Microbiological quality assessment of raw milk from a vending machine and of traditional Slovakian pasta filata cheeses

PETRA ŠIPOŠOVÁ – VERONIKA LEHOTOVÁ – ĽUBOMÍR VALÍK – ALŽBETA MEDVEĎOVÁ

Summary

Culture-based microbiological assessment of raw milk from a vending machine and of traditional Slovakian pasta filata cheeses made from raw milk was performed in this study. Unsatisfactory hygienic conditions of raw milk were indicated by the average counts of coliform bacteria and *E. coli* at a level of 3.00 ± 0.26 log CFU·ml^{-1} and 1.94 ± 0.56 log CFU·ml^{-1}, respectively. Similarly, higher counts of coliforms at a level of 3.74 ± 1.20 log CFU·g^{-1} on average were determined in pasta filata cheeses. The presumptive numbers of coagulase-positive staphylococci in raw milk were under the limit that is crucial for enterotoxins production. However, in pasta filata cheeses, the EU limit of 4 log CFU·g^{-1} for coagulase-positive staphylococci was exceeded in 53.8 % of samples. Counts of microscopic fungi in raw milk ranged from 2 log CFU·ml^{-1} to 4 log CFU·ml^{-1} and *Geotrichum candidum* was detected in 86.7 % of raw milk samples. The average counts of microscopic fungi in pasta filata cheeses were 3.85 ± 1.39 log CFU·g^{-1}. Despite the pasta filata cheese manufacture includes heat treatment (60–80 °C), the results of this study point to the need to pasteurize raw milk not only before consumption but also prior to production of Slovakian artisanal pasta filata cheeses.

Keywords

raw milk; pasta filata cheese; lactic acid bacteria; coliform bacteria; *Staphylococcus* spp.; microscopic fungi

Milk and dairy products are important staples of wholesome diet [1] and their production is also an essential part of rural economy. Slovakia belongs not only to the smallest milk producers in the European Union (EU) with an average annual production of raw cows' milk during last 10 years of approximately 900 000 t, but also to the countries with the lowest consumption of milk and dairy products [2, 3]. Despite that, consumption of milk and dairy products has been increasing since 2010 from 163 kg of milk and dairy products per person to 173 kg of milk and dairy products per person in 2019 [3].

To increase and boost consumption of dairy products, direct sale and distribution of raw milk to consumers was allowed in Slovakia in 1999 and, subsequently, also milk vending machines were established [4]. Nowadays, consumption of raw milk from vending machines is increasing also in most EU member states [5]. Moreover, raw milk from vending machines is widely used to prepare artisanal cheeses in households. However, microbiological quality of raw milk must meet the requirements of Commission Regulation (EC) No. 853/2004 [6] and also of Commission Regulation (EC) No. 1441/2007 [7].

As the microbiological composition of raw milk is affected by many factors (e.g. physiological state and health of animals, feeding, milking hygiene and sanitation procedures, weather, season) [1], it is also one of the important aspects causing shifts in quality and safety of both raw milk and dairy products [8]. It is well established that counts of bacteria prevail over numbers of microscopic fungi in milk. The dominant bacterial population is represented by lactic acid bacteria (LAB) that are taken as beneficial for subsequent production of dairy products and also for consumers of these products [1]. On the other hand, presence of spoilage and pathogenic microorganisms in

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milk can lead to defects of final products or to severe illnesses [8]. In 2018, raw cows' milk was the second most common commodity in EU contaminated with Shiga toxin-producing *E. coli* [9]. Although occurrence of pathogenic *E. coli* in Slovakian dairy products has decreased since 2011, those products still represent the main sources of *Staph. aureus* in food samples. Moreover, staphylococcal food poisoning causes 1 to 2 outbreaks per year in Slovakia [10]. In EU, the second highest number of food-borne outbreaks caused by bacterial toxins was related to *Staphylococcus* spp. in 2018, while milk and dairy products represented 1.2 % out of all tested food samples with positive detection of staphylococcal enterotoxins [9]. In that context, it is important to control the presence of contaminants like *E. coli* or *Staph. aureus* in milk and dairy products [4]. According to Commission Regulation (EC) No. 1441/2007 [7], the hygienic limit for *E. coli* in dairy products made from raw milk is 2 log CFU·g^{-1} and the limit for coagulase-positive staphylococci (CPS) in raw milk cheeses is 4 log CFU·g^{-1}.

Description of microbiological quality can provide necessary information for risk assessment and quality control of raw milk and dairy products. To improve awareness of microbiological quality of traditional Slovakian dairy products and to contribute to their higher quality and safety, we investigated selected microbial groups that are important from technological and safety point of view, including LAB, microscopic fungi, coliform bacteria and CPS, in raw milk from a vending machine and pasta filata cheeses made from raw milk.

MATERIALS AND METHODS

Sampling

Samples of raw cows' milk were obtained from the only one functional milk vending machine situated in Bratislava, Slovakia. The samples were collected at irregular time intervals from October 2018 to March 2020 in a total count of 15 samples. The milk samples were collected into sterile glass bottles (according to the instruction from the vending machine manual) and transferred in a cooling box within 30 min to the laboratory. Upon delivery, the microbiological analysis was immediately performed.

Samples of traditional pasta filata cheeses "Parenica", "Korbáčik", "Nite" or "Uzlíček", made from raw milk, were obtained from wholesale and retail chains in Bratislava. They were produced in various regions of Slovakia, producers being located in Hruštín, Dolné Plachtince, Jasenie and Necpaly. From the total of 39 cheese samples, 81 % were raw cows' milk cheeses and 19 % were raw ewes' milk cheeses. The cheese samples were collected and analysed in the time period from June 2017 to February 2020.

Microbiological analysis of samples

For microbiological analysis, the methods described in detail by Valík et al. [11] were followed, with determination of selected microbial groups as follows:

- presumptive numbers of lactococci were determined on M17 agar (Biolife, Milan, Italy), incubation being carried out aerobically at 30 ± 1 °C for 24 h,
- presumptive numbers of lactobacilli were determined on de Man, Rogosa and Sharpe (MRS) agar (Biokar Diagnostics, Beauvais, France), with incubation at 37 ± 1 °C for 48 h and at the presence of 5 % CO_2,
- coliform bacteria and *E. coli* were determined on Chromogenic Chromocult coliform agar (Merck, Darmstadt, Germany) with aerobic incubation at 37 ± 1 °C for 24 h,
- presumptive counts of CPS and *Staph. aureus* were determined on Baird-Parker agar (Merck) that was incubated aerobically at 37 ± 1 °C for 48 h,
- numbers of microscopic fungi were determined on yeast glucose chloramphenicol (YGC) agar (Biokar Diagnostics), incubation being carried out aerobically at 25 ± 1 °C for 72–120 h.

Simultaneously, presumptive counts of the microscopic fungus *Geotrichum candidum* in milk samples were determined based on the taxonomic aspect and its micromorphological and macromorphological properties [12].

Analyses were performed in duplicate and the results were expressed as the logarithm of colony forming units (CFU) per millilitre of milk or per gram of pasta filata cheese.

Statistical analysis

Statistical analysis was carried out using Microsoft Excel 365 (Microsoft, Redmond, Washington, USA). The results were presented as the average counts of microorganisms ± standard deviation. Also, the coefficient of variance (v_c), which is given by Eq. 1, was calculated.

$$v_c = \frac{s_d}{\bar{x}} \times 100 \tag{1}$$

where s_d is standard deviation and $\bar{x}$ is average value.

Statistical significance of the mean counts of microorganisms in winter and summer seasons was determined by Student's *t*-test with a least significant difference of 95 %.

RESULTS AND DISCUSSION

Microbiological analysis of raw milk from a vending machine

Raw milk is an optimal medium for a variety of microorganisms due to its high nutritional value, water activity and almost neutral *pH* [4]. The average *pH* value of raw milk samples analysed in our study was 6.72 ± 0.07 (v_c = 1.1 %; data not shown). Results of the microbiological analysis (Tab. 1) indicated that the dominant population of raw milk microbiota was represented by lactococci. As shown in Fig. 1A, there were 46.7 % of milk samples with presumptive counts of lactococci above 4 log CFU·ml^{-1}. Similar density of lactococci (3.96 ± 0.53 log CFU·ml^{-1}) in raw cows' milk from a vending machine were previously determined by Valík et al. [13]. Presumptive counts of lactobacilli in raw milk in our study were by approximately 1 log lower than the presumptive counts of lactococci. Overall, numbers of lactobacilli in 93.9 % of the milk samples were lower than 4 log CFU·ml^{-1}. Their average counts followed log-normal distribution and were consistent with results of Valík et al. [13] in the order of 3 log CFU·ml^{-1}. LAB density in raw milk may be affected by various factors. Mallet et al. [14] observed two-fold higher counts of presumptive lactobacilli in the spring sampling period (May–June) than in the winter samples (January–February). In our study, there was statistically no significant difference ($p < 0.05$) in presumptive counts of lactococci and lactobacilli determined during winter (October–March) and summer (April–July) months.

Raw milk is often contaminated by coliforms, including *E. coli*, that is considered as a commensal bacterial species of normal intestinal microbiome of humans and animals. Thus, in general, it is a relevant indicator of possible fecal contamination [4, 15, 16]. Higher level of coliforms (> 3 log CFU·ml^{-1}) in raw milk may indicate unsatisfactory hygienic conditions and inappropriate sanitary practices in the dairy environment, inadequate refrigeration or coliform mastitis in animals [16]. In the present study, counts of coliform bacteria ranged from 2.60 log CFU·ml^{-1} to 3.47 log CFU·ml^{-1} and counts of coliform bacteria in 40.0 % of milk samples from the vending machine were greater than 3 log CFU·ml^{-1} (Fig. 1B). Results presented by Kunová et al. [17] indicated better hygienic quality of raw milk from vending machines in Slovakia, since counts of coliforms were in all samples lower than 2 log CFU·ml^{-1}. Tremonte et al. [18] also determined counts of coliform bacteria lower than 3 log CFU·ml^{-1} in raw milk from Italian vending machines. However, those authors reported increased counts of coliforms after 24 h of raw milk storage at 4 ± 0.5 °C. This may be related to higher counts of coliforms determined in our study, since the cooling temperature of the vending machine in Bratislava was held at 4 °C and raw milk could be stored for more than 24 h. On the other hand, the indicator meaning of coliform bacteria needs to be also considered. Valík et al. [13] emphasized that raw milk was fecally contaminated, since they determined the density of coliforms in raw milk higher than 4 log CFU·ml^{-1} and they observed only minimal increase of coliform bacteria counts in raw milk after 24 h of storage at 6 ± 1 °C. *E. coli* is commonly found in milk at counts of < 2 log CFU·ml^{-1} [19]. However, in our study, 66.7 % of raw milk samples had presumptive counts of *E. coli* higher than

Tab. 1. Quantitative data on microorganisms in raw milk from a vending machine.

Microorganisms	$\bar{x}$ [log CFU·ml^{-1}]	s_d [log CFU·ml^{-1}]	v_c [%]	*min* [log CFU·ml^{-1}]	*max* [log CFU·ml^{-1}]
Presumptive lactococci	4.12	0.52	12.5	3.22	5.47
Presumptive lactobacilli	3.17	0.55	17.3	1.97	4.07
Coliform bacteria	3.00	0.26	8.8	2.60	3.47
Presumptive *E. coli*	1.94	0.56	28.8	0.48	2.63
Presumptive CPS	3.28	0.25	7.5	2.85	3.74
Presumptive *Staph. aureus*	2.43	0.34	14.1	1.60	2.89
Microscopic fungi	3.27	0.31	9.6	2.86	4.01
Presumptive *G. candidum*	1.21	0.67	55.7	< 1.00	2.18

$\bar{x}$ – average value, s_d – standard deviation, v_c – coefficient of variance, *min* – minimum value, *max* – maximum value, CPS – coagulase-positive staphylococci.

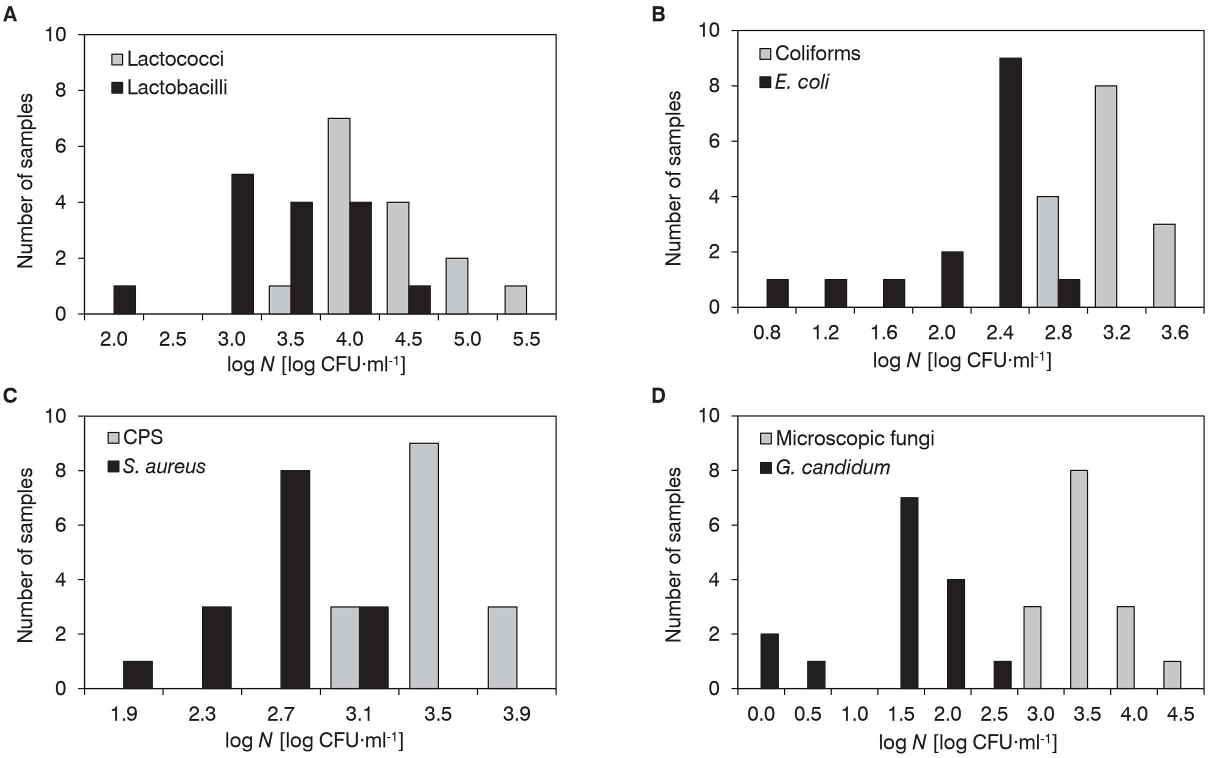

Fig. 1. Distribution of presumptive microbial counts in raw milk samples from a vending machine.

A – Lactococci and Lactobacilli, B – coliforms and *Escherichia coli*, C – coagulase-positive staphylococci and *Staphylococcus aureus*, D – microscopic fungi and *Geotrichum candidum*.
N – presumptive microbial counts, CPS – coagulase-positive staphylococci.

2 log CFU·ml^{-1} (Fig. 1B). In the study of Krahulcová et al. [20], counts of *E. coli* in raw milk from vending machines, located also in Bratislava, were determined in the range from 1.57 log CFU·ml^{-1} to 2.72 log CFU·ml^{-1}. Bogdanovičová et al. determined counts of *E. coli* from 1.00 log CFU·ml^{-1} to 3.30 log CFU·ml^{-1} in raw cows' milk in the Czech Republic [21].

CPS are commonly present in raw milk due to contamination from skin, mucosa membranes, teats and udders of milking animals that are the most important sources of staphylococci [22]. *Staphylococcus* spp., in particular *Staph. aureus*, are dominant agents of mastitis [4]. Density of *Staph. aureus* in properly drawn milk is usually from 2.00 log CFU·ml^{-1} to 2.30 log CFU·ml^{-1}. Increased counts of *Staph. aureus* in milk (mostly approximately 4 log CFU·ml^{-1}) are related to mastitis cases [22]. In our study, presumptive numbers of CPS ranged from 2.85 log CFU·ml^{-1} to 3.74 log CFU·ml^{-1} and the average presumptive counts of *S. aureus* were 2.43 ± 0.34 log CFU·ml^{-1} (Fig. 1C). In contrast to LAB and coliform bacteria, presumptive numbers of CPS (including *Staph. aureus*) were significantly different ($p < 0.05$) during winter and summer seasons. Although there is no limit for *Staph. aureus* counts in raw cows' milk set down in the EU regulations or Slovakian standards legislation, according to Czech and Slovenian legislation, there is limit value of 500 CFU·ml^{-1} [15, 21]. In the context of this, counts of presumptive *Staph. aureus* in milk were less than 500 CFU·ml^{-1} apart from 3 milk samples that were collected during summer months, in our study. Godič Torkar et al. [15] reported 58.8 % out of 51 raw milk samples with numbers of *Staph. aureus* exceeding the limit value of Slovenian legislation, the unsatisfactory samples being mostly collected in September. Bogdanovičová et al. [21] detected *Staph. aureus* in 26.9 % of raw cows' milk samples out of 175 samples, all with counts lower than 500 CFU·ml^{-1}. However, they did not observe any statistically significant association ($P > 0.05$; Chi-squared test for independence) between season and presence of this contaminant.

Occurrence of microscopic fungi in raw milk may be considered from different viewpoints. On the one hand, they are important in the production of cheeses and other dairy products, contributing to desirable organoleptic properties of final products. On the other hand, they can act as spoilage organisms or even represent a potential health risk relating to production of mycotoxins [23, 24]. Numbers of microscopic fungi in raw milk are usually in the range from 2 log CFU·ml^{-1} to 4 log CFU·ml^{-1} [1]. In the present study, the average counts of microscopic fungi in milk samples were 3.27 ± 0.31 log CFU·ml^{-1} (Fig. 1D) and there was statistically no significant difference ($p < 0.05$) in counts of microscopic fungi in relation to season. Lower counts of yeasts and moulds in raw cows' milk determined Godič Torkar et al. (mean value, 2.2 log CFU·ml^{-1}) [15] and Kunová et al. (1.26 ± 0.85 log CFU·ml^{-1}) [17]. Microscopic fungus *G. candidum* is a ubiquitous microorganism that is naturally present in raw milk, but usually at a low concentration below the level of 2 log CFU·ml^{-1}. Since *G. candidum* is of great importance to the ripening of traditional ewes' lump cheeses and secondary to Slovakian cheese "Bryndza" [12, 23], we investigated also presumptive counts of *G. candidum* in raw milk samples from a vending machine. *G. candidum* was detected in 86.7 % of raw milk samples, its average density being 1.21 ± 0.67 log CFU·ml^{-1}. As for all microscopic fungi, there was statistically no significant difference ($p < 0.05$) in counts of *G. candidum* in dependence on season.

Microbiological analysis of pasta filata cheeses made from raw milk

In Slovakia, production and consumption of pasta filata cheeses is a matter of national gastronomical heritage. Pasta filata cheeses are characterized by a unique manufacturing technology, which involves steaming and stretching of acidified cheese curd of pH 4.9–5.2 in hot water at a temperature of 60–80 °C. As a result of this process, the final products gain specific plastic and fibrous texture. This thermo-mechanical treatment may substantially affect distribution and viability of pasta filata cheese microbiota [25, 26]. The results of microbiological analysis of pasta filata cheese samples (summarized in Tab. 2) demonstrate the expected highest density of LAB in comparison with other microbial groups, with counts of presumptive lactococci in the order of 6 log CFU·g^{-1}. However, counts of presumptive lactobacilli were by more than 2 log CFU·g^{-1} lower than counts of presumptive lactococci. Higher levels of presumptive lactococci (in the order from 6 log CFU·g^{-1} to 8 log CFU·g^{-1}) and presumptive lactobacilli (in the order from 6 log CFU·g^{-1} to 7 log CFU·g^{-1}) were determined by Tomáška et al. [25] in Slovakian pasta filata cheeses. However, the increased counts of LAB may be related to the origin of raw milk since cheeses in that study were made from raw ewes' milk. According to Onipchenko et al. [26] and Aponte et al. [27], counts of LAB are not significantly affected by steaming during pasta filata cheese-making. On the other hand, Pappa et al. [28] reported a heat-inactivation effect of cheese stretching in hot water on mesophilic LAB.

Significant inhibition and reduction of coliform bacteria counts due to steaming process was reported by Onipchenko et al. [26] and Pappa et al. [28]. Also Mucchetti et al. [29] determined low levels of coliforms (< 2 log CFU·g^{-1}) in Greek pasta filata cheeses made from raw ewes' milk. On the other hand, considerable levels of coliforms (in order from 3 log CFU·g^{-1} to 6 log CFU·g^{-1}) observed Tomáška et al. [25] in Slovakian pasta filata cheeses made from raw ewes' milk. In our study, 74.4 % of cheese samples contained coliforms at counts above 3 log CFU·g^{-1}. Higher density of coliforms may indicate post-heat processing contamination, since coliform bacteria, including *E. coli*, are considered as heat-sensitive microorganisms [30]. However, some strains of *E. coli*

Tab. 2. Quantitative data on microorganisms monitored in raw milk-based pasta filata cheeses.

Microorganisms	$\bar{x}$ [log CFU·g^{-1}]	s_d [log CFU·g^{-1}]	v_c [%]	*min* [log CFU·g^{-1}]	*max* [log CFU·g^{-1}]
Presumptive lactococci	6.69	1.05	15.8	4.00	8.57
Presumptive lactobacilli	4.35	1.00	22.9	1.00	6.08
Coliform bacteria	3.74	1.20	32.1	1.70	5.79
Presumptive *E. coli*	1.85	0.99	53.6	0.70	4.73
Presumptive CPS	3.94	0.99	25.2	0.70	5.82
Presumptive *Staph. aureus*	3.10	1.02	32.9	0.70	5.70
Microscopic fungi	3.85	1.39	36.0	0.70	6.87

$\bar{x}$ – average value, s_d – standard deviation, v_c – coefficient of variance, *min* – minimum value, *max* – maximum value, CPS – coagulase-positive staphylococci.

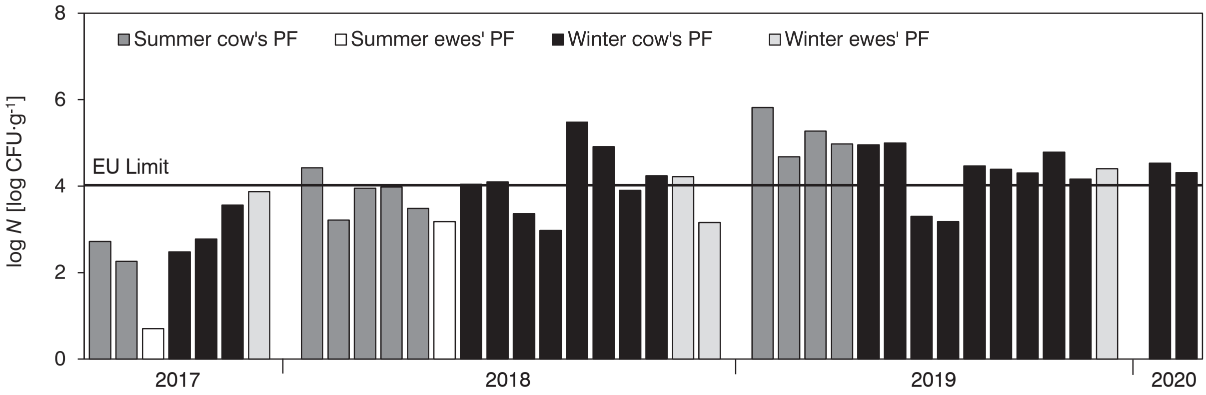

Fig. 2. Presumptive counts of coagulase-positive staphylococci in pasta filata cheeses made from raw milk.

Limit EU for coagulase-positive staphylococci in raw milk cheeses is 10^4 CFU·g^{-1} [7].
N – presumptive microbial counts, PF – pasta filata cheeses.

can be more heat resistant [31]. According to Li and Gänzle [30], decimal reduction time at 60 °C (D_{60}-value) for *E. coli* is more than 6 min. In the present study, 33.3 % of pasta filata cheese samples contained *E. coli* at counts from 2 log CFU·g^{-1} to 3 log CFU·g^{-1}. Despite the possible heat resistance of certain *E. coli* strains, it is also important to consider their indicator importance in connection with hygienic conditions and raw milk quality during the production, manipulation and cooling conditions during storage of pasta filata cheeses.

Considering the limit value of CPS in raw milk cheeses set by Commission Regulation (EC) No. 1441/2007 [7], 53.9 % of cheese samples were of unsatisfactory quality (Fig. 2). It is obvious that the average counts of CPS in pasta filata cheeses had and increasing tendency over years (2.53 log CFU·g^{-1} in 2017, 3.80 log CFU·g^{-1} in 2018 and 4.76 log CFU·g^{-1} in 2019), which points to the need for further monitoring of CPS numbers and to setting of appropriate measures if necessary. Moreover, one cheese sample in 2018 and 2 samples of pasta filata cheeses in 2019 contained presumptive CPS at counts higher than 5 log CFU·g^{-1}, which is the limit content for potential production of staphylococcal enterotoxins [22]. In Europe, about 10 % of raw milk cheeses represent a considerable potential threat to human health because of staphylococcal enterotoxins occurrence [32]. Mucchetti et al. [29] reported stretching and steaming of cheeses as a critical control point for reducing staphylococcal contamination. However, staphylococci are much more heat resistant than coliforms and their D_{60} value ranges from 4.8 min to 6.5 min [4, 33]. Also, higher counts of CPS and *Staph. aureus* may be related to post-steaming contamination, especially in case of manually prepared cheeses. Density of microscopic fungi after the steaming process was reported to remain in the range from 0 log CFU·g^{-1} to 4 log CFU·g^{-1} [26]. Tomáška et al. [25] reported the level of yeasts and moulds in Slovakian pasta filata cheeses to be in the range from 1 log CFU·g^{-1} to 5 log CFU·g^{-1}. Mucchetti et al. [29] determined microscopic fungi from 2 log CFU·g^{-1} to 4 log CFU·g^{-1} in Sicilian pasta filata ewes' cheeses. In the present study, the average counts of microscopic fungi were almost 4 log CFU·g^{-1}. However, there were 18.0 % of pasta filata cheeses with counts of microscopic fungi higher than 5 log CFU·g^{-1}. Based on colony morphology, yeasts prevailed over moulds and presence of *G. candidum* was observed in 7.7 % of cheese samples. Fungal contamination of dairy products generally occurs after the heat-treatment process and microscopic fungi causing spoilage usually originate from air, brine, equipment, and surfaces [34].

CONCLUSIONS

Microbiological quality of raw milk from a vending machine and of pasta filata cheeses was characterized in this work. The results suggest the need to improve quality and safety of traditional raw milk dairy products in Slovakia, as the counts of indicator microorganisms were too high in some cases. Based on this, heat treatment of raw milk from vending machines should be applied not only before consumption but also prior to using it in pasta filata cheesemaking in households. Simultaneously, it is inevitable to keep to high hygienic standards during raw milk cheese manufacture.

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