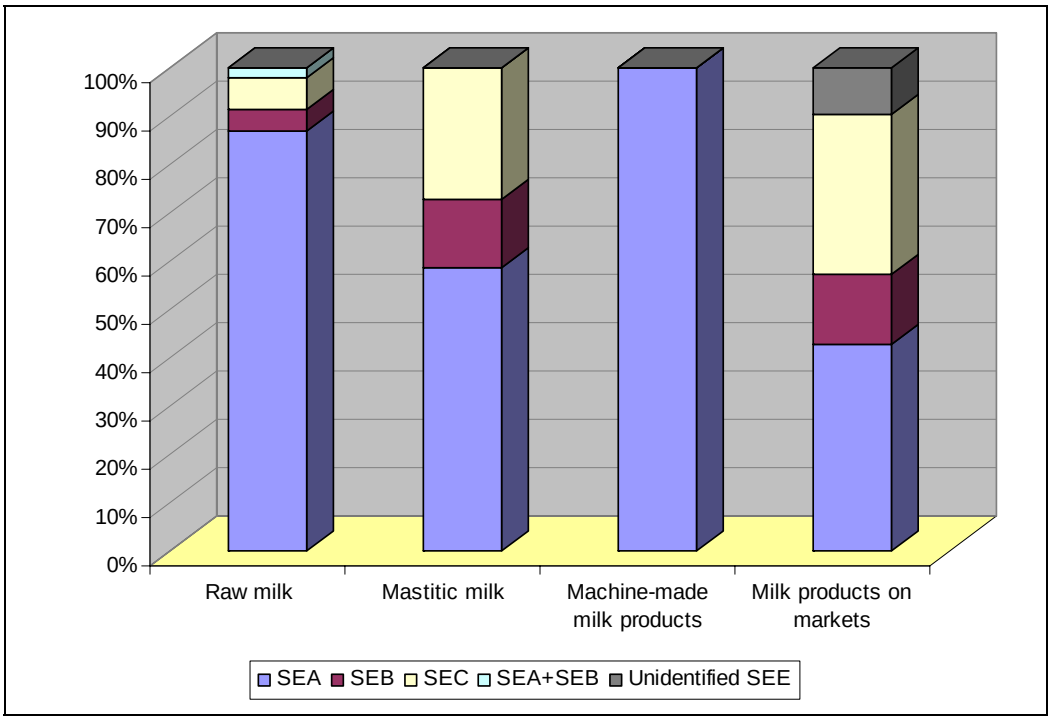

Figure 1. **The prevalence of *Staphylococcus aureus* enterotoxin serotypes in Latvia**

The occurrence of *Staphylococcus aureus* in commercially produced milk is low (8.8%). Staphylococcus was not found in the cultured products. It could be connected with influence of different chemical and biological factors on growth and reproduction of staphylococcal cells and formation of enterotoxins. According to the results of some authors (Asperger H., 1994; Halpin-Dohnalek M.I., Marth E.H., 1989), acidic conditions (pH < 4.0) and presence of *Lactobacillus acidophilus, L.casei, Streptococcus thermophilus, Bifidobacteria* and yeasts inhibits growth of staphylococcus and formation of enterotoxins. It is known that lowest value of pH for growth of *S.aureus* cells is 4.0 and for synthesis of enterotoxins – 4.8. Acidic conditions and presence of other microorganisms are important factors inhibiting the growth of staphylococcus in cultured milk products so these products could be regarded as safe in relation to the enterotoxin contamination.

It should be noted that the occurrence of enterotoxin-forming strains in milk products is low. It should be taken into account that during the processing the non-pasteurised milk is influenced by mechanical (separation, dilution) and thermal factors (pasteurisation). As result the main part of bacteria are destroyed and the final bacterial contamination level of the product is low. The staphylococcal enterotoxins are thermally stable compounds which are not decomposed during the processing of the milk. Thus in case of use of non-pasteurised milk contaminated with *S.aureus* (> 10^5 CFU/ml) there exists a high risk of contamination of final product with enterotoxins in concentrations which may represent a serious risk to the consumers. The results obtained in this work show that the occurrence of *S.aureus* in milk products is low (8.8% from analysed samples). The low risks connected with this kind of products are confirmed by absence of data about cases of infections in Latvia caused by presence of staphylococcal enterotoxins in processed milk products.

Occurrence of enterotoxin-forming *S.aureus* in milk available on market is low. It could be assumed that the milk entering the commercial chain mostly has been heated. The heating diminishes the initial concentration of bacteria in milk although the occurrence of *S.aureus* in milk samples is quite high – 43.9% of cases. It should be noted that aggregate milk from the farm contains milk from different cows and hygienic conditions and occurrence of sub-clinical mastitis should be controlled carefully.

The content of *S.aureus* enterotoxins in samples of curd and sour cream is higher in comparison with milk. It is known that curd and sour cream is the concentrate of milk fats and proteins. In process of concentrating the contents of bacteria including the enterotoxin-forming staphylococcus increases as well. Commercial production commonly is connected with separation of bacterial cells with separators and centrifuge. Small farms usually are not applying these technologies. Staphylococcus are resistant against drying so un-clean vessel could be the additional source of contamination. (Sinell 1980; Halphin-Dohnalek 1989; Miwa 2001). Importance of the personal hygiene is high especially for the cases when hand work is involved during the production of the curd. It should be noted that staphylococcus are resistant against heating at low temperatures (< 55^o C) (Adams et.al, 1995). If the heating of the milk in process of preparation of curd was not sufficient to destroy all bacteria and the level of initial contamination was high,

the risk of presence of enterotoxin-forming strains is very substantial. The conclusion could be drawn that the quality of milk products is depending on quality of raw materials, conditions of processing and conditions of storage before and during the realization.

Evaluation of the obtained results shows that the primary source of contamination of milk products with S.aureus in Latvia is the milk obtained on farms with cows infected by *S.aureus* caused mastitis. These assumptions are correlating with data obtained by other authors (Cenci-Goga et.al, 2003; Loncarevic et.al., 2005).

It should be noted that enterotoxin-forming *S.aureus* strains are described in the literature as one of the main causes of food infection, especially strains of *S.aureus* forming enterotoxin A (Evenson et.al, 1988; Yamashita et.al, 2003; Ikeda et.al, 2005). This assumption is confirmed by results of researches showing prevalence of staphylococcal enterotoxin A (SEA) both in bovine mastitis milk and milk products. Some publications emphasize the prevalence of SEB, SEC un SED serotypes (Tollersrud et.al, 2000; Stephan et.al, 2001; Nagase et.al, 2002) but the regional difference strongly influences staphylococcus populations as indicated by different researchers (Annemüller et.al, 1999; Stephan et.al, 2001; Joo et.al, 2001). In order to determine the geographical localization of staphylococcus in Latvia the molecular biological methods should be used in further researches. This method allows analysing of isolated strains with high level of precision. The determination of type of coding gene would be possible as well. This method has been used for the last 10 year and obtained results are published by several authors (Lange et.al, 1996; Akineden et.al, 2001; Lim et.al, 2004; Becker et.al, 2004; Zschock et.al, 2005). The determination of *S.aureus* strains able to produce several serotypes of enterotoxins simultaneously as well as unidentified toxins gives the sound reason for continuation of the work in this field.

Taking into account the occurrence of *S.aureus* in analyzed samples and prevalence of enterotoxin A in bovine mastitis milk the conclusion could be made that milk of the cows infected by mastitis is the primary source of contamination of milk products with enterotoxin-forming staphylococcus.

Conclusions

1. Enterotoxin–forming *S.aureus* strains in Latvia mostly are found in milk of mastitis bovine (77.3% of the cases). The occurrence is less specific for the non-pasteurised milk (29.7%), industrially produced milk products (24.1%) and in market realization existing milk products (15.2%).
2. Contamination of non-pasteurised milk and milk products with enterotoxin–forming *S.aureus* etiologically can be relevant with mastitis occurrence in Latvia.
3. Staphylococcus enterotoxin A (SEA) is prevailing in Latvia in milk of mastitis bovine and milk products.
4. Milk of mastitis bovine is a primary contamination source of milk and milk product with enterotoxin–forming staphylococcus in Latvia, what is confirmed by SEA prevalence.

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