Seasonal dynamics and possible development of total count of microorganisms in sheep's milk

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Abstract

The work is focused on the evaluation of seasonal dynamics of the total count of microorganisms in sheep's milk and on the proposal of developing cut-off values for standard limitation for the next fifteen years for hygienic safety support. The total count of microorganisms was measured between years 2012–2014 (n = 4,746). The results were statistically evaluated using medians, geometric means, arithmetic means, means of log values ($\log_{10}$) and standard deviations for the cut-off limit determination. This model was patterned on the maximum of total count of microorganisms in the tested percentiles 95 (statistical conventional interval), 91, 90, 80 and 70% and on the result of medians (from 109 to 148 × 10^3 cfu/ml). These cut-off limits were divided into three classes (I to III) of standard quality, and the model of dynamics for their gradual implementation time was created as follows: initiation period (class I = ≤ 800; II = from 801 to 1,300; III = 1,301 to 4,000; non-standard = > 4,000 × 10^3 cfu/ml); second period (class I = ≤ 550; II = 551 to 800; III = 801 to 1,300; non-standard = > 1,300 × 10^3 cfu/ml); third period (standard class = ≤ 800 × 10^3 cfu/ml); fourth period (standard class = ≤ 550 × 10^3 cfu/ml); and the last legislative period (≤ 300 × 10^3 cfu/ml as a real hygienic limit). The limit for milk without heat treatment corresponds to the real value of the median 200 × 10^3 cfu/ml. The work is important for the procedures in the development of methods for the control and safety of raw food material.

Bulk ovine milk, conventional interval, real intervals, TCM cut-off value

Sheep breeding in the Slovak Republic has had a long tradition. In the years 2012, 2013, and 2014 totals of 399, 389, and 396 thsd sheep were registered, respectively, of which 139, 141, and 140 thsd represented dairy sheep, respectively (Gálik 2014). Raw sheep's milk is processed either directly by manufacturers on the farm or purchased and processed by dairy plants. There are currently three bigger dairies and several smaller ones for sheep's milk processing in the Slovak Republic. Typical products made from sheep's milk are various national cheese specialities, such as the Bryndza cheese. The quality of raw sheep's milk is controlled by independent testing laboratories. The total count of microorganisms (TCM) is a basic and mandatory indicator for evaluation of raw sheep's milk quality (Regulation EC No. 853/2004). According to this Regulation, the food companies are not allowed to bring to the market raw milk exceeding the permissible value of antibiotic residues listed in the Annex to Regulation EEC no. 2377/90 and ES 324/2004. Another basic indicator the somatic cell count (SCC) is defined in this Regulation only for raw cow's milk. According to Regulation 853/2004 raw milk shall not exceed the limits of TCM (calculated as rolling geometric mean over a two-month period, with at least two samples per month) for small ruminants - 1,500,000 in 1 ml, and shall not exceed the limit 500,000 in 1 ml if the milk

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is intended for direct consumption or processing without heat treatments. The reference method for TCM is plate count cultivation method at 30 °C for 72 h (ISO 4833-1). A rapid alternative method (flow cytometry) has been introduced for routine control of milk quality (Tomáška et al. 2014).

Microbial quality of raw sheep's milk is of particular importance when its processing does not include pasteurisation. The TCM is only a raw estimation not giving detailed information about possible pathogen bacteria contamination in milk; however, samples with lower TCM are expected to be safer. Yet, Fotoua et al. (2011) found that although 97% of the tested raw sheep's milk samples met the EU legislation limits for TCM, 24% of them contained *Staphylococcus aureus*. Similarly, D'Amico and Donnelly (2010) evaluated quality of various raw milks used for cheese production on small-scale farms, and in 38% samples *Staphylococcus aureus* was detected. Muehlherr et al. (2003) examined 63 samples of ewe's bulk-tank milk in Switzerland and TCM varied from 200 to 18 × 10^6 cfu/ml (the mean was 85 × 10^4 cfu/ml). Gonzalo et al. (2006) investigated TCM in sheep's raw milk in relation to the type of breed and milking in the Spanish region of Castilla-Leon. They evaluated 9,353 bulk milk samples in total for one year and observed higher results for hand milking and bucket-milking machines (geometric means were 202 × 10^3 and 206 × 10^3 cfu/ml, respectively) compared to the parlour system (dead-ended milkline 125 × 10^3 cfu/ml, looped milkline system 102 × 10^3 cfu/ml). Variability of TCM among the breeds ranged from 176 × 10^3 cfu/ml (Awassi) to 117 × 10^3 (Churra).

The presented work is focused on the evaluation of seasonal dynamics of the total count of microorganisms, and on the possible development of TCM cut-off values for standard (market) limitation of TCM values and thus for support and improvement of the quality and safety of sheep's milk. The cut-off limit proposal was designed on the basis of statistical values of three reference files.

Materials and Methods

Origin of samples

The samples originated from the Testing Laboratory Examinala for milk quality measurement in Slovakia (Dairy Research Institute, Žilina, Slovakia). There the results were evaluated of bulk milk samples from the breeds Improved Walachian sheep, Tsigai, Lacaune, East Friesian sheep, and Slovak milk sheep. The TCM investigation was blind-performed in terms of breed. The results recorded during January–December of the years 2012, 2013, and 2014 (n = 1,587, 1,742 and 1,667, respectively) were used for TCM investigation (Tables 1–3).

Analytical method

The bacterial count was determined by a method based on laser flow cytometry (Tomáška et al. 2006) using BactoScan FC (Foss, Hillerød, Denmark). The method was accredited according to criteria of ISO 17025, regularly controlled via internal (pilot samples) and external (Huefner standards; MIH Milchwirtschaftliches Institut Dr. J. Huefner, Hergarz, Germany) and by participation in interlaboratory comparisons (MIH Milchwirtschaftliches Institut Dr. J. Huefner). The method was also regularly compared using the reference method (ISO 4833-1).

Statistical method

The logarithmic (log_{10}) transformation of TCM data was performed because of absence of normal frequency distribution of these values (Reneau et al. 1986; Hanuš et al. 2009). The arithmetic mean is not a suitable parameter for sets of TCM data and therefore the geometric means were used. In general, the basic statistical characteristics as medians (m), geometric means (gx), arithmetic means (x), means of log values (log) and standard deviations (sd) were calculated using MS Excel (Microsoft, Redmond, Washington, USA). Also selections of values in TCM files were performed along selected real percentiles (in %) for the possibility to estimate the dynamics of TCM values and propose the next realistic development of TCM cut-off limits for future hygienic safety support.

Results

Analysis of TCM dynamics

The results of TCM during the three-year monitoring (2012, 2013, and 2014) are included in Tables 1–4. The geometric means amounted to 178, 195, and 235 × 10^3, arithmetic

Table 1. Monthly total count of microorganisms during the year 2012.

Month	n	m	gx	x ± sd	x log ± sd
I	5	331	341	455 ± 296	2.5324 ± 0.3320
II	1	325	325	325 ± 0	2.5119 ± 0.0000
III	43	68	98	255 ± 518	1.9901 ± 0.5333
IV	251	97	140	600 ± 1,527	2.1475 ± 0.6410
V	294	124	154	534 ± 1,361	2.1872 ± 0.5752
VI	260	184	221	554 ± 1,230	2.3438 ± 0.5169
VII	234	123	173	662 ± 1,638	2.2371 ± 0.5839
VIII	260	134	175	564 ± 1,351	2.2419 ± 0.5704
IX	216	208	265	930 ± 1,863	2.4231 ± 0.6479
X	12	117	163	475 ± 1,037	2.2123 ± 0.5096
XI	9	163	186	206 ± 98	2.2691 ± 0.1928
XII	2	157	146	157 ± 58	2.1630 ± 0.1674
I - XII	1.587	134	178	615 ± 1,471	2.2516 ± 0.5926

n = number of samples; m = median; gx = geometric mean; x = arithmetic mean; sd = standard deviaton; log = mean of log values

Table 2. Monthly total count of microorganisms during the year 2013.

Month	n	m	gx	x ± sd	x log ± sd
I	8	97	99	103 ± 29	1.9959 ± 0.1215
II	17	127	240	1,117 ± 2,239	2.3800 ± 0.6704
III	103	93	138	606 ± 1,613	2.1396 ± 0.6080
IV	274	128	167	626 ± 1,552	2.2230 ± 0.6007
V	271	169	192	548 ± 1,292	2.2837 ± 0.5455
VI	274	207	225	560 ± 1,277	2.3514 ± 0.5031
VII	289	160	217	695 ± 1,500	2.3368 ± 0.5842
VIII	262	137	171	587 ± 1,516	2.2338 ± 0.5657
IX	210	196	244	642 ± 1,375	2.3876 ± 0.5264
X	18	278	388	1,137 ± 1,759	2.5892 ± 0.6559
XI	9	144	126	162 ± 135	2.1018 ± 0.2874
XII	7	39	70	119 ± 138	1.8470 ± 0.4136
I - XII	1.742	159	195	613 ± 1,445	2.2902 ± 0.5656

n = number of samples; m = median; gx = geometric mean; x = arithmetic mean; sd = standard deviaton; log = mean of log values

means were 615, 613, and 704 × 10^3, and medians were 134, 159, and 190 × 10^3 cfu/ml, respectively. The monthly values of TCM means and medians in each year are relatively balanced within the season, except for the months with a smaller number of cases (January and February, and then October, November and December). The comparative and typical seasonal trends of TCM development between the years are also not very evident, even during a more reliable stage of the season (March to September). The explanation may be the higher dependence of TCM on the work and technology during milking than on the environmental temperature or the lactation dynamics. From the results it is evident that the hygienic milk quality has not improved during the monitored years. A seasonal trend was not confirmed in the decisive months during the overall evaluation of the three-

Table 3. Monthly total count of microorganisms during the year 2014.

Month	n	m	gx	x ± sd	x log ± sd
I	11	128	126	159 ± 96	2.1004 ± 0.3192
II	30	48	125	995 ± 1,965	2.0964 ± 0.8608
III	61	104	168	733 ± 1,724	2.2243 ± 0.6446
IV	249	121	165	512 ± 1,268	2.2181 ± 0.5469
V	264	160	207	632 ± 1,335	2.3159 ± 0.5880
VI	267	182	243	802 ± 1,653	2.3849 ± 0.6183
VII	268	188	245	727 ± 1,569	2.3900 ± 0.5662
VIII	257	227	309	762 ± 1,457	2.4896 ± 0.5248
IX	226	288	337	781 ± 1,391	2.5272 ± 0.5323
X	21	301	277	750 ± 1339	2.4418 ± 0.6066
XI	5	92	117	306 ± 443	2.0696 ± 0.5812
XII	8	102	150	520 ± 883	2.1775 ± 0.6809
I - XII	1.667	190	235	704 ± 1,471	2.3711 ± 0.5855

n = number of samples; m = median; gx = geometric mean; x = arithmetic mean; sd = standard deviaton; log = mean of log values

Table 4. Monthly total count of microorganisms during the years 2012–2014.

Month	n	m	gx	x ± sd	x log ± sd
I	24	127	143	200 ± 198	2.1556 ± 0.3377
II	48	96	161	1,024 ± 2,050	2.2055 ± 0.8015
III	207	95	136	570 ± 1,502	2.1335 ± 0.6101
IV	774	117	157	581 ± 1,459	2.1969 ± 0.5986
V	829	139	182	570 ± 1,331	2.2598 ± 0.5725
VI	801	192	229	638 ± 1,405	2.3601 ± 0.5487
VII	791	153	212	696 ± 1,565	2.3253 ± 0.5813
VIII	779	167	209	637 ± 1,446	2.3209 ± 0.5667
IX	652	237	280	786 ± 1,563	2.4477 ± 0.5745
X	51	211	275	822 ± 1,466	2.4398 ± 0.6201
XI	23	155	145	211 ± 238	2.1602 ± 0.3578
XII	17	99	110	312 ± 643	2.0397 ± 0.5638
I - XII	4,996	161	202	644 ± 1,463	2.3049 ± 0.5830

n = number of samples; m = median; gx = geometric mean; x = arithmetic mean; sd = standard deviaton; log = mean of log values

year reference period. We can only state that the highest mean monthly value of TCM was recorded in September, 280 and 786 × 10^3 cfu/ml (geometric and arithmetic mean); whereas the lowest values were recorded in March, 136 and 570 × 10^3 cfu/ml (Table 4).

The overall evaluation was also performed in the conventional interval of 95% (real percentile) of the cases belonging to the file for possible model estimate of the real cut-off limits of standard microbiological quality of sheep's milk (Table 5). As evident, the monthly geometric means, medians, also arithmetic means and standard deviations decreased significantly. It means an important shift to the better quality. The relevant means of TCM were 335 and 168 × 10^3 (arithmetic and geometric means) and 148 × 10^3 (median). That means an improvement of the means by 48, 16.8, and 8.1%, respectively, compared to the original sample set (Tables 4 and 5). The highest limit value TCM reached 3,981 × 10^3 cfu/ml in this modified file for milk of non-standard quality (Table 6).

Table 5. Total count of microorganisms values performed in the real percentile of 95% as a statistically conventional interval.

Month	n	m	gx	x ± sd	x log ± sd
I	23	126	132	172 ± 147	2.1220 ± 0.3031
II	46	85	136	755 ± 1,628	2.1337 ± 0.7393
III	197	91	112	255 ± 516	2.4750 ± 0.4876
IV	735	108	129	264 ± 434	2.1113 ± 0.4813
V	788	132	152	293 ± 443	2.1820 ± 0.4709
VI	761	182	193	341 ± 459	2.2851 ± 0.4515
VII	751	143	176	368 ± 647	2.2450 ± 0.4777
VIII	740	160	175	330 ± 492	2.2428 ± 0.4647
IX	619	221	237	464 ± 700	2.3739 ± 0.4899
X	48	196	228	524 ± 827	2.3587 ± 0.5442
XI	22	149	131	166 ± 118	2.1187 ± 0.3069
XII	16	92	89	157 ± 174	1.9518 ± 0.4543
I - XII	4,746	148	168	335 ± 528	2.2258 ± 0.4821

n = number of samples; m = median; gx = geometric mean; x = arithmetic mean; sd = standard deviaton; log = mean of log values

Table 6. The selection of total count of microorganisms values performed along real percentiles (in %) including a statistically conventional interval (percentile) of 95%.

%	n	m	gx	x ± sd	x log ± sd	max (in 1000 cfu/ml)
95	4,746	148	168	335 ± 528	2.2258 ± 0.4821	3,981
91	4,555	140	151	247 ± 281	2.1778 ± 0.4293	1,500
90	4,496	137	146	231 ± 248	2.1651 ± 0.4173	1,312
85	4,247	128	131	186 ± 161	2.1162 ± 0.3755	765
80	3,997	121	118	157 ± 116	2.0731 ± 0.3437	534
70	3,497	109	100	123 ± 72	1.9985 ± 0.2996	307

n = number of samples; m = median; gx = geometric mean; x = arithmetic mean; sd = standard deviaton; log = mean of log values

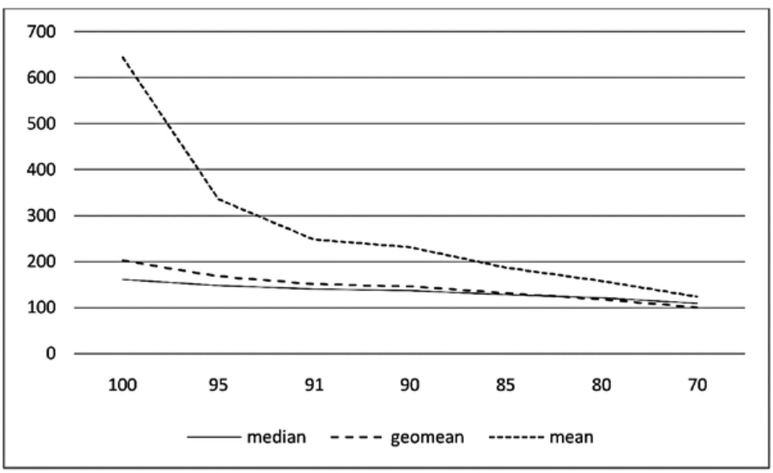

Fig. 1. The frequency distribution of total count of microorganisms in the tested percentiles (confidence interval at a probability level of 100, 95, 91, 90, 80 and 70%).

Subsequently, the evaluation was carried out for other stricter real percentile intervals of 90, 85, 80, and 70% (Table 6). The highest potential TCM cut-off limits were then 1,312, 765, 534 and 307 × 10^3 cfu/ml among these modified (reduced) files. The percentile of 91% was also included in these intervals because it corresponds to the mentioned literary value which is acceptable for microbiological quality of raw sheep's milk (Regulation EC No. 853/2004), and it is equivalent to the potential limit value of TCM for standard milk quality ≤ 1,500 × 10^3 cfu/ml. Comparing the total and subsequent modified files (100 vs. 95, 91, 90, 85, 80, and 70%), the following improvements of the microbiological quality for the means were recorded: 644 vs. 335, 247, 231, 186, 157, 123 (arithmetic mean), 202 vs. 168, 151, 146, 131, 118, 100 (geometric mean) and 161 vs. 148, 140, 137, 128, 121, 109 × 10^3 (median) cfu/ml, respectively (Fig. 1). It follows from these results that with decreasing numbers of samples, the differences between the arithmetic and geometric mean and their relevant medians were decreasing simultaneously. The frequency distribution of TCM values was gradually normalized in the model files (Fig. 1).

Model development for new normative cut-off limits of TCM

Definition of basic hygiene (microbiological, TCM) qualitative cut-off limits can be estimated by the evaluation of reduction of TCM intervals in the whole period (2012–2014). These limits can be divided into three classes of standard quality (I to III), and subsequently a model of dynamics can be created for their gradual time implementation. This model can serve for financial differentiation of potential supplier-customer contracts, for legislative modification and thus for the support of future hygiene and safety of raw sheep's milk:

- initiation period (3 years): class I = ≤ 800; II = from 801 to 1,300; III = 1,301 to 4,000; non-standard = > 4,000 × 10^3 cfu/ml;
- following period (3 years) after previous combination: class I = ≤ 550; II = 551-800; III = 801 to 1,300; non-standard = > 1,300 × 10^3 cfu/ml;
- for a further period of ca 4 years after the implementation of previous legislation: standard class = ≤ 800 × 10^3 cfu/ml;
- for a further period of ca 5 years after the implementation of previous legislation: standard class = ≤ 550 × 10^3 cfu/ml;
- for a further period after the implementation of previous legislation: standard class = ≤ 300 × 10^3 cfu/ml as a real hygienic limit.

This final legislative limit is higher than the limit for milk intended for the production of milk products by a process that does not involve heat treatment (≤ 300 vs. ≤ 500 × 10^3 cfu/ml). Currently there is considerable difference between these limits. The proposed modification would also constitute a reduction in the limit for milk without heat treatment to 200 × 10^3 cfu/ml, which is the real value of median in the evaluated group. This value can be introduced parallel to the limit for raw sheep's milk in the last step, i.e. after 15 years.

This mentioned proposal is patterned on the maximum of TCM in the tested percentiles (real percentiles as conventional interval at a probability level of 95% and the others as 90, 80, and 70%) and on the results of medians (from 109 to 148 × 10^3 cfu/ml). The proposal provides an objective image of possible development that can be further modified according to concrete actual requirements of the time periods, but with regard to reality of quality.

Discussion

The results show a high variability of TCM which clarify that sheep's milk is microbiologically more complex than cow's milk (Tomáška et al. 2006). The above expressed high variability is a good proof of normal frequency distribution absence in the used data files. Therefore, the use of data transformation (Ali and Shook 1980; Reneau et al. 1986; Wiggans and Shook 1987; Hanuš et al. 2009), geometric means and medians as right mean values is an authorized step for relevant derivation of future quality limits in this work. For this reason, the arithmetic means and relevant standard deviations were only secondary indices in this type of statistical evaluation for foodstuff legislation purposes as previously have recommended also Shook (1982), Raubertas and Shook (1982) and Janů et al. (2007). The results of TCM which are used for a new model development are comparable with the results published by Muehlherr et al. (2003). They mentioned similar results of TCM mean of 850 × 10^3 cfu/ml but lower median of 4.78 log cfu/ml (60 × 10^3 cfu/ml) for 63 bulk milk samples. Gonzalo et al. (2006) described similar results for geometric means depending on different milk systems which ranged between 102 and 202 × 10^3 cfu/ml. They observed a significant relation between TCM and bulk tank milk cell counts (BTSCC). According to these results, they even propose a program to improve the health indicators of sheep's milk, by also introducing somatic cells. The program should be implemented at the same time.

Classification of sheep's milk, and consequent payment according to classes (extra and scale of other classes) currently exists in some European countries. Pirisi et al. (2007) compared the quality of sheep's and goat's milk in relation to the financial interests of breeders in some countries in their book. In Greece, sheep's milk is divided into four quality classes according to TCM. The first three classes involve extra money for the quality: class AA < 200,000, class A equals 200,000 to 500,000, class B equals 500,000 to 1,500,000 and class G > 1,500,000 cfu/ml. Only sheep's milk with TCM < 500,000 cfu/ml involves extra money in Sardinia. In contrast, milk with TCM > 3,000,000 is penalized. In some regions of France the occurrence of coliform bacteria in sheep's milk, fat and protein are controlled. Many of the above mentioned limits correspond in good way to the dynamics of the development of the sheep's milk quality control proposal in this paper. Furthermore, in case of other small (goat) ruminants milk, the raw milk is classified into four categories in the Poitou Charentes Region of France: TCM ≤ 50,000 (extra quality milk, class R); class A = 50,001 to 100,000, class B = 100,001 to 200,000, class C > 200,000 cfu/ml. The SCC for extra milk quality is < 1,000,000 in 1 ml. Also fat and protein are checked. In Spain, there is the highest extra money for goat milk containing TCM < 50,000, then for milk with TCM from 50,000 to 150,000 and then from 150,000 to 500,000 cfu/ml. Milk with TCM > 500,000 cfu/ml is penalized. Also the fat content is determined for payment purposes. In Norway, the following classes for goat's milk in terms of TCM are valid: elite class E < 20,000; class 1 from 21,000 to 30,000; 2 from 31,000 to 50,000; 3 > 50,000 cfu/ml; SCC for E class < 1,500,000 in 1 ml. Our proposed limits can be used in the Czech Republic for the new normative TCM limit. They are based on a large set of TCM results and on the tested percentiles (real intervals at the level of 95, 90, 80, and 70%) and on the experience with the payment system in other countries.

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