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EXAMINATION OF THE PRESENCE OF CERTAIN SPECIFIC BACTERIA IN RAW COW MILK

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Abstract

The following research was conducted to evaluate the quality of raw cow's milk at the level of individual milk producers, which is considered the first step for the production of safe and high-quality milk. For testing raw milk for the presence of certain specific bacteria, were taken 45 samples of fresh raw cow's milk. Raw cow's milk samples are analyzed for the possible presence of certain specific pathogens such as E. coli, coagulase-positive staphylococci, Enterobacteriaceae, and Total Coliform bacteria. An increase in the total number of bacteria and the number of somatic and contamination with some specific pathogenic bacteria leads to a change in organoleptic markers in raw milk. The changes that occur in raw milk are conditioned by the number and type of microbiological agents. Most often these changes refer to just one mistake, a mistake in the taste, smell, aroma, or consistency of the dairy product. However, in cases of greater microbial contamination, all organoleptic errors can occur simultaneously. In addition, every slightest change due to the presence of microbiological causes of spoilage reduces the nutritional value of the product. Despite its importance for overall quality, control of spoilage microorganisms is not mandatory for the dairy industry, and the products are controlled by an insignificant number of producers. These tests aim to prove the presence of certain specific types of pathogenic bacteria in raw milk.

Keywords: raw milk, E. coli, coagulase-positive staphylococci, Enterobacteriaceae, and Total Coliform bacteria.

1. Introduction

Milk is a food with high nutritional value and an ideal environment for the growth of many microorganisms. Fresh milk is easily spoiled and becomes unsuitable for processing and human consumption (FAO, 2001). The milk obtained from the udder of healthy animals is free of pathogenic bacteria, but some animals in grazing conditions may suffer from subclinical mastitis and excrete the causative agents in milk, such milk contaminates most of the milk in the milk tank. In addition, raw milk can be contaminated by equipment, animal skins, the environment, or water used for cleaning, etc. (FAO, 2008). Foodborne illness is a widespread global problem. Considerable literature mentions some epidemics that have occurred as a result of consuming contaminated milk which according to appearance, taste, and smell, is completely normal, but is contaminated with several harmful bacteria (Centers for Control and Prevention of Disease, CDC, 2009). The milk contaminated with high levels of spoilage bacteria usually becomes unsuitable for further processing because it does not meet the consumer requirements in terms of nutritional value, hygienic quality, and sensory characteristics (Nanu et al., 2007).

Escherichia coli is an important conditionally pathogenic bacterium, widespread in the farm environment that causes inflammation of the udder, known as coli-mastitis (Hogan and Smith, 2003). The bacterium E. coli is the most common contaminant of raw and processed milk (Quinn et al., 2002). It is a reliable indicator of fecal contamination of water and food, such as milk and dairy products (Todar, 2008). The examined milk containing E. coli is considered hygienically and healthily defective. Dairy products obtained from raw milk contaminated with this bacterium must not be placed on the market for general consumption.

The presence of ***coliform bacteria*** in food of animal origin indicates environmental and fecal contamination, as these microorganisms are abundant in the food environment (Shojaei and Yadollahi, 2008). Coliform bacteria are the second group of Gram-negative bacteria that often contaminate raw milk. Their presence in milk indicates fecal contamination and contamination with soil and water. When coliform bacteria are present in large numbers they cause errors in the appearance and taste of milk due to the formation of gas and increased acidity. Typical representatives of this bacterial group are Escherichia, Enterobacter, Citrobacter, Klebsiella, Proteus, and Serratia spp. (Walker, 1988; Ledford, 1998). Because some species of coliform bacteria belong to the group of enteropathogenic bacteria, their presence in raw milk is potentially dangerous to human health (Desmarcheller and Fegan, 2002). A count of more than 100 CFU/ml of coliform bacteria in raw milk is an indicator of unsatisfactory hygiene practices (Chambers, 2002).

Coagulase-positive staphylococci are a group of bacteria that can be found in contaminated raw milk. Detection of bacteria in milk samples is evidence of mammary gland inflammation or mastitis. In addition to S. aureus, S. intermedius and S. hyicus have been shown to cause mastitis in recent years. S. aureus is one of the major causes of foodborne illness in humans. The bacterium S. aureus is most associated with intoxication due to its ability to produce a variety of potent enterotoxins (Balaban and Rasooly, 2000; Le Loir et al., 2003). Identical strains of S. aureus are occasionally isolated from dairy cows and milking cows (Jorgensen et al., 2005a), but strains derived from bovine mastitis generally represent a genetically different group than human strains, indicating host specificity (Van Leeuwen et al., 2005).

Enterobacteriaceae or intestinal bacteria are part of the normal microflora of the digestive system in humans and animals. Enterobacteriaceae within good manufacturing practice are used as indicators of the efficiency of plant remediation and as indicators of the efficiency of the heat treatment process (Baylis et al., 2011). Enterobacteriaceae are good indicators of the adequacy of the production process at the end of the production process but are not reliable in assessing the correctness of the product during the shelf life. The reason lies in the fact that some Enterobacteriaceae can multiply in food under refrigeration conditions, so their established number does not necessarily reflect the initial contamination or indicate an inappropriate storage temperature.

2. Material and methods

2.1. Examination material: To examine the presence of certain specific bacteria in raw cow's milk a total of 45 samples were taken from farmers, to determine the origin of the microbiological contamination in the milk. Samples were taken over a period of three consecutive months (December, January, and February). Samples for examination were taken to identify specific microorganisms in raw milk (Escherichia coli, coagulase-positive staphylococci, bacteria of the genus Enterobacteriaceae, and total coliform bacteria).

Samples intended for examination of specific microorganisms in milk were taken as follows: milk samples were taken under aseptic conditions before regular milking. The udder was first washed with lukewarm soap and water, especially the teat, and wiped clean with a clean cloth. Disinfection of the tops of the teat was performed with cotton wool soaked in 70% ethanol. First, the more distant quarters were disinfected, then the closer ones. Three streams of milk were squeezed from each quarter of the udder before sampling. After disinfection, milking was performed in special sterile plastic cups with a quantity of 60 ml. After sampling, samples of fresh raw milk were placed in a hand-held refrigerator (temperature 4°C) and transported directly to an authorized laboratory for bacteriological examination. Raw milk samples were stored at 4°C until the beginning of the laboratory examination.

2.2. Methods for the examination of raw milk samples: The samples intended for the determination of specific bacteria were analyzed in the laboratory "ANIMA-VET" which is accredited by the standard ISO 17025/2005 by the Accreditation Institute of the Republic of Macedonia (IARM) with certificate number LT - 033. The samples were analyzed after internationally recognized ISO standards, as follows:

1. Counting of E. coli according to the standard MKS ISO 16649-2:2008. A horizontal method is used to count β-glucuronidase-positive Escherichia coli and ISO 16649-1. Colony counting technique at 44°C using 5-bromo-4-chromo-3-indolyl β -glucuronide is used.
2. Coagulase-positive staphylococci will be counted according to the standard MKS EN ISO 6888-2/A1:2008. A horizontal method is used to count coagulase-positive staphylococci (aptaphylococcus aureus and other species). Agar medium with rabbit plasma fibrinogen will be used for the growth of these bacteria.
3. Counting of Enterobacteriaceae will be realized by the standard ISO 21528-2:2004, using horizontal methods for detection and counting of Enterobacteriaceae - method of counting colonies.
4. Determination of total coliform bacteria will be performed by the ISO 4832:2006 standard with a horizontal method for counting coliforms - colony counting technique.
The results of the research will be tabulated and will be processed according to statistical methods applied in the scientific research field.

3. Results and discussion

3.1. Examination of specific pathogenic bacteria in raw milk: To determine the degree of contamination of raw milk with specific pathogenic bacteria as well as to determine the causes of that contamination, samples were taken for analysis from milk producers.
Table 1 shows the results of the statistical processing of certain specific microorganisms.
The arithmetic mean of ***Escherichia coli*** colonies in milk in the analyzed samples was $\bar{x}$=82.55 CFU/ml, where the minimum and maximum values ranged from 0 to 1,230 CFU/ml. The standard deviation of E. coli colonies in raw milk is SD = 220.93 CFU/ml, while the coefficient of variation is very high and CV = 267.62%.
Coagulase-positive staphylococci in raw milk in the analyzed samples ranged from 0 to 70,000 CFU / ml, with an average mean of $\bar{x}$=2,690.44 CFU/ml. The standard deviation for bacterial colonies of positive staphylococci was SD = 10,952.52CFU/ml colonies in milk. While the coefficient is the highest, compared to other specific bacteria, and is CV = 407.09%.
From the results shown in Table 1, Enterobacteriaceae in raw milk ranges from 100 CFU/ml to 4,240,000 CFU/ml. The arithmetic mean of the raw milk test for the number of Enterobacteriaceae in raw milk was $\bar{x}$ = 2.650x103 CFU/ml, with a standard deviation of SD = 682,291.81 CFU/ml and coefficient of variation of CV = 257.46%.
The average value of the total ***coliform bacteria*** in the examined samples is $\bar{x}$=600,295.55 CFU/ml, while the minimum and maximum values are 900 CFU/ml and 5,140,000 CFU/ml, respectively, with a standard deviation of SD = 1,187,986, 91 CFU/ml and a coefficient of variation of CV = 198.90% which was the lowest of all specific bacteria examined.

Table 1. Tabular presentation of the analyzed specific bacteria in raw milk (N = 45)

Type of microorganisms	Examined parameters				
	Min	Max	x̄	SD	CV
E. coli (CFU/ml)	0	1.230	82,55	220,93	267,62%
Coagulase positive staphylococci (CFU/ml)	0	70.000	2.690,44	10.952,52	407,09%
Enterobacteriaceae (CFU/ml)	100	4.240.000	265.012,67	682.291,81	257,46%
Total coliform bacteria (CFU/ml)	900	5.140.000	600.291,55	1.187.986,91	198,90%

According to the obtained results shown in Table 2, it can be seen that out of 45 examined samples, in 20, or expressed in percentage 44% of the samples, no bacterial colony of the type E. coli was found. A small number of bacterial colonies were detected in 18 samples or 40%. Bacterial colonies >100 CFU/ml were found in 7 samples or expressed as a percentage of 15%. In total, 38 samples of fresh raw milk meet the hygienic criteria set by the Rulebook on special requirements relating to microbiological criteria for food (Official Gazette of RM, no. 100/2013).

Table 2. Tabular representation of the number of E. coli colonies (CFU/ml) shown by categories

E. coli CFU/ml	**Number of samples (N=45)**	**Percent (%)**
0	20	44,44 %
from 10 – 100	18	40,00 %
over 100	6	13,33 %
over 1000	1	2,22 %

From the results shown in Table 3 in 23 samples of raw milk, no bacterial colony was found, while 16 samples did not exceed the number of bacteria according to the Rulebook, while 6 samples contained bacterial colonies more than the allowed limits prescribed by the additional requirements for raw milk. The total number of copies that meets the criteria of the rulebook is 39 copies. Raw cow milk according to the rulebook from the special requirements of the "Official Gazette of RM" 26/2012, should additionally meet the requirements for the presence of Staphylococcus aureus/ml in raw milk. However, in our case, the results obtained are considered satisfactory because the number of bacteria does not exceed the permissible limits of 500 CFU/ml in 39 analyzed samples. In addition, 6 samples of raw milk have unsatisfactory results because it exceeds the maximum value of 2,000 CFU/ml in more than one sample of raw milk. In two samples of raw milk, the number of bacteria was determined between the minimum value for the number of bacteria (m = 500 CFU/ml) and the maximum value for the number of bacteria (M = 2000 CFU/ml).

Table 3. Tabular representation of the number of colonies of coagulase-positive staphylococci (CFU / ml) shown by categories

CFU/ml of coagulase-positive staphylococci	Number of samples (N=45)	Percent (%)
From. 0 – 500	39	86,66 %
From. 501 - 2000	2	4,44 %
Over. 2000	4	8.88 %

Table 4 shows the results of the analyzed samples for the presence of Enterobacteriaceae in raw cow's milk. According to the obtained results in terms of the number of Enterobacteriaceae, it can be concluded that all samples contain a large number of bacterial colonies, that is, the number of colonies ranges from 100 CFU/ml to 4.24 x 104 CFU/ml.

Table 4. Tabular representation of the number of colonies of Enterobacteriaceae (CFU / ml) shown by categories

CFU/ml на Enterobacteriaceae	Number of samples (N=45)	Percent (%)
From. 101 - 1000	6	13,33 %
From. 1.001 – 10.000	9	20,00 %
From. 001 – 100.000	17	37,78 %
From. 100.001 – 1.000.000	9	20,00 %
Over. 1.000.000	4	8,89 %

Table 5 shows the results of the examined samples for the presence of total coliform bacteria in raw cow's milk. According to the obtained results in terms of the number of coliform bacteria, it can be concluded that all samples do not meet the criteria of hygienic correctness, because they are contaminated with a large number of bacterial colonies, starting from 900 CFU/ml, up to the maximum number of colonies in the sample which is 5.14x104 CFU/ml.

Table 5. Tabular representation of the number of coliform bacterial colonies (CFU/ml) shown by categories

CFU/ml of total coliform bacteria	Number of samples (N=45)	Percent (%)
To. 1.000	1	2.22 %
From. 1.001 – 10.000	9	20,00 %
From. 10.001 – 100.000	16	35,56 %
From. 100.001 – 1.000.000	11	24,44 %
From. 1.000.001 – 10.000.000	8	17,78 %

4. Discussion

In determining the total number of Escherichia coli colonies in milk (Table 2), we found that 25 samples (55.56%) had more than 10 colonies per ml of milk. According to Hogan and Smith, (2003), E. coli is an important conditionally pathogenic bacterium, widespread in the farm environment that causes inflammation of the udder, known as cold mastitis. Quinn et al. (2002) also found that E. coli is the most common contaminant in raw and processed milk. It is a reliable indicator of fecal contamination of water and food, such as milk and dairy products (Todar, 2008). The examined milk containing E. coli is considered hygienically and healthily defective. E. coli is a pathogen isolated from raw milk. It reaches due to fecal contamination as a result of unhygienic milking. However, it is also isolated in pasteurized milk products, which have subsequently been contaminated (Keene et al. 1997).

When determining ***coagulase-positive staphylococci*** in raw milk in the examined samples, we found that most (86.66%) contain from 0 - 500 CFU / ml (Table 3). Coagulase-positive staphylococci are a group of bacteria that can be found in contaminated raw milk. Staphylococci are among the most common causes of bacterial infections in humans, and Staphylococcus aureus is the most important pathogen of staphylococci. In veterinary medicine, 3 types of staphylococci are important as pathogens of mastitis: S. aureus, S. intermedius, and S. hyicus (Devriese, 1990). S. aureus and S. intermedius are coagulase-positive, while coagulase formation is variable in S. hyicus, mostly negative or weakly positive (Kloss and Schleifer, 1986).

Staphylococcus aureus causes serious disease in humans and animals and is the most common etiological agent of infectious bovine mastitis (Omoe et al., 2002). According to researchers Balaban and Rasooly (2000) and Le Loir et al. (2003), S. aureus is most commonly associated with intoxications due to its ability to produce a variety of potent enterotoxins. Identical strains of S. aureus are occasionally isolated from dairy cows and milking hands (Jorgensen et al., 2005a), but strains derived from bovine mastitis represent a genetically different group than human strains, indicating host specificity (Van Leeuwen et al., 2005).

According to the obtained results of the total number of bacterial colonies of *Enterobacteriaceae* in raw milk, it was determined that all the examined samples had the presence of these microorganisms (Table 4). The presence of *Enterobacteriaceae* in milk is an indicator of fecal contamination, respectively insufficient hygiene during milk production, storage, and handling. Milk in which the presence of enterobacteria has been found is considered hygienic and unhealthy. Isolated by microbial swabs from surfaces, staff hands and utensils indicate fecal contamination and insufficient cleaning, washing, and disinfection (Ewing, 1986). According to Baylis et al., (2011) Enterobacteriaceae are used within good manufacturing practice as indicators of plant remediation efficiency and as indicators of the efficiency of the heat treatment process. Enterobacteriaceae make up 5-33% of the psychotropic microflora present in raw milk. Their optimum growth temperature (> 30°C) tends to be higher than that of pseudomonas, but they adapt well to cooling temperature growth (Muir, 1996a). However, Enterobacteriaceae can sometimes, in other circumstances, remain dominant at storage temperatures above 8 ° C (Varnam & Sutherland, 2001). Species of the genera Enterobacter and Klebsiella are most commonly associated with coliform decay, while Escherichia spp. only occasionally show sufficient growth to produce a defect (Frank, 1997).

The minimum number of bacterial colonies of total coliform bacteria in raw milk in the tested samples was min = 900CFU / ml, while the maximum number n was max = 5.140.000CFU / ml.

Table 5 presents the results of the examined samples for proving the total coliform bacteria in raw cow's milk. According to the obtained results in terms of the number of coliform bacteria, it can be concluded that not all samples meet the criteria of hygienic correctness. According to Shojaei and Yadollahi, (2008), the presence of coliform bacteria in food of animal origin indicates environmental and fecal contamination, as these microorganisms are often present in the food environment. Coliform bacteria are the second group of Gram-negative bacteria that often contaminate raw milk. When coliform bacteria are present in large numbers, they lead to defects in sensory characteristics, such as changes in the appearance and taste of milk due to gas

production and increased acidity (Samaržija et al., 2007). Typical representatives of this bacterial group are Escherichia, Enterobacter, Citrobacter, Klebsiella, Proteus, and Serratia spp. (Walker, 1988; Ledford, 1998). Because some species of coliform bacteria belong to the group of enteropathogenic bacteria, their presence in raw milk is potentially dangerous to human health (Desmarcheller and Fegan, 2002). Compared to psychotropic bacteria, coliform bacteria in raw chilled milk grow more slowly, but still significantly better than many other bacteria present in milk (Samaržija, 2012). Chambers, (2002) considers that the number of more than 100 CFU / ml of coliform bacteria in raw milk is an indicator of unsatisfactory hygienic practice. This contamination is the result of additional contamination in the process of milking, collecting, and storing raw milk. Coliform bacteria can produce biofilm and their appearance indicates unhygienic milk production (Moatsou and Moschopoulou, 2014).

5. Conclusion

Based on the results obtained from the examined raw milk samples, we can conclude the following:

1. With E. coli and coagulase-positive staphylococci there is a small percentage of samples containing these bacteria, while in terms of Enterobacteriaceae and total coliform bacteria all samples (45) show high contamination of raw milk with these bacteria.
2. Of the total samples examined (45) for the determination of the total number of bacterial colonies of E. coli, only 7 samples have a higher number of bacterial colonies in raw milk than allowed, while 38 samples of fresh raw milk meet the hygienic criteria set by the standard.
3. Coagulase-positive staphylococci in the examined samples were found in a small number of samples, which indicates that raw milk is derived from cows that do not have subclinical mastitis. Out of a total of 45 samples, 39 are below the minimum threshold according to the regulation for additional requirements for staphylococci. A total of 6 samples show a sign of contamination of raw milk more than allowed.
4. Demonstration of Enterobacteriaceae has a purpose to prove whether raw milk is contaminated or not, as they are a large group of bacteria with many representatives. In 45 samples examined in the laboratory, no sample meets the criteria for hygienic correctness of raw milk.
5. According to the obtained results n terms of the number of coliform bacteria, it can be concluded that all samples do not meet the criteria of hygienic safety of the examined milk, because they are contaminated with a large number of bacterial columns, starting from 900 CFU/ml, up to the maximum number of colonies in the sample which is 5.14×104CFU/ml.
6. As a general conclusion it can be mentioned that the contamination of raw milk with microorganisms comes as a result of improper handling of milk after milking, poor milking hygiene, conditions for transport, storage and storage of raw milk, and most importantly the lack of milk storage milk freezers at several individual milk producers.

References

[1]. Balaban, N., Rasooly, A. 2000. Staphylococcal enterotoxins. International Journal of Food Microbiology 61: 1–10.
[2]. Baylis, C., Uyttendaele, M., Joosten H., Davies A. (2011). Enterobacteriaceae and their significance to the food industry.
[3]. Centers for Disease Control and Prevention, CDC. 2009. Surveillance for foodborne disease outbreaks. The United States, 2006, Morbidity Mortality Weekly Rep. 58: 609-615.
[4]. Chambers, J. V. (2002). The microbiology of raw milk. In R. K. Robinson (Ed.), Dairy microbiology handbook: The microbiology of milk and milk products, (3rd ed) (pp. 39–91). John Wiley and Sons, Inc. New York, USA.
[5]. Desmarcheller, P., Fegan, N. (2002): Eescherichia coli. Encyclopedia od Dairy Science, Vol. 4, Edited by Roginski, H., Fuquay W.J., Fox, F.P., Academic Press, 948-954.

[6]. FAO 2001. The lactoperoxidase system of milk preservation. Regional Lacto-peroxidase Work-shop in West Africa; Burkina Faso: 17-19.
[7]. FAO 2008. Milk Hygiene, In Milking, Milk Production Hygiene and Udder Health, FAO Animal Production and Health Papers-78, FAO Corporate Document Repository 1-7 pp.
[8]. Hogan, J., Smith, K.L., 2003. Coliform mastitis. Vet.Res. 34, 507-519.
[9]. Jørgensen, H.J., Mørk, T., Caugant, D.A., Kearns, A., Rørvik, L.M., 2005a. Genetic variation among Staphylococcus aureus strains from Norwegian bulk milk. Applied and Environmental Microbiology 71, 8352–8361.
[10]. Le Loir, Y.; Baron, F., Gautier, M. 2003. Staphylococcus aureus and food poisoning. Genetics and Molecular Research 2, 63–76.
[11]. Ledford, R.A. (1998): Raw Milk and Fluid Milk Products. Applied Dairy Microbiology 41. 55-64.
[12]. Nanu, E., Latha, C., Sunil, B., Thomas, M., Venon, K.V. 2007. Quality assurance and public health safety of raw milk at the production point. American Journal of Food Technology 2: 145–152.
[13]. Quinn, P.J., Carter, M.E., Markey, B., Carter, G.R.2002. Clinical veterinary microbiology. Grafos, Spain.
[14]. Shojaei, Z.A. and Yadollahi, A. (2008): Physicochemical and microbiological quality of raw, pasteurized and UHT milk in shops. Asian J. Sci. Res. 1: 532-538.
[15]. Todar, K. 2008. Pathogenic E. coli. Available http://ww.textbookofbacteriology .net/e. coli.him accessed on: 20th October, 2009.
[16]. van Leeuwen WB, Melles DC, Alaidan A et al. (2005). Host-and tissue-specific 491 pathogenic traits of Staphylococcus aureus. J Bacteriol 187: 4584-4591.
[17]. Walker, S.J. (1988): Major Spoilage Microorganisms in Milk and Dairy Products. Journal of the Society of Dairy Technology 41, 91-92.