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Research Article

The prevalence of bacterial contaminants in artisanal cheese sold in informal markets. The case of Kosovo

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Abstract

The aim of this study was to examine the prevalence of bacterial contaminants (*Escherichia coli, Staphylococcus aureus, Listeria monocytogenes and Salmonella* spp.) in 116 artisanal cheese samples sold in informal markets of Kosovo. The results provide evidence for a presence of the aforementioned contaminants in the artisanal cheese, with levels of positive samples of 64.7%, 39.7% and 3.4% for *Escherichia coli, Staphylococcus aureus* and *Listeria monocytogenes*, respectively. *Salmonella* spp. could not be detected. It also could be shown that the contamination of the cheeses with *Escherichia coli* and *Staphylococcus aureus* was higher during the summer season with *Staphylococcus aureus* counts up to 10^6 CFU/g, a level sufficient for the potential production of enterotoxins. The number of samples contaminated with *Listeria monocytogenes* was, however, higher during the winter season. Microbiological analyses relied on the ISO standards methods, an automated Enzyme Linked Fluorescent Assay for detection of *Listeria monocytogenes* was used in addition. The findings of this study strongly suggest that institutions responsible for public health, need to increase attention and control measures in order to improve the safety of foods sold in informal markets, particularly with regard to artisanal cheese which is a frequently consumed product.

Keywords: artisanal cheese, bacterial contaminants, informal market

Abbreviations CPS – coagulase-positive staphylococci; ELFA – enzyme linked fluorescent assay; GHP – good hygienic practices; GMP – good manufacturing practices; SPSS – statistical package for social sciences; VIDAS – vitek immuno diagnostic assay system

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Introduction

Considering the widespread consumption of artisanal dairy products around the world, there is a growing concern regarding their safety and microbiological quality. This is especially the case with countries, such as Kosovo, where the production process of artisanal cheeses, which are most often white soft or semi-soft cheeses, is often based on traditional methods passed from generation to generation with minimal precautions regarding food safety standards. In general, homemade, or artisanal cheeses sold in informal markets in Kosovo are produced from unpasteurized milk or milk which is only partially pasteurized. The most frequent source of cheese contamination comes from raw, insufficiently pasteurized milk or post-pasteurization contamination (Little et al. 2008). In Europe, most outbreaks related to cheese consumption are associated with the presence of *Salmonella* spp., *Staphylococcus aureus* (*S. aureus*), *Listeria monocytogenes (L. monocytogenes)*, and *Escherichia coli (E. coli)* (Little et al., 2008; Guzman-Hernandez et al. 2016). Thus, the present paper aimed on examining the potential prevalence of these bacterial contaminants in artisanal cheese purchased from informal shops in Kosovo, as well as the factors associated with the contamination of this type of cheese by these bacteria.

Salmonella spp. are mainly transmitted by food and represented the number one cause of foodborne outbreaks in the EU in 2018, with 9600 human cases and 2227 hospitalizations (Costanzo et al. 2020) being reported. In the United States of America, they represented the second major cause of foodborne disease and were responsible for most hospitalizations and deaths (Scallan et al. 2011). Contamination of raw milk with *Salmonella* occurs mainly by fecal wastes of the animals (Heredia et al. 2018). Although infected people mostly show gastrointestinal symptoms, manifested as mild food poisoning symptoms, some patients experience more serious health conditions (Chiu et al. 2004). In addition, antibiotic resistant strains of *Salmonella* are still a topic of major concern (Eng et al. 2015).

S. aureus is a gram-positive bacterium very common in both humans and animals. Many foodborne outbreaks are reported to be associated with consumption of cheese contaminated with *S. aureus* (De Buyser et al. 2001; Hummerjohann et al., 2014). Healthy adults are a main reservoir of this microorganism, therefore contamination during the processing and handling of food may occur (Jorgensen et al. 2005; Kerouanton et al. 2007; Sakwinska et al. 2011). In addition, animals with clinical or subclinical mastitis can spread staphylococci through their milk (Pexara et al. 2016). During cheesemaking, *S. aureus* can multiply considerably (Medveďová et al. 2006) and numbers of *Staphylococcus aureus (S. aureus)* in cheese up to 10^7 CFU/g or higher must be considered as a potential risk for the production of enterotoxins (Delbes et al. 2006).

L. monocytogenes is a ubiquitous microorganism which can cause serious disease, particularly immunocompromised people as well as pregnant women being at high risk. Listeriosis in humans is mainly acquired by eating contaminated food which makes it a serious public health concern (Mead et al. 1999; Boggs et al. 2001; Lunden et al. 2004; Costanzo et al. 2020). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food- borne outbreaks in 2017 (EFSA) states that there were 1635 human listeriosis cases associated with food-borne outbreaks and a prevalence rate of 0.9% in soft and semisoft cheeses made from raw or low-heat treated milk and 0.1% in hard cheeses made from raw or low-heat-treated milk.

E. coli is part of normal microbiota in the digestive tract of humans and warm-blooded animals (Bélanger et al. 2005), but the presence of *E. coli* in foods, especially cheese, suggests either that the milk has not been pasteurized or mishandled during or after the production. In addition, pathogenic strains of *E. coli* like O157:H7 are associated with many outbreaks due to consumption of contaminated cheese (Gaulin et al. 2012; Langer et al. 2012; McCollum et al. 2012), particularly if made from raw milk (Stephan et al. 2008).

In general, impure equipment used in production, lack of personal hygiene during and after the production as well as lack of heat treatment of milk, influence the presence and level of pathogenic microorganisms in cheese (El-Kholy et al. 2014). Therefore, all the pathogens mentioned above tend to be a threat to human health, if precautions such as those based on GMP (Good manufacturing

practice) and GHP (Good hygienic practices) are not taken into consideration. Beside these practices, there seem to be other external factors influencing the rate of contamination. For instance, according to Oliver et al. (2005), among other factors, the rate of contamination in milk is greatly influenced by the season of the year. That is, during summer when the temperatures and humidity index are higher there is a higher tendency for the dairy animals to deal with mastitis- which is the inflammation of the udder, resulting in greater shed of milk borne pathogens (Morse et al. 1988).

While many studies have examined the prevalence of such pathogens in cheese, and specifically in artisanal cheese (De Buyser et al. 2001), there is a paucity of research when it comes to studies examining the issue at hand in Kosovo. It is important to conduct research in Kosovo, especially knowing the high demands for this type of cheese imposed by the population in this country, which highlights the importance of sufficient control measures. Like it was previously mentioned, most of the cheeses for sale in the informal market, are limitedly controlled by the relevant institutions, that are responsible for public health. Therefore, the first aim of the current paper was to determine the prevalence of bacterial contaminants (*E. coli, S. aureus, L. monocytogenes and Salmonella spp*.) in artisanal cheeses sold in local informal markets based in Kosovo.

Furthermore, the second aim of this paper was to evaluate the effect of the season of cheese production (i.e. summer vs. winter) as well as origin of the milk (i. e. *cow, sheep, goat*) on the level of microbiological contamination in artisanal cheese.

An important issue in improving food safety is the availability of assays providing rapid and reliable results. Particularly the long time needed from the taking the sample to providing the result using the ISO 11290-1:2017 method for *L. monocytogenes* (Bernardi et al. 2015) forced the development of other methods like Enzyme Linked Fluorescent Assay (ELFA), which currently is a convenient, fast, highly sensitive and specific method for the detection of *L. monocytogenes* in foods (Ueda and Kuwabara 2010). Therefore, the last aim of this study was to compare the results from the two standard methods, that is - ISO 11290-1:2017 and VIDAS LMO2 (AFNOR nº BIO-12/11-03/04), for the detection of *L. monocytogenes*.

Materials and Methods

Sample collection. One-hundred and sixteen cheese samples were collected in open-air markets (also known as informal markets) located in the six largest cities of Kosovo – namely, Peja, Prishtine, Prizren, Gjilan, Gjakove and Mitrovice. The collection and analyses of cheese samples has been done during summer season (57 samples) and during the winter season (59 samples). Cheese samples were identified by ID number, date of collection, origin of cheese (*cow, sheep, goat*) and the name of city where it was taken. Samples (300 – 400 g each), were placed in sterile plastic bags (*WHIRL pack)*, added into containers with ice and sent to the laboratory within two hours after the collection. Analysis was done within twenty-four hours.

Table 1. Distribution of samples based on season and the origin of cheese milk

Origin of the cheese milk	Number of samples		
	Summer	Winter	TOTAL
Cow	19	26	45
Sheep	21	14	35
Goat	17	19	36
Total	57	59	116

Microbiological and immunological analyses. For each cheese sample, the following ISO methods were used:

Horizontal method for the enumeration of *coagulase-positive staphylococci* was applied according to ISO 6888-2/99.

Horizontal method for the enumeration of *beta-glucuronidase-positive E. coli* was applied according to ISO 16649-2:2001.

Horizontal method for the detection of *Salmonella* spp. was applied according to ISO 6579:2002.

Horizontal method for the detection of *L. monocytogenes* was applied according to ISO 11290-1:2017.

The detection of the *L. monocytogenes* in cheese samples was done also by Enzyme Linked Fluorescent Assay method (ELFA) using VIDAS LMO2 kit (AFNOR n° BIO-12/11-03/04) in the automated fluorescent immunoassay instrument (miniVIDAS-bioMerieux). For pre-enrichment 25 g of cheese sample, which were mixed and homogenised with 225 ml Half-Fraser broth, were incubated for 24-26 hours at 30±1°C. After the pre-enrichment, subculturing took place, where 0.1 ml of the solution was placed in 10 ml Fraser broth and incubated for 24-26 hours at 37±1°C. After subculturing, a 500 µl (0.5 ml) aliquot of unheated Fraser broth was placed in the miniVIDAS (Vitek Immuno Diagnostic Assay System) using LMO2 kit. Samples found to be positive using the LMO2 kit, were confirmed using isolation on chromogenic media from Ottaviani Agostini Agar. Blue colonies with halo after the 24 hours of incubation at 37±1°C indicated the presence of *L. monocytogenes*.

Statistical analyses. Results obtained from the microbiological and immunological tests were analysed with the Statistical Package for Social Sciences (SPSS), using chi-square testing (*L. monocytogenes and Salmonella spp*.) and ANOVA (*Coagulase-positive staphylococci and Beta-glucuronidase-positive E. coli*).

Results and Discussion

Coagulase-positive staphylococci (CPS). The results showed that from a total of 116 samples, 46 (39.7%) contained CPS. Furthermore, the highest score of CPS was 7.1×10^6 CFU/g, whereas the lowest score was 4.9×10^2 CFU/g.

To compare the samples on the level of contamination depending on the season (*summer* vs. *winter*) an independent sample t-test was performed. The analyses showed a significant difference between the two seasons t (114) = 2.63, $p < 0.05$, where higher scores of contaminations were present during the summer (M = 543817.54, SD = 1543826.07) as compared to the winter season (M = 13401.01, SD = 76025.67). To compare the samples on the level of contamination depending on the animal category from which the cheese was derived (*cow, sheep, goat*) a one-way ANOVA test was performed with CPS contamination as a dependent variable and animal category as an independent variable. The results show a non-significant difference between the animal categories F (2,113) = 1.49, p = 0.229, $\eta^2 = 0.02$. To examine the interaction between the season and the animal category, a two-way ANOVA was conducted with season and animal category as independent variables and CPS level of contamination as a dependent variable, however, the results showed a non-significant interaction F (2,110) = 1.29, p = 0.278, $\eta^2 = 0.02$.

Furthermore, a bivariate Pearson correlation was conducted for CPS *and Beta-glucuronidase-positive E. coli*, to estimate the relationship between them. The results showed a significant positive correlation r (114) = .23, $p < 0.05$.

Beta-glucuronidase-positive *Escherichia coli.* The results showed that from a total of 116 samples, 75 (64.7%) were positive for *beta-glucuronidase-positive E. coli*. Furthermore, the highest score of *beta-glucuronidase-positive E. coli* was 7.9×10^6 CFU/g, whereas the lowest score was 8.1×10^2 CFU/g.

To compare the level of contamination depending on the season (*summer* vs. *winter*) an independent sample t-test was performed. The analyses showed a significant difference between the two seasons t (114) = 2.51, $p < 0.05$, where higher scores of contaminations were present during the summer (M = 357254.56, SD = 1059125.76) as compared to the winter season (M = 10765.08, SD =23861.74). To compare the samples on the level of contamination depending on the animal category from which the cheese was derived from (*cow, sheep, goat*) a one-way ANOVA test was performed with *beta-glucuronidase-positive E. coli* contamination as a dependent variable and animal category as an independent variable. The results showed a non-significant difference between the animal categories F (2,113) = 0.91, p = 0.402, $\eta^2 = 0.01$. To examine the interaction between the season and the animal category, a two-way ANOVA was conducted with season and animal category as independent variables and level of *beta-glucuronidase-positive E. coli* contamination as a dependent variable, however, the results showed a non-significant interaction F (2,110) = 1.42, p = 0.245, $\eta^2 = 0.02$.

***Listeria* monocytogenes.** The results from both analytical methods, ISO 11290-1:2017 and AFNOR nº BIO-12/11-03/04 indicated that from a total of

116 samples, 4 (3.4 %) of them were positive for *L. monocytogenes*. To compare the number of positive samples with *L. monoctyogenes* depending on the season (*summer* vs. *winter*), a chi-square test of independence was performed. The analyses showed a significant relation between *season* and *L. monocytogenes* X^2 (1, N = 116) = 4.01, $p < 0.05$, where *L. monocytogenes* were more likely to be present in the winter season (6.8% positive, 93.2% negative) as compared to the summer season (0% positive, 100% negative). To compare the number of positive samples with *L. monoctyogenes* depending on the animal category from which the cheese was derived from (*cow, sheep, goat*), a chi-square test of independence was performed. The results showed a significant interaction between animal category and *L. monocytogenes* X^2 (2, N = 116) = 6.53, $p < .05$, where *L. monocytogenes* was more present in cows (8.9%) as compared to *sheep and goat* (both 0%). Lastly, same as by ISO 11290-1 method, the automated miniVIDAS assay (LMO2 kit) showed the same sensitivity and specificity.

Table 2. Listeria monocytogenes results based on "AFNOR nº BIO-12/11-03/04" method

Origin of the cheese milk	Number of samples		Positive samples w/L.Monocytogenes VIDAS LMO2 (AFNOR n° BIO-12/11-03/04)	
	Summer	Winter	Summer	Winter
Cow	19	26	0	4
Sheep	21	14	0	0
Goat	17	19	0	0
total	57	59	0	4
Grand total	116		4	

***Salmonella* spp.** The results showed that from a total of 116 samples, all of them were negative for the presence of *Salmonella* spp.

The overall results for the presence of bacterial contaminats examined in this study are presented in Fig. 1 while the results for the level of contamination of the samples with CPS and *E. coli* are presented in Fig. 2.

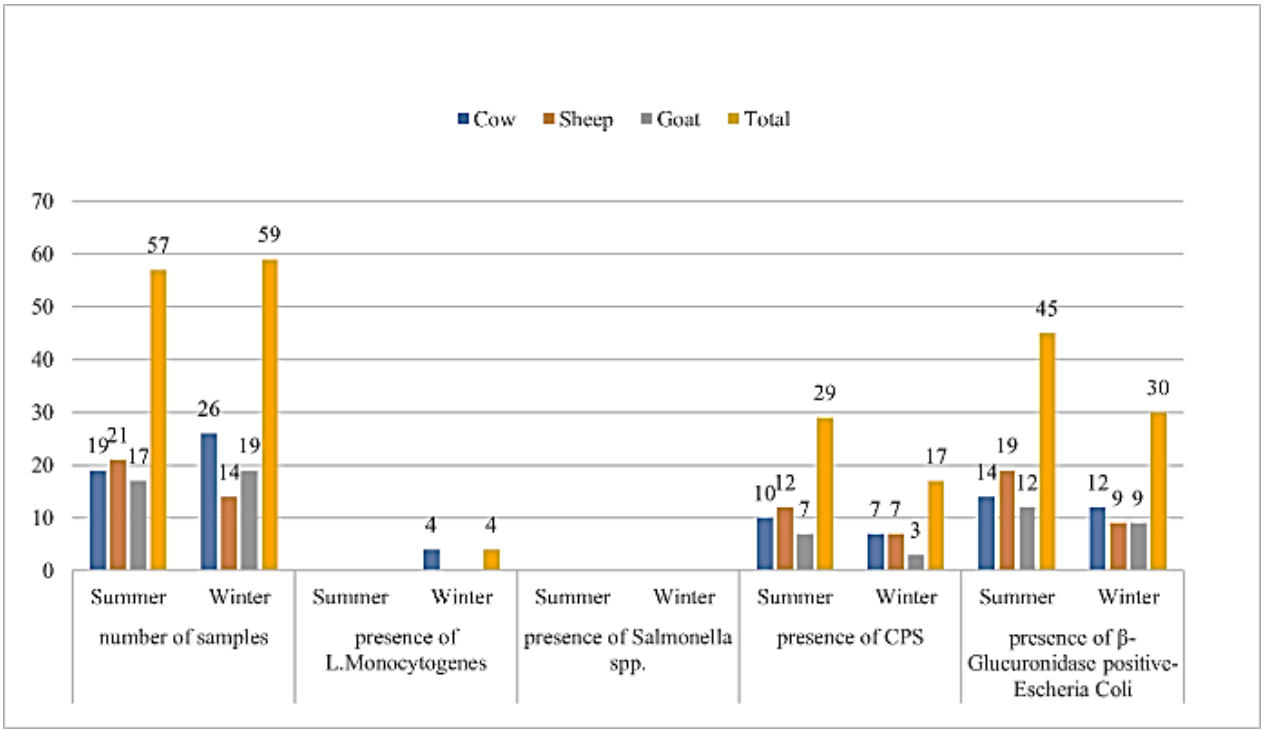

Figure 1. Presence of pathogenic bacteria according to the season and origin of cheese milk

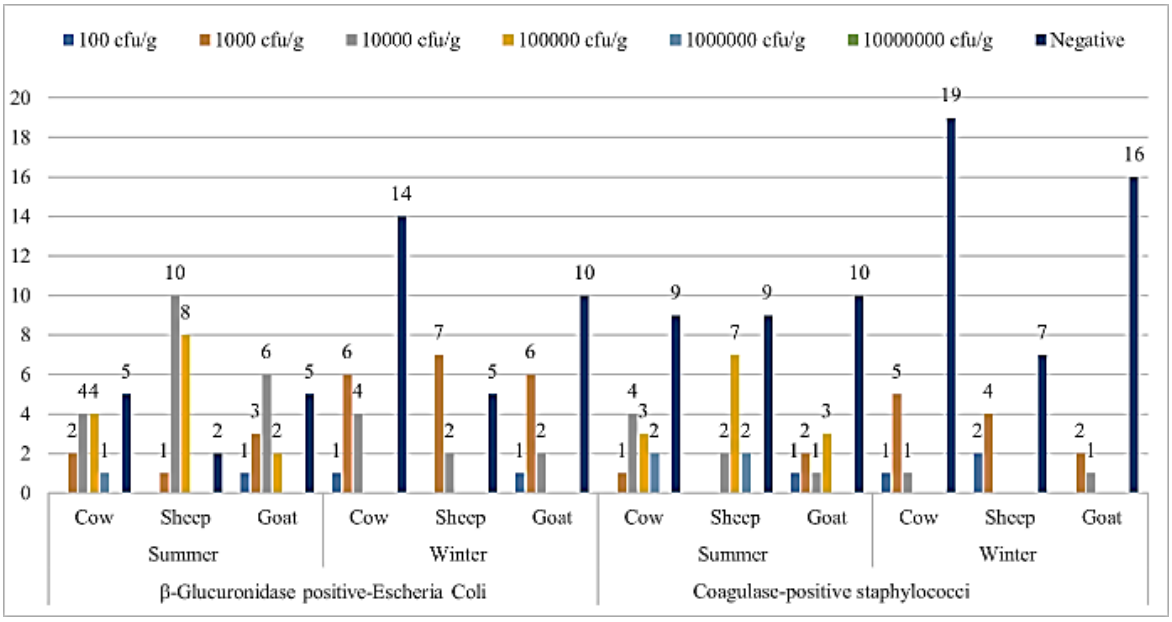

Figure 2. Level of contamination of the cheese samples with *E. coli and S. aureus* regarding the origin of the cheese milk and the season

Based on the current literature suggesting that raw milk used to produce artisanal cheese is a potential hazard which leads to foodborne outbreaks (Verraes et al. 2015), the presence of bacterial contaminants in the cheese samples used in the present study could be expected. Furthermore, it was hypothesized that due to the weather conditions during the summer the prevalence of bacterial contaminants will be higher, and it will be more pronounced in cows. Lastly, based on literature and several studies (Bernardi et. al. 2015; Oktay and Heperkan 2006; Silberiagel et al. 2004) it was hypothesized that the two methods for detection of *L. monocytogenes* would show a different pattern of results.

Firstly, as expected, the presence of *E. coli* (64.7% positive), *S. aureus* (39.7% positive) and *L. monocytogenes* (3.4% positive) could be expected in the artisanal cheese samples. Astonishingly, no sample contained *Salmonella.* Although there is limited literature examining the microbiological quality and presence of pathogens in artisanal and farmstead cheeses made from raw milk, the findings of the present study seems to be similar with previous studies (Rola et al. 2016; Jørgensen et al. 2005) in which they concluded that the contamination level of the cheese with *S. aureus* increased during the manufacture and this was attributed to the fact that the hands of the cheese makers were the source of contamination, because in 66% of the swabs taken from the hands of cheese makers *S. aureus* was present. Similar results as in our study have been reported also by Barreto De Deus et al. (2017). They concluded that the level of contamination on the final product depends on contamination of milk during processing, storage, personal hygiene, and sanitary habits of the food handlers**.** Regarding *Salmonella*, similar results with our study have been reported also by another study (De Reu et al. 2007), where no *Salmonella* were detected in any cheese samples. This absence could be due to the endogenous microflora, which may inhibit the growth of *Salmonella*. The prevalence of *L. monocytogenes* was similar with the previous study conducted by Cokal et al. (2012) in which they explained that the reason for the contamination must be due to the use of raw milk, production techniques, and the contaminated production environment. Also, the results for the presence of *E. coli* in our study are similar to those of a study conducted in Egypt by Ombarak et al. (2016) and in Italy by Zago et al. (2007), where it was concluded that although the level of contamination of the raw milk cheeses with non-pathogenic strains of *E. coli* was high, these strains may contribute to the different organoleptic characteristics of these products.

Secondly, we examined the presence of pathogenic bacteria as an effect of the season and origin of the raw milk. At the descriptive level (also as seen in Table 1), from the 116 samples, 57 of them were collected in the summer, whereas 59 were collected in the winter. Also, 36 of them were of *goat* origin, 35 were of *sheep* origin and 45 were of *cow* origin. For the CPS, the results indicated that this pathogen was significantly more often present in cheese sold in summer as compared to winter. Similar results were also found in a previous study by Sánchez-Gamboa et al. (2018) in Chihuaha cheese, but only when this cheese was at the beginning of ripening stage. However, at the end of the ripening stage, samples were negative for *S. aureus*. As this aspect was beyond the scope of this study, future studies should examine the role of the ripening time and its effects on the growing pattern of different microbiological agents. Somewhat unexpectedly though, we found no significant effect of the origin of the raw milk cheese on the levels of CPS in cheese samples. Also, there was no two-way interaction between the season and the origin of the raw milk cheese on the presence of CPS. We did, however, find a positive correlation between the levels of CPS and *E. coli*, which we anticipated knowing that both are considered also as hygienic indicators (De Reu et al. 2004).

For *E. coli*, the results showed that there was a significantly higher presence of these bacteria during summer as compared to the winter. Contrary to our findings, the study of Can and Elmali (2017) found higher prevalence of *E. coli* during the winter season as compared to the summer season, but authors concluded that the finding could be attributed to the production techniques of the traditional Turkish cheese named Carra. Carra cheese is usually matured by being buried in soil after insertion in a jug. Usually, it is consumed during the winter season (around January), hence, isolation of *E. coli* from cheese is more likely to occur in colder months. Particularly with view on artisanal cheeses, differences in findings across studies may often be attributed to production procedures and the type of cheese. Furthermore, our study found no significant effect of the origin of raw milk, and no significant interaction between the season and the origin of the raw milk, in the presence of *E. coli*.

Contrarily, for *L. monocytogenes*, the results showed that this pathogen was significantly more present during the winter as compared to the summer. This could be due to the fact that during the winter in Kosovo the main feed for the cows is silage. Spoiled silage and low silage quality are the main factors influencing the presence of *L.monocytogenes* in ruminants and therefore in milk (Queiroz et al. 2018). In a study conducted by Ho et al. (2007), *L. monocytogenes* was isolated from 38% of silage samples. Similar results are reported by El Marnissi et al. (2013) where the highest presence of *L. monocytogenes* was observed during the winter and autumn compared to summer and fall. Furthermore, our study found that *L. monocytogenes* was significantly more present in cheese made from cow milk as compared to sheep and goat milk cheese.

Finally, we compared the results for the presence of *L. monocytogenes* using two different analytical methods and found no discrepancy between them. In detail, both methods detected 4 positives out of 116 samples. This finding is in line with the study of Aznar and Solís (2006) who also found no differences in the results between the two methods. Noteworthy, in addition to using the two methods that we utilized in our study, the authors of the former study also used a PCR method and concluded that PCR seemed to be more sensitive in detecting the presence of *L. monocytogenes*. However, for practical reasons, this method could not be conducted in the present study.

Conclusions

To conclude, the overall results suggest substantial evidence for the presence of bacterial pathogens in artisanal type of cheese- a cheese that is widely used in Kosovo. Also, the findings show that both the season and the origin of the cheese milk seem to influence the level of the contamination in cheese. Finally, beside using standard microbiological procedures, alternative techniques for the detection of specific pathogens enable reduced assay time without losing sensitivity and specificity. Importantly, our study implies that artisanal cheeses sold in informal markets in Kosovo, are often not safe for human consumption. Thus, institutions that deal with public health, especially those dealing with food safety should increase controlling and monitoring routines in those informal markets.

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