Original scientific paper
DOI: /10.5513/JCEA01/20.4.2277
Journal of Central European Agriculture, 2019, 20(4), p.1076-1088

The investigation of suitability of raw milk consumption from vending machines in Croatia

Istraživanje prikladnosti konzumacije sirovog mlijeka iz mljekomata u Hrvatskoj

Nataša MIKULEC[1], Jasminka ŠPOLJARIĆ[1] (✉), Šimun ZAMBERLIN[1], Mateja KRGA[1], Biljana RADELJEVIĆ[1], Dijana PLAVLJANIĆ[1], Iva HORVAT KESIĆ[1], Nevijo ZDOLEC[2], Vesna DOBRANIĆ[2], Neven ANTUNAC[1]

[1] University of Zagreb Faculty of Agriculture, Department of Dairy Science, Svetošimunska 25, 10000 Zagreb, Croatia

[2] University of Zagreb Faculty of Veterinary Medicine, Department of Hygiene, Technology and Food safety, Heinzelova 55, 10000 Zagreb, Croatia

✉ Corresponding author: jspoljaric@agr.hr

ABSTRACT

The consumption of raw milk from vending machine in Croatia has increased in recent years. However, its health safety is insufficiently investigated and controlled. The objective of this study was to investigate the suitability of raw milk consumption, from 28 vending machines from 3 counties in Croatia during one-year research period, based on the physicochemical and hygiene quality. Also, the simulation of psychrothrophic bacteria growth has been conducted in order to determine its optimal storage time because of their unfavorable effect on the milk quality. The physicochemical composition of milk samples (n=320) was determined by the infrared spectrometry (chemical composition) and cryoscopic method (freezing point), while hygiene quality was estimated with a total bacterial count (TBC) and a somatic cell count (SCC). Microbiological analyses included determining the presence of *Enterococcus* spp., *Pseudomonas* spp. and the pathogenic species. Satisfactory quality of raw milk was established in 95% (chemical quality) and 70% unadulterated samples (without added water). The prescribed hygiene quality was met by 36.6% (TBC) and 71.9% (SCC) samples. Of total 320 samples, the presence of bacteria in genus *Enterococcus* spp. and *Pseudomonas* spp. was confirmed in 93.4% and 96.9% samples, respectively, and bacteria *Escherichia coli*, *Staphylococcus aureus*, *Lysteria monocytogenes* was confirmed in 32.2%, 27.5% and 3.8% samples. The finding of *Salmonella* spp. and *Yersinia enterocolitica* was negative. At the end of the study improvement of the microbiological quality of raw milk from vending machines was established. The results indicate the need for systematic control and obligate thermal treatment of raw milk before consumption.

Keywords: chemical composition, consumption, hygiene quality, raw milk, vending machine

SAŽETAK

Konzumacija sirovog mlijeka iz mljekomata u Republici Hrvatskoj posljednjih godina je povećana. Međutim, njegova zdravstvena sigurnost nije dovoljno istražena i kontrolirana. Cilj rada bio je istražiti prikladnost konzumacije sirovog mlijeka iz 28 mljekomata, tijekom jednogodišnjeg razdoblja u 3 županije u RH, na osnovu njegove fizikalno-kemijske i higijenske kvalitete. Također, provedena je simulacija rasta psihrotrofnih bakterija, u cilju određivanja optimalnog vremena pohrane zbog njihovog nepovoljnog utjecaja na kvalitetu mlijeka. Metodama infracrvene spektrometrije i krioskopije, uzorcima sirovog mlijeka (n=320) određen je fizikalno-kemijski sastav, dok je higijenska kvaliteta određena ukupnim

brojem bakterija (TBC) i brojem somatskih stanica (SCC). Mikrobiološke analize uključivale su određivanje prisutnosti *Enterococcus* spp., *Pseudomonas* spp. te patogenih vrsta. Zadovoljavajuća kvaliteta sirovog mlijeka utvrđena je u 95% (kemijska kvaliteta) i 70% nepatvorenih uzoraka (bez dodane vode). Propisanoj higijenskoj kvaliteti udovoljilo je 36,6% (TBC) i 71,9% (SCC) uzoraka. Prisustvo bakterija iz roda *Pseudomonas* spp. utvrđena je u 93,4% odnosno 96,9% uzoraka, a bakterija *Escherichia coli*, *Staphylococcus aureus*, *Lysteria monocytogenes* u 32,2%, 27,5% odnosno 3,8% uzoraka. Nalaz *Salmonella* spp. i *Yersinia enterocolitica* bio je negativan. Na kraju istraživanja utvrđeno je poboljšanje mikrobiološke kvalitete sirovog mlijeka iz mljekomata. Rezultati ukazuju na potrebu sustavne kontrole te obveznu termičku obradu sirovog mlijeka iz mljekomata prije konzumacije.

Ključne riječi: higijenska kvaliteta, kemijski sastav, konzumacija, mljekomat, sirovo mlijeko

INTRODUCTION

The increasing trend for the consumption of raw milk from vending machines seen in most European Union Member States has arisen in recent years due to consumer demand for more natural milk, with a higher nutritional value and reacher flavour that is not subject to prior processing (Oliver et al., 2009; Giacometti et al., 2012a; Kunová et al., 2017). Consumption of raw milk is also on the rise in Croatia, despite the fact that due to its composition, raw milk is a very suitable medium for the growth and development of numerous microorganisms, including certain pathogens (Croatian Food Agency, 2016). The microbiological quality of raw milk is the most important and most variable parameter of overall quality. In general, if the milk is obtained from healthy dairy cows, it will contain a negligible number of bacteria that can be considered the natural bacterial population such as: *Micrococcus* spp., *Streptococcus* spp., *Lactococcus* spp. and *Corynebacterium* spp. The European and national legislation have prescribed microbiological standards for the purpose of the product health suitability Commission Regulation (2005/2073/EC) and the Guidance on microbiological food criteria (Ministry of Agriculture, Fisheries and Rural Development of Republic of Croatia, 2011). If the conditions of Good Hygiene Practices (GHP) are not met during milking and following milking, the milk can be contaminated from a range of sources: air, water, bedding, soil, forage, milking apparatus, milk storage and transport equipment and humans. Cooling and storage of raw milk in the vending machines at a temperature of 2-6 °C for longer than 24 hours, favours the growth of psychrotrophic bacteria, including certain pathogenic bacteria such as *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Campylobacter jejuni* (Giacometti et al., 2012a; Giacometti et al., 2012b; Bianchi et al., 2013a; Tremonte et al., 2014). The risks of consuming raw milk from vending machines has been the subject of numerous studies in many countries around the world, particularly in relation to the health safety of consumers, especially chronically ill persons with compromised immunity, infants, small children and pregnant women (Baars, 2013; Bianchi et al., 2013b; Medvedova et al., 2013). Dalzini et al. (2016) pointed out the importance of controlling quality and the microbiological suitability of raw milk intended for sale in vending machines. The recommendation “raw milk must be boiled prior to consumption” should be emphasized on the vending machine, as stated by many authors (Anonymous, 2012; Giacometti et al., 2012a; Bianchi et al., 2013; Giacometti et al., 2013; Medvedova et al., 2013; Doležalová et al., 2014; Dalzini et al., 2016).

Due to the potential threats to human health, regular controls of the physicochemical composition and microbiological safety of raw milk from vending machines is necessary. Pursuant to the national Food Act (OG 115:2018), consumers may not be misled and are entitled to the right that purchased and consumed foods do not threaten their health and economic interests. In the Republic of Croatia, control of raw milk is carried out in accordance with the Ordinance on the determination of the composition of raw milk (OG 27:2017), Ordinance on the inspection of raw milk intended for public consumption (OG 84:2016) and Act on Official Controls Performed in Compliance with Regulations on Food, Feed, and Animal

Health and Welfare (OG 14:2014). These controls include determining: the total bacterial count, somatic cell count, presence of inhibitory substances and the basic chemical composition of milk from family farms, who sell their raw milk via their own vending machines.

The sale of raw milk via vending machine and the absence of recommendation by the manufacturer (OG 84:2015) regarding the required heat treatment of raw milk before consumption represent a potential risk to consumer health. The raw milk sold via vending machine is considered as consumable milk and must meet the quality prescribed by the Commission Regulation (2013/1308/EC) and national Ordinance on the determination of the composition of raw milk (OG 27:2017). Regarding to its hygienic quality there can be applied valid microbiological criteria for raw milk 100 000 cfu/mL and 400 000 SCC/mL prescribed in Commission Regulation (2004/853/EC) and the national Ordinance (OG 27:2017).

The quality of the physicochemical parameters of raw milk from vending machines according to available literature sources was studied by Stetca et al. (2014), Tremonte et al. (2014) and Croatian Food Agency (2016) while its hygienic quality and suitability during storage in vending machine has been studied by more authors Giacometti et al. (2012a, 2012b), Bianchi et al. (2013a, 2013b), Giacometti et al. (2013), Stetca et al. (2014), Tremonte et al. (2014), Dalzini et al. (2016), Croatian Food Agency (2016), Godic Torkar et al. (2017) and Kunova et al. (2017).

More effective control of the physical, chemical, microbiological, hygiene and sanitary aspects should be performed on raw milk produced on farms, for the purpose of reducing the health risks to consumers (Stetca et al., 2014; Rossi et al., 2018).

Food business entities must ensure that milk must be quickly cooled to a temperature no more than 6 °C until the time of processing (Commission Regulation 2004/853/EC). Considering the manner of storing raw milk (cooling) until the time of sale, the potential microbiological risk in the sense of souring are the previously mentioned psychrotrophic bacteria. After hygienic milking, raw milk typically contains between several hundred to several thousand bacteria per millilitre (Frank and Hassan, 2003). First class raw milk may contain up to 100 000 cfu/mL (OG 27:2017). However, the criteria for the number of psychrotrophic bacteria in Croatia is not defined, even though different EU Member States list different values, from 5 000 cfu/mL (or 3.69 log cfu/mL) for premium quality raw milk (Samaržija et al., 2012), 10 000 cfu/mL in Spain (Reguillo et al., 2018) to 50 000 cfu/mL in Czech and Slovakia (Cempírková and Mikulová, 2009; Vietoris et al., 2016). An increasing number of psychrotrophic bacteria in the microbial population of raw milk stored in the vending machines will negatively influence on its quality. Low temperatures and longer storage of milk favour the growth of psychrotrophic bacteria that break down protein and milk fat with their proteolytic and lipolytic enzymes which leads to a change in the taste of milk and dairy products: bitter, rancid, unclean and fruity (Sørhaug and Stepaniak, 1997; Samaržija et al., 2012).

The objective of this study was (i) to investigate the suitability of raw milk from vending machines for consumption over a one-year period, from the territory of 3 counties in Croatia, in relation to physicochemical and hygiene quality, (ii) to investigate the presence of *Enterococcus* spp., *Pseudomonas* spp. and pathogenic species: *Escherichia coli*, *Staphylococcus aureus*, *Lysteria monocytogenes*, *Salmonella* spp., *Yersinia enterocolitica* and (iii) to conduct the simulation of psychrothrophic bacteria growth in laboratory conditions, in order to determine its optimal storage time in vending machine.

MATERIALS AND METHODS

Location and number of vending machines

The raw milk samples (n=320) from the 28 vending machines were sampled for the determination of the physicochemical composition and microbiological analysis in the 3 counties (the City of Zagreb, Zagreb County and Sisak-Moslavina County). In these 3 counties in Croatia during the research there were situated about 70% of total number of vending machines, according to the scientific opinion of the Croatian Food Agency

(2016). The number of tested vending machines was variable and depended on various unpredictable factors, such as changes in ownership of the vending machines, closure of existing and opening of new vending machines at new locations, empty container at the time of sampling. From the 28 vending machines at the beginning of the research, there were only 17 vending machines (n=204) investigated through the whole one-year research period.

Sampling

Raw milk samples were taken at one-month intervals, from December 2015 to January 2017. Raw milk from the vending machines was sampled in early morning hours into a sterile bottle (0.5 L) and transported in mobile coolers at a temperature of +4 °C (±2 °C) within 2 h in the laboratory. Some of vending machines didn´t have the data of filling and emptying so this kind of data records could not be carried out. All samples were stored in the refrigerator at a temperature of +4 °C (±2 °C) until the time of analysis. All analyses were started within 24 hours of sampling.

Analysis

The content of dry matter, milk fat, protein, lactose and non-fat dry matter (NFDM) in milk were determined using the infrared spectrometry method (HRN ISO 9622:2017) on the MilkoScan FT 120 instrument (Foss Electric, Denmark). The freezing point of milk was determined using the cryoscopic method (HRN EN ISO 5764:2010) on the Cryostar 1 instrument (Funke Gerber, Germany). The addition of water in milk was calculated according to Sabadoš (1996). The somatic cell count (SCC) in milk was determined using the fluoro-opto-electronic method (HRN EN ISO 13366-2:2007) on the Fossomatic Minor instrument (Foss Electric, Denmark) and total bacterial count (TBC) using the flow cytometry method (HRN EN ISO 21187:2008, HRN EN ISO 4833-1:2013) on the Bactoscan FC instrument (Foss Electric, Denmark). Analysis of the physicochemical composition and hygiene quality of raw milk was conducted by accredited methods according to the norm HRN EN ISO/IEC 17025:2007, in the Reference Laboratory for Milk and Dairy Products of the Department of Dairy Science, Faculty of Agriculture University of Zagreb.

Additional microbiological analysis included enumeration of *Staphylococcus aureus* (HRN EN ISO 6888-1:2004), *Escherichia coli* (HRN ISO 16649-2:2001), *Pseudomonas* spp. (HRS ISO/TS 11059:2009) and *Enterococcus* spp. (Compass *Enterococcus* Agar, Biokar diagnostics, 2011) and detection of *Salmonella* spp. (HRN EN ISO 6579-1:2017), *Yersinia enterocolitica* (HRN EN ISO 10273:2003) and *Listeria monocytogenes* (HRN EN ISO 11290-1:1999). The presence of *Listeria monocytogenes* was additionally evaluated by the Molecular Detection System *Listeria monocytogenes* (3M, SAD), which combines isothermal DNA amplification and bioluminescence detection. The analyses were performed at the Department for Hygiene, Technology and Food Safety, Faculty of Veterinary Medicine University of Zagreb.

During eight months, the simulation of the psychrotrophic bacteria growth in raw milk was carried out (in 11 cycles). Samples of raw milk (from the selected farm) immediately after milking were delivered to the laboratory mobile coolers. The milk volumes of 50 mL were transferred into sterile vials for each time of storage (0, 24, 48 and 72 h) and stored at a temperature of +4 °C (±2 °C). The number of psychrotrophic bacteria in raw milk samples after 0, 24, 48 and 72 h of storage was determined (HRN ISO 6730:2010).

One-way analysis of variance (ANOVA) was used to analyse the data, Bartlett's test for equal variances testing and Tukey's test for multiple comparisons at 95% confidence interval. The data was analysed using computer program GraphPrism 5 (GraphPad Software, 2008).

RESULTS AND DISCUSSION

The results of the analysis of total 320 milk samples (from the 28 vending machines) are shown in Table 1, regardless of sampling dynamics.

Table 1. Prescribed chemical composition of the raw milk from the vending machines according to legal regulations*

Component (n=320)	Value according to legal regulations*	Average value (± SE)	CV (%)	Min – Max	Share of samples comply with legal regulations* (%)
Milk fat (%)	3 – 5.5	4.24 ± 0.03	11.08	2.06 - 5.52	98.44
Protein (%)	2.5 – 4	3.48 ± 0.01	6.61	2.65 - 4.13	97.5
NFDM (%)	Min 8.5	8.98 ± 0.02	3.45	7.14 - 10	94.69

* Ordinance on the determination of the composition of raw milk (OG 27:2017). NFDM – non-fat dry matter, SE – standard error, CV – coefficient of variation

From the results in Table 1, it can be concluded that in most cases, the raw milk from the vending machines complied with the requirements for chemical composition according to the Ordinance on the determination of the composition of raw milk (OG 27:2017). According to the OG 27/2017 prescribed chemical composition for raw milk is minimum 3% milk fat, minimum 2.5% proteins and minimum 8.5% NFDM. Of the total number of samples (320), only 5 samples were not compliant, due to lower content of the milk fat in 3 and higher freezing point value in 2 samples. Pursuant to Commission Regulation (2013/1308/EC), 95% of the raw milk samples belonged to the group of whole milks, with an average content of milk fat over 3.5%. The data of dry matter and lactose content are not prescribed by the regulations and are not shown.

The raw milk from the vending machines contained, on average, 3.48% proteins. A total of 8 samples were not compliant with the OG 27:2017 due to a higher protein content of over 4%. This could be due to unbalanced nutrition or an excessive share of concentrates in the animal's diet. The range of milk fat and proteins in raw milk in other EU Member States: Romania (Stetca et al., 2014), Czech Republic (Hanuš et al., 2008; Zájác et al., 2015), Hungary (Jancsó, 2015) were within the values laid down in the national Ordinance (OG 27:2017).

The assessment of raw milk adulteration can be made on the basis of the content of NFDM. In 94.69% of cases, the raw milk from the vending machines satisfied the requirements of the national OG 27:2017, i.e. 17 milk samples contained less than 8.5% NFDM (Table 1). During the research period, the content of NFDM was quite uniform. Confirmation of milk fraud by the addition of water was conducted using the reference cryoscopic method (Table 2).

The assessment of chemical quality of raw milk from vending machine was performed by determination of adulteration with added water by determination of freezing point of milk. The prescribed value of the freezing point of raw milk is -0.517 °C (OG 27:2017). Raw milk from the vending machine intended for consumption is considered consumable milk. The prescribed freezing point may not be greater than 2% of the freezing point of raw milk (including technological water) prescribed in the Ordinance on milk and dairy products (OG 64:2017).

A higher freezing point was determined in 91 milk samples, while 229 samples had freezing points lower than the reference value and were compliant according to national legislation (OG 27:2017). During the research period, the values of the freezing point varied from the minimum of -0.585 °C to a maximum of -0.3796 °C. Non-adulteration factors which affecting the freezing point of raw milk are breed of cow, stage of lactation, season, feed, morning and evening milk, climate, milk from different quarters etc. (Bulletin FIL-IDF, 1983). The mean values of the freezing point of raw milk from individual vending machines (17) during one-year research period are shown in Figure 1.

Of the total 320 analysed samples of raw milk, an addition higher than 2% of water was found in 16 samples. The adulteration, ie the significant addition of water (25.31%) was found in just one milk sample from the vending machine 18. In the case of discovery of

adulterated milk with more than 2% of added water, the manufacturers are obliged to take measures according to the prescribed legal regulations.

Total bacterial count and somatic cell count as indicators of hygienic quality of raw milk from the vending machines are shown in Table 3.

Table 2. Freezing point values of raw milk from the vending machines according to legal regulation*

Parameter (n=320)	FP value according to legal regulation* (°C)	Average value (± SE)	CV (%)	Min – Max (°C)
Freezing point	Not higher than -0.517	-0.519 ± 0.001	1.93	-0.585 to -0.3796

* Ordinance on the determination of the composition of raw milk (OG 27:2017). FP – freezing point, CV – coefficient of variation, SE – standard error.

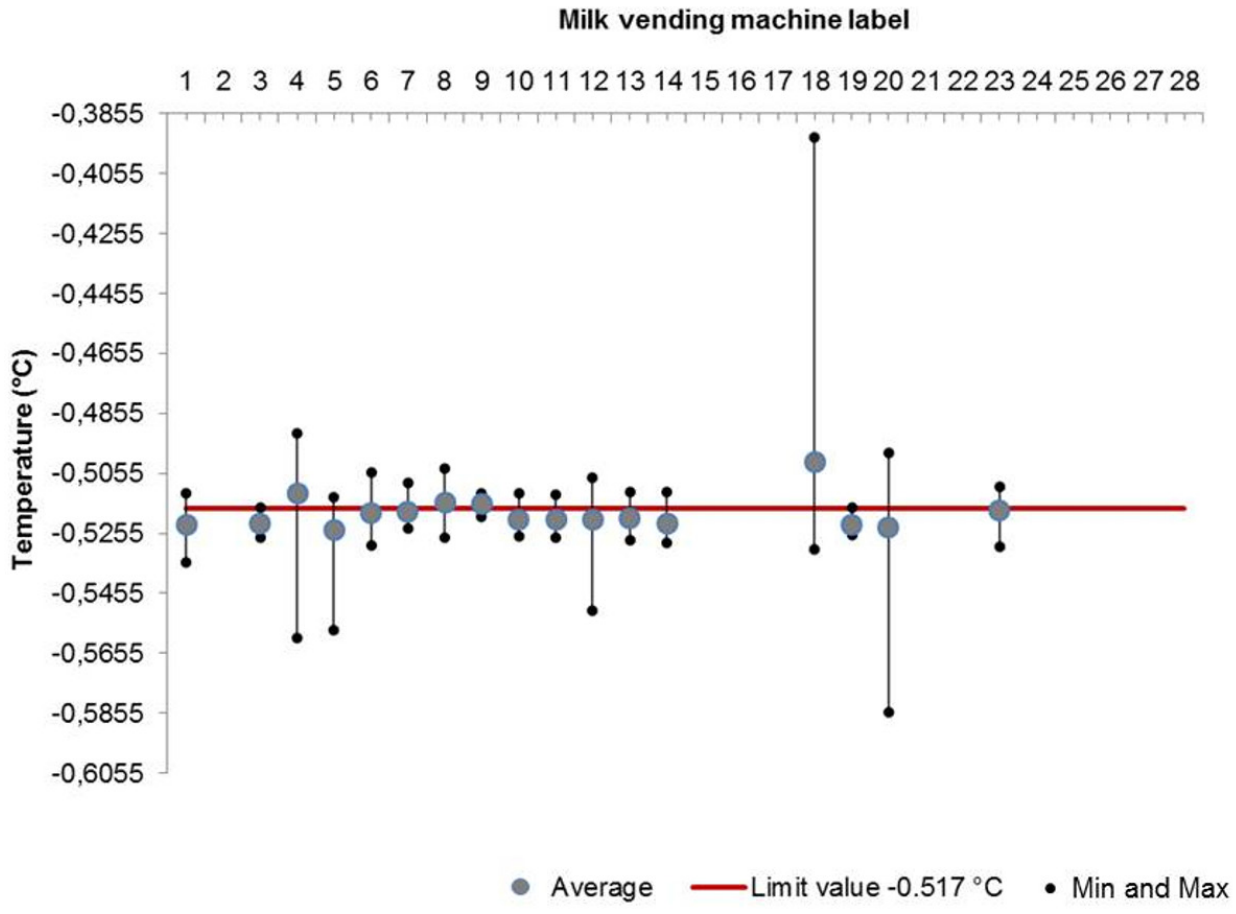


Figure 1. The freezing point values of raw milk from individual vending machines

Table 3. Total bacterial count and somatic cell count of raw milk from the vending machines

TBC* (cfu/mL)	No.	Share (%)	SCC** (per mL)	No.	Share (%)
	320			320	
≤100 000	117	36.6	≤400 000	230	71.9
>100 000	203	63.4	>400 000	90	28.1

*TBC – total bacterial count, cfu - colony forming unit; **SCC – somatic cell count.

Of the 320 individual raw milk samples, analysed for the total bacterial count, 36.6% samples contained less than 100 000 cfu/mL, and 71.9% samples contained less than 400 000 cell/mL. The requirements for raw milk (Class 1; ≤100 000 cfu/mL; ≤ 400 000 cell/mL) were used to determine the hygienic correctness of raw milk from vending machines (OG 27:2017). These criteria were met by 107 samples. Of that number, 81.3% samples were from milk producers member of dairy - livestock cooperatives´, that apply GHP in the production and processing of milk while other 18.7% milk producers had worse milk quality.

Better quality of raw milk from vending machines was confirmed in Slovenia, where the share of milk samples with a total bacterial count of over 100 000 cfu/mL was 39.2%. The cause of this is inadequate hygiene measures, storage conditions or improper cooling of milk. The share of milk samples with a somatic cell count in excess of 400 000 cell/mL was also lower, at 21.6% (Godic Torkar et al., 2017). The criteria of 100 000 cfu/mL was met by 91% samples of raw milk from vending machines in Slovakia (Vietoris et al., 2016). In relation to the prescribed criteria of raw milk in Slovenia and Croatia, the requirements in Italy are stricter (50 000 and 300 000 cell/mL). The share of milk from vending machines in Italy exceeding the bacterial count of 50 000 cfu/mL was 44.8%, and exceeding the somatic cell count of 300 000 cell/mL was 18% (Giacometti et al., 2012b).

The findings of selected bacteria in raw milk from the vending machines during the research period is shown in Figure 2.

Of the total 320 analysed samples of raw milk from 28 vending machines, 299 were confirmed to contain *Enterococcus* spp., and 310 samples contained *Pseudomonas* spp. Sources of milk contamination by *Enterococcus* spp. include humans, the external part of the udder and teats and by *Pseudomonas* spp. remnant water in the milking machine, milk pipes or coolers, unclean udders, inadequate cleaning of surfaces of dairy farm equipment for the receipt, transport and storage of milk (Frank and Hassan, 2003). The presence of *Escherichia coli* was confirmed in 103 samples during the research period. The most common sources of contamination are: humans, faeces, water, milking equipment and unclean equipment. Its presence in milk is an indicator of faecal contamination in milk production, during and after the completion of milking. In terms of bacterial indicators of contamination - *E. coli* and *Enterococcus* spp., results of this research are within expectations of this type of production, with a high variability of value, mostly related to the hygiene practice of individual farms (Zdolec et al., 2016). This is also apparent in the results of previous research of raw milk microbiota sampled on Croatian dairy farms (Dobranić et al., 2016).

One of the most common causes of mastitis in cows is the bacteria *Staphylococcus aureus*, which was confirmed in 88 samples of raw milk samples, and possible sources of contamination include: faeces, external part of udder and teats and skin. Bianchi et al. (2013b) confirmed the presence of *S. aureus* in 40% samples, while Godic Torkar et al. (2017) found even higher values (54.5% of samples in raw milk having a somatic cell count over 400,000 cell/mL).

In the conditions of cooling milk in the vending machine, *L. monocytogenes* can grow and reproduce at low temperatures of milk storage. In humans, this bacterium can cause a range of disorders (gastroenteritis, encephalitis, miscarriage). Its presence in raw milk was confirmed in 12 samples. Dalzini et al. (2016) reported the ability of *L. monocytogenes* to survive and grow in various types of foods (milk and cheese), even at low temperatures. An incidence of *L. monocytogenes* of 1.66% was found in raw milk samples, and in 0.5% of raw milk samples from vending machines. Sources of contamination with *L. monocytogenes* can be faeces and silage used in dairy cow nutrition, in which pathogenic bacteria reproduce mainly in unfavourable fermentation conditions (pH>5 to 5.5) (Husu, 1990). Due to the different expression of results (only qualitative) it is difficult to compare the literature data from different countries. For the purpose of preventing the growth of other pathogenic microorganisms in raw milk, it should be

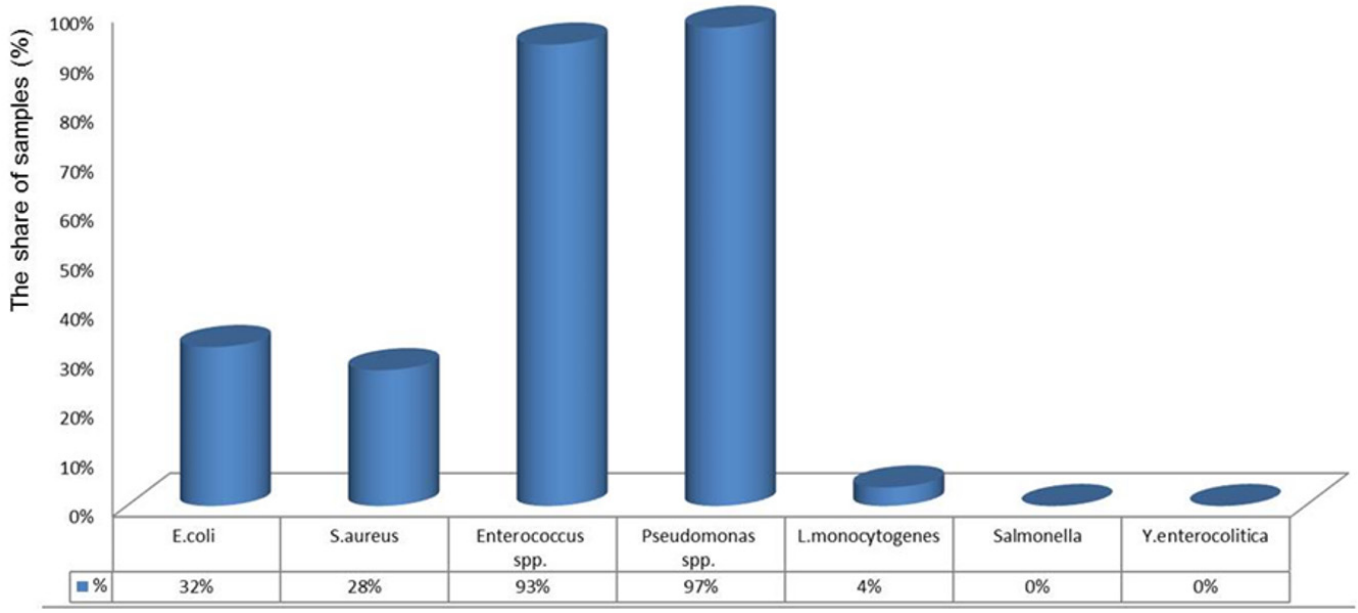


Figure 2. The share of raw milk samples positive for selected bacteria (Ministry of Agriculture, Fisheries and Rural Development of Republic of Croatia, 2011 and Commission Regulation, 2005/2073/EC)

stored at temperatures from 0 to +4 °C, and consumers must be informed of the importance of cooking milk prior to consuming it.

All 320 raw milk tested samples were *Salmonella* spp. and *Y. enterocolitica* negative. The appearance of *Salmonella* spp. in raw milk from vending machines is up to 1% (Giacometti et al., 2012a; Bianchi et al., 2013a; Giacometti et al., 2013). The consumption of raw milk increases the risk of contamination with the bacteria *Y. enterocolitica*, which is capable of surviving longer periods of time at refrigeration temperatures. The incidence of *Y. enterocolitica* in raw milk varied from 1.2 to 6.1% (Oliver et al., 2009), while its incidence in raw milk from vending machines has not been determined.

In the case of positive findings of spoilage agents and pathogenic species, the milk producers received expert advisory assistance, which has resulted in improvements in the food safety of raw milk. Based on the results of the microbiological analysis of raw milk from vending machines, its mandatory cooking prior to consumption is recommended. This recommendation was also made by other authors (Italian Ministry of Health, 2009; Giacometti et al., 2012a; Bianchi et al., 2013a; Medvedová et al., 2013; Doležalova et al., 2014; Tremonte et al., 2014; European Food Safety Authority, 2015; Vietoris et al., 2016; Croatian Food Agency, 2016).

Results of raw milk quality from only 17 vending machines (204 milk samples) that were controlled during the whole research period are shown in Table 4.

The content of milk fat in raw milk of 17 vending machines varied from 3.84% to 4.81%. Only milk from two vending machines 8 and 10 contained a significant higher content of milk fat ($P<0.05$) compared to all other. The content of protein and non-fat dry matter in milk varied from 3.28% and 8.63% (vending machine 18) to maximum 3.83% and 9.37% (vending machine 8). The lowest content of lactose (4.38%) was also found in the vending machine 18, which resulted in a significant higher average freezing point value (-0.502 °C) compared to all other vending machines ($P<0.05$). Obtained results for vending machine 18 indicate the frequent occurrence of milk adulteration by addition of water. The physicochemical quality of milk sampled from vending machines throughout the whole research period complied with prescribed regulations (OG 27:2017), with the exception of four vending machines in which the freezing point values were higher than prescribed.

DOI: /10.5513/JCEA01/20.4.2277

Table 4. Results of raw milk from vending machines controlled during the whole research period

Vending machine label (n=204)	Dry matter (g/100 g)	Milk fat (g/100 g)	Protein (g/100 g)	Lactose (g/100 g)	NFDM (g/100 g)	Freezing point (°C)	TBC (log_{10}/mL)	SCC (log_{10} /mL)
	$\bar{x}$ ± SE	$\bar{x}$ ± SE	$\bar{x}$ ± SE	$\bar{x}$ ± SE	$\bar{x}$ ± SE	$\bar{x}$ ± SE	$\bar{x}$ ± SE	$\bar{x}$ ± SE
1	13.02±0.15^{A}	3.86±0.17$^{a.A}$	3.61±0.03^{A}	4.58±0.02$^{a.A}$	9.16±0.06^{a}	-0.523±0.002$^{a.A}$	4.24±0.17^{A}	4.98±0.06^{A}
3	13.37±0.09^{A}	4.19±0.07$^{a.A}$	3.62±0.03^{A}	4.59±0.02$^{a.A}$	9.18±0.02^{a}	-0.522±0.001$^{a.A}$	4.49±0.15^{A}	5.35±0.03$^{B.C}$
4	12.79±0.09^{A}	4.08±0.05$^{a.A}$	3.32±0.04^{B}	4.42±0.02$^{a.B}$	8.71±0.04^{b}	-0.512±0.005^{a}	6.71±0.14$^{B.c}$	5.69±0.04$^{B.D}$
5	12.98±0.19^{A}	4.07±0.15^{a}	3.42±0.08^{B}	4.51±0.02^{a}	8.91±0.09^{b}	-0.525±0.003$^{a.A}$	6.31±0.20$^{B.e}$	5.54±0.08$^{B.D}$
6	13.13±0.21^{A}	4.29±0.15^{a}	3.42±0.05^{B}	4.41±0.05$^{a.B}$	8.84±0.09^{b}	-0.519±0.002^{a}	6.35±0.22$^{B.e}$	5.74±0.07$^{B.D}$
7	13.31±0.11^{A}	4.31±0.08^{a}	3.39±0.03^{B}	4.66±0.01$^{a.A.C}$	9±0.04$^{b.A}$	-0.518±0.001^{a}	4.84±0.21^{A}	5.2±0.06^{C}
8	14.19±0.09^{B}	4.81±0.05$^{a.B}$	3.83±0.06^{A}	4.59±0.03$^{a.A}$	9.37±0.05^{a}	-0.515±0.002^{a}	5.24±0.17$^{B.d}$	5.29±0.06^{C}
9	13.21±0.07^{A}	4.35±0.06^{a}	3.48±0.05^{B}	4.41±0.02$^{a.B}$	8.86±0.04^{b}	-0.516±0.001^{a}	4.83±0.21^{A}	5.56±0.06$^{B.D}$
10	13.51±0.12^{B}	4.48±0.1$^{a.B}$	3.49±0.03^{B}	4.58±0.02$^{a.A}$	9.03±0.04$^{b.A}$	-0.521±0.001$^{a.A}$	4.96±0.21^{A}	5.31±0.06^{C}
11	13.41±0.14^{A}	4.26±0.13$^{a.A}$	3.61±0.05^{A}	4.57±0.02$^{a.A}$	9.15±0.04^{a}	-0.521±0.001$^{a.A}$	5.46±0.21$^{B.d}$	5.3±0.06^{C}
12	13.06±0.19^{A}	4.37±0.14^{a}	3.3±0.05^{B}	4.44±0.01$^{a.B}$	8.69±0.07$^{b.B}$	-0.518±0.002^{a}	5.26±0.19$^{B.d}$	5.66±0.06$^{B.D}$
13	12.84±0.16^{A}	4±0.13$^{a.A}$	3.44±0.05^{B}	4.43±0.02$^{a.B}$	8.84±0.06^{b}	-0.52±0.002$^{a.A}$	5.49±0.17$^{B.d}$	6.07±0.05$^{B.E}$
14	13.38±0.1^{A}	4.24±0.09$^{a.A}$	3.60±0.05^{A}	4.58±0.02$^{a.A}$	9.14±0.04^{a}	-0.522±0.001$^{a.A}$	5.57±0.23$^{B.d}$	5.4±0.11$^{B.C}$
18	12.69±0.22^{A}	4.06±0.1$^{a.A}$	3.28±0.06^{B}	4.38±0.08$^{a.B}$	8.63±0.13$^{b.B}$	-0.502±0.010$^{a.B}$	6.20±0.16$^{B.e}$	5.66±0.05$^{B.D}$
19	12.92±0.11^{A}	3.92±0.11$^{a.A}$	3.44±0.04^{B}	4.57±0.04$^{a.A}$	9±0.03$^{b.A}$	-0.523±0.001$^{a.A}$	5.22±0.09$^{B.d}$	5.31±0.14^{C}
20	12.75±0.09^{A}	3.84±0.07$^{a.A}$	3.42±0.08^{B}	4.5±0.04$^{a.A.D}$	8.91±0.09^{b}	-0.524±0.006$^{a.A}$	5.01±0.32^{D}	5.2±0.1^{C}
23	13.48±0.18^{B}	4.38±0.11^{a}	3.53±0.07^{B}	4.57±0.03$^{a.A}$	9.1±0.08^{a}	-0.518±0.001^{a}	6.36±0.18$^{B.e}$	5.41±0.07$^{B.C}$

NFDM – non-fat dry matter, TBC – total bacterial count; SCC – somatic cell count, $\bar{x}$ - average value, SE - standard error; different exponents marked with small letters in the same column indicate that there is a statistically significant difference (P<0.05); different exponents marked with capital letters in the same column indicate that there is a statistically significant difference (P<0.05)

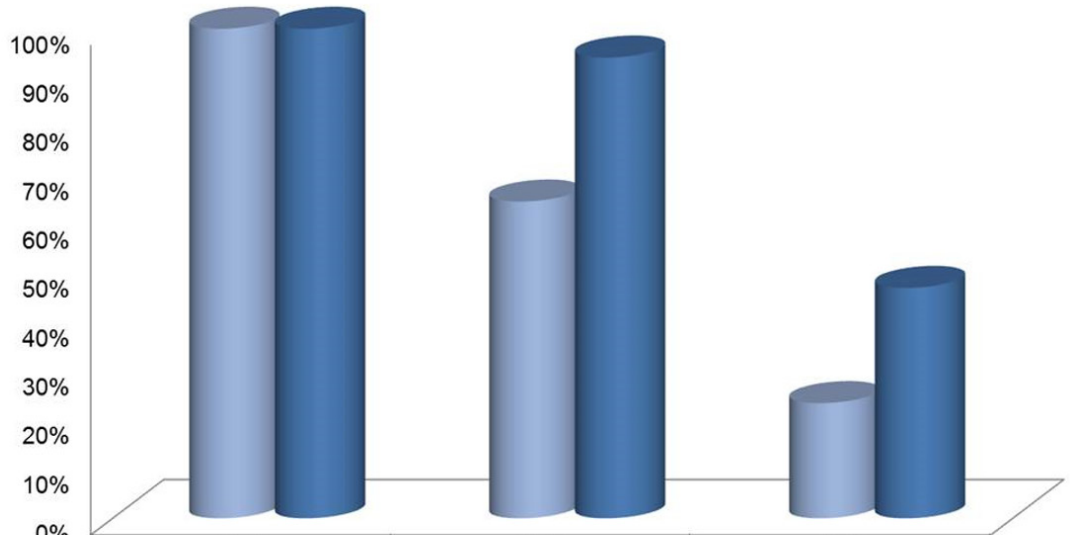
The prescribed criteria for total bacterial count was met in five vending machines and for somatic cells count in twelve vending machines, while the both criteria of hygienic quality were met in only five vending machines. The presence of one or more investigated pathogenic bacteria species was found in raw milk of all 17 vending machines analysed throughout the whole research period, indicating its unacceptable health safety.

The results of the physicochemical and hygiene qualities of raw milk from the 17 vending machines which were continuously evaluated from the beginning to the end of the research period are shown in Figure 3.

In comparing the results of raw milk from 17 vending machines, at the beginning to the end of the research period, can be concluded that the raw milk met the requirements of the national Ordinance (OG 27:2017) for its chemical composition (100%). At the end of the research period, there was a 29.41% improvement in the physical quality of raw milk, expressed as the value of the freezing point, and a 23.53% improvement in the hygiene quality, expressed as the TBC and SCC, in comparison to the beginning. This improvement is the result of the education of the milk producer by the Croatian Advisory Service.

A simulation of psychrotrophic bacteria growth in raw milk was performed after 0, 24, 48 and 72 hours (Figure 4) for the purpose of proposing the optimum storage time of raw milk in vending machines at temperatures of +4 °C (±2 °C).

Exponential growth of psychrotrophic bacteria is evident with increase storage time of milk. After 48 hours of storage of raw milk, the number of psychrotrophic bacteria increased by 16% over the 24-hour storage time, prescribed by the Ordinance on the registration of entities and the registration and approval of entities in food operations (OG 84:2015). Reguillo et al. (2018) got similar results for different storage times (24 h and 48 h) of raw milk in vending machines where the number of psychrotrophic bacteria significantly increased by 18%.



	satisfies CHEMICAL COMPOSITION	satisfies FREEZING POINT	satisfies HYGIENIC QUALITY
Beginning of the research (2015)	100.00%	64.71%	23.53%
End of the research (2017)	100.00%	94.12%	47.06%

Figure 3. Raw milk quality from the 17 vending machines according to legal regulation (OG 27:2017)

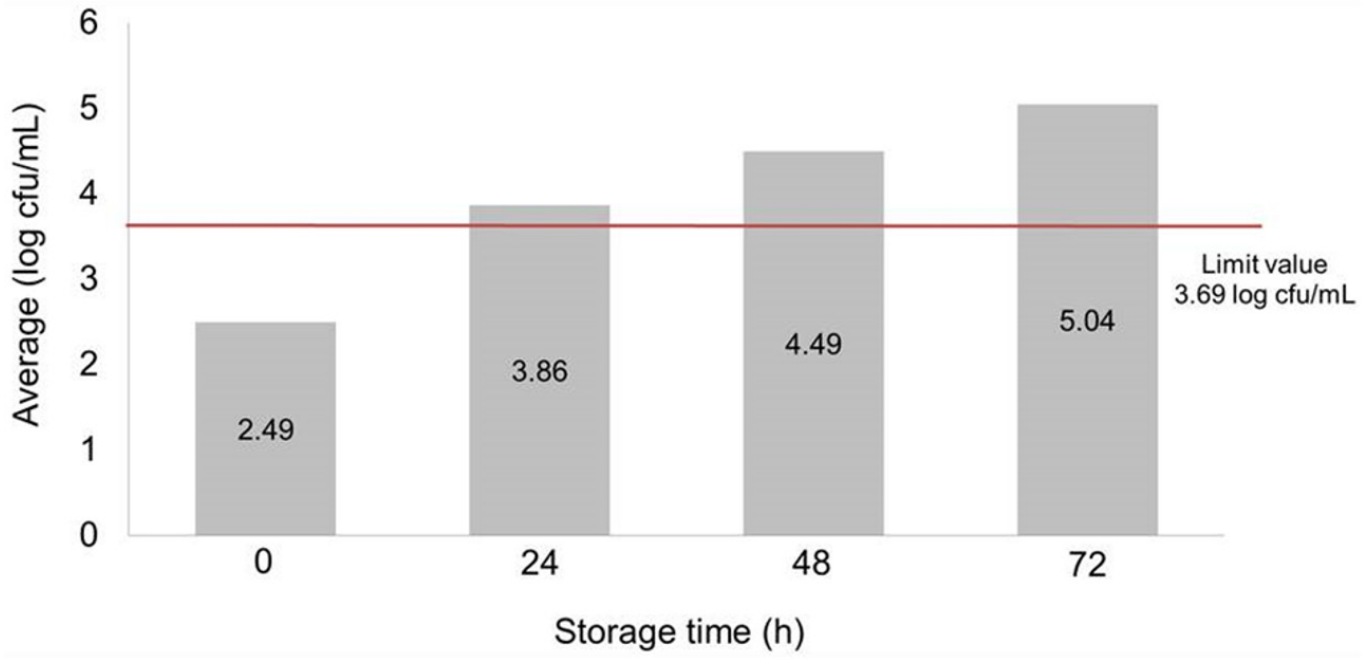


Figure 4. Simulation of psychrotrophic bacteria growth in raw milk

CONCLUSIONS

The chemical composition of raw milk from vending machines was of satisfactory quality, pursuant to the legal regulation. Determining of freezing point of raw milk it has been proven cases of adulteration with added water. Hygienic quality of raw milk was not satisfactory because of established high values of the TBC and SCC. At the end of research study there was established the improvement in the hygiene quality of the raw milk for 23.53% what is result of the education of the milk producers and regular controls. The presence of selected pathogenic bacteria, except *Salmonella* spp., *Y. enterocolitica*, was established in the raw milk from vending machines. Based on the determined number of the psychrotrophic bacteria in the raw milk, it was confirmed, that its optimal storage time in the vending machines at +4 °C (±2 °C) is 24 h. On the basis of these results, it is evident that due to the appearance of pathogenic bacteria, the raw milk can´t be consumed as raw and must be boiled prior to consumption. The official control of raw milk from vending machines would improve the current system of raw milk control in Croatia, which would influence an increase of consumer trust and improved competitiveness of those producers abiding by the principles of GHP.

ACKNOWLEDGMENTS

This study was conducted as part of the research project entitled: Innovative technology in the vending machines control in order to increase the quality and competitiveness of milk producers (Inovativna tehnologija u kontroli mljekomata u cilju povećanja kvalitete i konkurentnosti proizvođača mlijeka) financed by the Ministry of Agriculture, Agricultural Research Council (Project No. 2015-13/69).

REFERENCES

Act on Official Controls Performed in Compliance with Regulations on Food, Feed, and Animal Health and Welfare (2014) The Official Gazette, No. 14. Zagreb. Available at: https://www.zakon.hr/z/642/Zakon-o-slu%C5%BEbenim-kontrolama-koje-se-provode-sukladno-propisima-o-hrani,-hrani-za-%C5%BEivotinje,-o-zdravlju-i-dobrobiti-%C5%BEivotinja-

Anonymous (2012) The dangers of raw milk: Unpasteurized milk can pose a serious health risk. U.S. Food and Drug Administration. Food Facts, 1-2.

Baars, T. (2013) Milk consumption, raw and general, in the discussion on health or hazard. Journal of Nutritional Ecology and Food Research, 1, 91-107. DOI: https://dx.doi.org/10.1166/jnef.2013.1027

Bianchi, D.M., Barbaro, A., Gallina, S., Vitale, N., Chiavacci, L., Caramelli, M., Decastelli, L. (2013a) Monitoring of foodborne pathogenic bacteria in vending machine raw milk in Piedmont, Italy, Food Control, 32 (2), 435–439. DOI: https://dx.doi.org/10.1016/j.foodcont.2013.01.004

Bianchi, D.M., Gallina, S., Bellio, A., Chiesa, F., Civera, T., Decastelli, L. (2013b) Enterotoxin gene profiles of *Staphylococcus aureus* isolated from milk and dairy products in Italy. Letters in Applied Microbiology, 58, 190-196. DOI: https://dx.doi.org/10.1111/lam.12182

Bulletin FIL-IDF (1983) Measurement of extraneous water by the freezing point test. Document 154. Brussels, Belgium.

Cempírková, R., Mikulová, M. (2009) Incidence of psychrotrophic lipolytic bacteria in cow´s raw milk. Czech Journal of Animal Science, 54 (2), 65-73. DOI: https://dx.doi.org/10.17221/1667-CJAS

Commission Regulation 2004/853/EC of 29 April 2004 Laying down specific hygiene rules for on the hygiene of foodstuffs.

Commission Regulation 2005/2073/EC of 15 November 2005 Microbiological criteria for foodstuffs.

Commission Regulation 2013/1308/EC of 17 December 2013 of the European Parliament and of the Council establishing a common organization of the markets in agricultural products and repealing.

Biokar diagnostics (2011) Compass *Enterococcus* Agar. Allone: Biokar diagnostics.

Dalzini, E., Bernini, V., Bertasi, B., Daminelli, P., Losio, M.N., Varisco, G. (2016) Survey of prevalence and seasonal variability of *Listeria monocytogenes* in raw cow milk from Northern Italy. Food Control, 60, 466-470. DOI: https://dx.doi.org/10.1016/j.foodcont.2015.08.019

Dobranić, V., Kazazić, S., Filipović, I., Mikulec, N., Zdolec, N. (2016) Composition of raw cow's milk microbiota and identification of enterococci by MALDI-TOF MS. Veterinarski arhiv, 86, 581–590. Available at: https://hrcak.srce.hr/166060

Doležalová, H., Pícha, K., Navrátil, J., Bezenková, A. (2014) Factors that influence the selling of milk through milk vending machines. Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis, 62 (4), 641-650. DOI: http://dx.doi.org/10.11118/actaun201462040641

European Food Safety Authority (EFSA) (2015) Scientific opinion on the public health risks related to the consumption of raw drinking milk. EFSA Journal, 13 (1), 3940. DOI: https://dx.doi.org/10.2903/j.efsa.2015.3940

Food Act (2018) The Official Gazette, No. 115. Zagreb. Available at: https://www.zakon.hr/z/1719/Zakon-o-hrani-2015-2018 (in Croatian)

Frank, J.F., Hassan, A.N. (2003) Microorganisms associated with milk. In: Roginski, H., Fuquay, J.W., Fox, P.F. eds. Encyclopedia of dairy sciences. Cambridge: Academic Press.

Giacometti, F., Serraino, A., Finazzi, G., Daminelli, P., Losio, M.N., Tamba, M., Garigliani, A., Mattioli, R., Zanoni, G.R., Riu, R. (2012a) Field handling conditions of raw milk sold in vending machines: Experimental evaluation of the behaviour of *Listeria monocytogenes*, *Escherichia coli* O175:H7, *Salmonella typhimurium* and *Campylobacter jejuni*. Italian Journal of Animal Science, 11, 132-136. DOI: https://dx.doi.org/10.4081/ijas.2012.e24

Giacometti, F., Serraino, A., Finazzi, G., Daminelli, P., Losio, M.N., Arrigoni, N., Piva, S., Florio, D., Riu, R., Zanoni, R.G. (2012b) Sale of raw milk in Northern Italy: Food safety implications and comparison of different analytical methodologies for detection of foodborne pathogens. Foodborne Pathogens and Disease, 9 (4), 293-297. DOI: https://dx.doi.org/10.1089/fpd.2011.1052

Giacometti, F., Bonilauri, P., Serraino, A., Peli, A., Amatiste, S., Arrigoni, N., Bianchi, M., Bilei, S., Cascone, G., Comin, D., Daminelli, P., Decastelli, L., Fustini, M., Mion, R., Petruzzelli, A., Rosmini, R., Rugna, G., Tamba, M., Tonucci, F., Bolzoni, G. (2013) Four-year monitoring of foodborne pathogens in raw milk sold by vending machines in Italy. Journal of Food Protection, 76 (11), 1902-1907. DOI: https://dx.doi.org/10.4315/0362-028X.JFP-13-213

Godic Torkar, K., Kirbiš, A., Vadnjal, S., Biasizzo, M., Galicic, A., Jevšnik, M. (2017) The microbiological quality of Slovenian raw milk from vending machines and their hygienic – technical conditions. British Food Journal, 119 (2), 377-389. DOI: https://dx.doi.org/10.1108/BFJ-06-2016-0291

GraphPad Software (2008) GraphPad Prism 5.00 for Windows. San Diego, CA: GraphPad Software. Available at: www.graphpad.com

Ministry of Agriculture, Fisheries and Rural Development of Republic of Croatia (2011) Guidance on microbiological food criteria. 3rd revised edition. Zagreb: Ministry of Agriculture, Fisheries and Rural Development of Republic of Croatia. Available at: https://edoc.site/mikrobioloki-vodi-pdf-free.html (in Croatian)

Hanuš, O., Vegricht, J., Frelich, J., Macek, A., Bjelka, M., Louda, F., Janu, L. (2008) Analysis of raw cow milk quality according to free fatty acid content in Czech Republic. Czech Journal of Animal Science, 53 (1), 17-30. DOI: https://dx.doi.org/10.17221/2717-CJAS

HRN EN ISO 11290-1:1999 Microbiology of the food chain — Horizontal method for the detection and enumeration of *Listeria monocytogenes* and of *Listeria* spp. - Part 1: Detection method. Zagreb: Croatian Standards Institute.

HRN EN ISO 10273:2003 Microbiology of the food chain - Horizontal method for the detection of pathogenic *Yersinia enterocolitica*. Zagreb: Croatian Standards Institute.

HRN EN ISO 6888-1:2004 Horizontal method for the detection and enumeration of coagulase-positive staphylococcus - Part 1: *Staphyloccocus aureus* and other species. Zagreb: Croatian Standards Institute.

HRN EN ISO 13366-2:2007 Milk - Enumeration of somatic cells - Part 2: Guidance on the operation of fluoro-opto-electronic counters. Zagreb: Croatian Standards Institute.

HRN EN ISO 21187:2008 Milk - Quantitative determination of bacteriological quality - Guidance for establishing and verifying a conversion relationship between routine method results and anchor method results. Zagreb: Croatian Standards Institute.

HRN EN ISO 5764:2010 Milk - Determination of freezing point - Thermistor cryoscope method (Reference method). Zagreb: Croatian Standards Institute.

HRN EN ISO 4833-1:2013 Microbiology of the food chain - Horizontal method for the enumeration of microorganisms - Part 1: Colony count at 30 °C by the pour plate technique. Zagreb: Croatian Standards Institute.

HRN EN ISO 6579-1:2017 Microbiology of the food chain - Horizontal method for the detection, enumeration and serotyping of *Salmonella* - Part 1: Detection of *Salmonella* spp. Zagreb: Croatian Standards Institute.

HRN EN ISO/IEC 17025:2007 General Requirements for the Competence of Testing and Calibration Laboratories. Zagreb: Croatian Standards Institute.

HRN ISO 16649-2:2001 Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of beta-glucuronidase-positive *Escherichia coli* - Part 2: Colony-count technique at 44 °C using 5-bromo-4-chloro-3-indolyl beta-D-glucuronide. Zagreb: Croatian Standards Institute.

HRS ISO/TS 11059:2009 Milk and milk products – Method for the enumeration of *Pseudomonas* spp. Zagreb: Croatian Standards Institute

HRN ISO 6730:2010 Milk - Enumeration of colony-forming units of psychrotrophic microorganisms - Colony-count technique at 6.5 °C. Zagreb: Croatian Standards Institute.

HRN ISO 9622:2017 Milk and liquid milk products - Guidelines for the application of mid-infrared spectrometry. Zagreb: Croatian Standards Institute.

Croatian Food Agency (2016) O javno zdravstvenom riziku vezanom za konzumaciju sirovog mlijeka. Znanstveno mišljenje. Osijek: Hrvatska Agencija za Hranu. Available at: https://www.hah.hr/wp-content/uploads/2015/10/znanstveno-misljenje-o-javno-zdravstvenom-riziku-vezanom-za-konzumaciju-sirovog-mlijeka-novo.pdf

Husu, J.R. (1990) Epidemiological Studies on the occurrence of *Listeria monocytogenes* in the feces of dairy cattle. Journal of Veterinary Medicine, 37, 276-282. DOI: https://dx.doi.org/10.1111/j.1439-0450.1990.tb01059.x

Italian Ministry of Health (2009) Ordinance by the Ministry of Health. Misure urgenti in materia di produzione, commercializzazione e vendita diretta di latte crudo per l'alimentazione umana. Rome, Italy: Italian Ministry of Health.

Jancsó, A. (2015) The practice of direct selling and the quality of directly sold raw bovine milk. Dissertation. Mosonmagyaróvár, Hungary: University of West Hungary Faculty of Agriculture and Food Science. Available at: http://doktori.nyme.hu/524/3/Jancso_Andras_angol_t.pdf

Kunová, S., Golian, J., Zeleňáková, L., Lopašovský, L., Čuboň, J., Haščik, P., Kačániová, M. (2017) Microbiological quality of fresh and heat-treated cow´s milk during storage. Potravinarstvo Slovak Journal of Food Sciences, 11 (1), 652-657. DOI: https://dx.doi.org/10.5219/799

Medvedova, A., Studeničova, A., Valik, L., Ladislav, O. (2013) Microbial and sensory quality of raw milk cheeses from the milk vending machines. Acta Chimica Slovaca, 6 (1), 49-54. DOI: https://dx.doi.org/10.2478/acs-2013-0009

Oliver, S.P., Boor, K.J., Murphy, S.C., Murinda, S.E. (2009) Food safety hazards associated with consumption of raw milk. Foodborne Pathogens Disease, 6 (7), 793-806. DOI: https://dx.doi.org/10.1089/fpd.2009.0302

Ordinance on the registration of entities and the registration and approval of entities in food operations (2015) The Official Gazette, No. 84. Zagreb.

Ordinance on the inspection of raw milk intended for public consumption (2016) The Official Gazette, No. 84. Zagreb.

Ordinance on the determination of the composition of raw milk (2017) The Official Gazette, No. 27. Zagreb.

Ordinance on milk and dairy products (2017) The Official Gazette, No. 64. Zagreb.

Reguillo, L., Hernandez, M., Barrientos, E., Perez-Rodriguez, F., Valero, A. (2018) Evaluation of the influence of frequency of milk collection and milking dayshift on the microbiological quality of raw milk. Journal of Food Quality, 1-7. DOI: https://dx.doi.org/10.1155/2018/1306107

Rossi, E.M., Barreto, J.F., Mueller, R., Cipriani, K., Biazussi, C., Valer, E., Tederke, J. Dorfey, D.P. (2018) Bacteriological quality of raw milk: a problem concerning many farmers. Food and Public Health, 8 (1), 1-7. DOI: https://dx.doi.org/10.5923/j.fph.20180801.01

Sabadoš, D. (1996) Kontrola i ocjenjivanje kakvoće mlijeka i mliječnih proizvoda. Zagreb: Hrvatsko mljekarsko društvo (Croatian Dairy Union).

Samaržija, D., Zamberlin, Š., Pogačić, T. (2012) Psychrotrophic bacteria and milk and dairy products quality. Mljekarstvo, 62 (2), 77-95.

Sørhaug, T., Stepaniak, L. (1997) Psychrotrophs and their enzymes in milk and dairy products: Quality aspects. Trends in Food Science and Technology, 8, 35-41.

Stetca, G., Ilea, A., Şuteu, L., Coldea, T. (2014) Raw milk hygiene at local markets and automatic milk dispenser machines. Bulletin UASVM Food Science and Technology, 71 (2), 161-164. DOI: https://dx.doi.org/10.15835/buasvmcn-fst:10822

Tremonte, P., Tipaldi, L., Succi, M., Pannela, G., Falasca, L., Capilongo, V., Coppola, R., Sorrentino, E. (2014) Raw milk from vending machines: effects of boiling, microwave treatment, and refrigeration on microbiological quality. Journal of Dairy Science, 97, 3314-3320. DOI: https://dx.doi.org/10.3168/jds.2013-7744

Vietoris, V., Zajác, P., Zubrická, S., Čapla, J., Čurlej, J. (2016) Comparison of total bacterial count (TBC) in bulk tank raw cow´s milk and vending machine milk. Carpathian Journal of Food Science and Technology, 8 (1), 184-191.

Zajác, P., Čapla, J., Vietoris, V., Zubrická, S., Čurlej, J. (2015) Effects of storage on the major constituents of raw milk. Potravinarstvo, 9 (1), 375-381. DOI: https://dx.doi.org/10.5219/518

Zdolec, N., Dobranić, V., Butković, I., Koturić, A., Filipović, I., Medvid, V. (2016): Antimicrobial susceptibility of milk bacteria from healthy and drug-treated cow udder. Veterinarski arhiv, 86, 163–172. Available at: https://hrcak.srce.hr/156704