Microbiological quality of Livno cheese

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Abstract

This study aimed to assess the microbiological quality of Livno cheese and milk as its raw material. It also investigated potential differences in microorganism presence and quantity between milk and cheese to understand the impact of milk processing on microbiological quality. A total of 15 raw milk and 15 Livno ripened cheese samples were analysed for the presence of *Salmonella* spp. and *Listeria monocytogenes*. Detection and quantification were performed for the following microorganisms: coagulase-positive staphylococci, aerobic mesophilic bacteria (for milk samples), *E. coli*, *Enterobacteriaceae*, sulfite-reducing clostridia (for cheese samples), yeasts and moulds. *Salmonella* spp. was not detected in any of the samples. Microbiological analysis of milk revealed varying levels of aerobic mesophilic bacteria, *E. coli*, *Enterobacteriaceae*, yeasts, moulds, and *L. monocytogenes*. Coagulase-positive staphylococci were detected in only two of 15 raw milk samples. In 15 Livno cheese samples, all tested microorganisms were below detectable levels except for *E. coli* (found in two samples) and *Enterobacteriaceae* (found in three samples). Statistical tests indicated significant differences in microbial presence and quantity between milk and cheese, except for coagulase-positive staphylococci. Given the importance of cheese microbiology for food safety and consumer health, this research provides valuable insights into the production and quality control of this traditional Bosnian cheese.

Key words: *foodborne pathogens; public health; Livno cheese; dairy industry; milk processing*

Introduction

The microbiological quality of highly consumed dairy products such as cheese, plays a crucial role in ensuring food safety and overall consumer health. Cheese production involves using various microorganisms, including bacteria, yeasts, and moulds, which contribute to the flavour, texture, and aroma of the final product. However, inadequate control, production, and trade can lead to the growth of harmful pathogens, such as *Salmonella* spp., *Listeria monocytogenes*,

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or *Escherichia coli*, posing significant risks to consumers. Stringent monitoring and control measures are necessary throughout production, from raw milk collection to ageing and packaging. These measures involve regular testing for the presence of pathogenic bacteria, ensuring proper sanitation practices, and implementing effective quality management systems.

Additionally, the microbiological quality of cheeses directly impacts their organoleptic properties. The presence of specific strains of bacteria and moulds contributes to the development of unique flavours and textures, enhancing the overall consumer experience. A well-maintained microbiological profile not only ensures the safety of cheeses but also promotes their distinct and desirable sensory attributes.

Livno cheese is one of the most popular indigenous cheeses in Bosnia and Herzegovina (B&H), consumed weekly by 64% of the consumers surveyed (Matić et al., 2014). Its production dates back to 1886, and the place of origin is the area of southwestern B&H, i.e., the wide area of the Livno Polje field (Kirin et al., 2003). Over time, the characteristics of Livno cheese changed primarily due to the transition from sheep's milk to cow's milk, or their mixture. A further important change occurred with the introduction of pasteurisation of milk for cheese making and the use of dairy cultures. In recent times, modern cheesemaking equipment has introduced and enabled new improvements, especially in the salting and ripening of cheese. However, the basic production parameters and characteristics of this cheese remained unique and recognisable (Kirin et al., 2003.). In regard to its properties, Livno cheese belongs to the hard, full-fat cheeses that ripen for 60–90 days (Kirin et al., 2003; Matić et al., 2014). Livno cheese of high quality on the cut has round, regularly spaced holes, medium size, yellowish colour and a well-formed rind (Sarić and Bijeljac, 2003).

Therefore, the primary objective of this study was to evaluate the microbiological quality of raw milk and the widely consumed Livno cheese manufactured from that milk. The second objective was to ascertain whether there was a difference in the presence or number of microorganisms between milk and Livno cheese in order to verify the impact of milk processing on microorganisms.

Material and methods

Sampling

The sampling and transport of cow's milk and cheese samples were carried out according to BAS EN ISO 7218:2008 Microbiology of food and animal feed – General requirements and guidance for microbiological examinations. After sampling, the samples were transported to the laboratory at temp. +4°C, and were immediately analysed. First, raw cow's milk was sampled, followed by a sampling of Livno cheese made from that milk after 66 days of ripening. A total of 15 milk samples (approximately 200 mL each) and 15 cheese samples (200 g each) were collected from the dairy located in the Livno Polje field in southwestern Bosnia and Herzegovina, where Livno cheese is traditionally manufactured.

Laboratory analysis

Sample preparation was performed in accordance with BAS EN ISO 6887-5:2022 Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 5: Specific rules for the preparation of milk and milk products. During sample preparation for

analysis, the surface of the container containing the sample at the point where the opening is performed was cleaned with a cotton swab soaked in 96% ethanol to prevent cross-contamination of the sample.

For the detection of *Salmonella* spp. and *L. monocytogenes*, 25 g or 25 mL of the sample, or 10 g or 10 mL for the detection of other microorganisms, was weighed into a sterile Erlenmeyer flask.

Before use, buffered peptone water (BPW) (Condalab, Spain) was tempered to room temperature, and then added according to the ratio of 9 x mg or 9 x V mL, i.e., 90 mL BPW to 10 g/mL sample, or 225 ml BPW to 25 g/mL sample. The flasks were then put on an orbital shaker (J.P. SELECTA, Spain) for 15 minutes.

If the microbiological examination required decimal dilutions, 1 mL of the initial dilution was transferred using a sterile pipette or micropipette to a test tube containing 9 mL BPW. Then, the test tube was covered to perform homogenisation with a Vortex mixer (5 to 10 s) to obtain the dilution (10^{-2} or 0.01). Subsequent dilutions were successively prepared in the previously described manner (10^{-3}, 10^{-4}, etc.).

All 30 samples were analysed for the presence of *Salmonella* spp. and *L. monocytogenes*. Detection and quantification were performed for the following microorganisms: coagulase-positive staphylococci, aerobic mesophilic bacteria (for milk samples), *E. coli*, *Enterobacteriaceae*, sulfite-reducing clostridia (for cheese samples), yeasts and moulds. Analyses were performed in the Laboratory for Microbiology of Food and Animal Feed of the Veterinary Institute, Faculty of Veterinary Medicine, University of Sarajevo.

Microbiological analysis of milk and cheese was carried out in accordance with the valid editions of the following ISO standards:

— BAS EN ISO 6579-1:2018/A1:2022 Microbiology of the food chain — Horizontal method for the detection, enumeration and serotyping of Salmonella — Part 1: Detection of *Salmonella* spp.
— BAS EN ISO 11290-1:2018 Microbiology of the food chain — Horizontal method for the detection and enumeration of Listeria monocytogenes and of *Listeria* spp. — Part 1: Detection method;
— BAS EN ISO 6888-1:2023 Microbiology of the food chain - Horizontal method for the enumeration of coagulase-positive staphylococci (Staphylococcus aureus and other species);
— BAS EN ISO 21528-2:2018 Microbiology of the food chain - Horizontal method for the detection and enumeration of Enterobacteriaceae — Part 2: Colony-count technique;
— BAS EN ISO 4833-1:2014 Microbiology of the food chain — Horizontal method for the enumeration of microorganisms — Part 1: Colony count at 30°C by the pour plate technique;
— BAS ISO 16649-2:2008 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;
— BAS ISO 21527-1:2009 Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of yeasts and moulds — Part 1: Colony count technique in products with water activity greater than 0.95.
— BAS ISO 15213:2008 Microbiology of the food chain — Horizontal method for the detection and enumeration of Clostridium spp. — Part 1: Enumeration of sulfite-reducing *Clostridium* spp. by colony-count technique.

To perform the method for detecting and enumerating the mentioned microorganisms, the original dehydrated media and their supplements, reagents and chemicals prescribed by the applied ISO standards were used.

Statistical analysis

Statistical analysis was performed using statistical tools (MS Excel and R Studio®). Fisher's exact test was performed instead of Pearson's chi-square test due to the smaller number of samples to determine whether there are differences in the measured parameter (the presence of a certain microorganism) in the two groups (raw milk and Livno cheese). The Student's T-test (two-tailed) was performed to determine whether there is a significant difference between the average number of microorganisms between milk and Livno cheese.

Results

In the total of 15 raw milk samples, *Salmonella* spp. was not detected (Table 1). The quantities of other tested microorganisms (cfu) per mL milk are presented in Figure 1. The average count of aerobic mesophilic bacteria, *E. coli*, *Enterobacteriaceae* and yeasts was 5.41 $\log_{10}$ cfu/mL, 2.96 $\log_{10}$ cfu/mL, 4.99 $\log_{10}$ cfu/mL and 3.40 $\log_{10}$ cfu/mL, respectively. Moulds were detected in 13 of 15 samples, with an average count of 2.41 $\log_{10}$ cfu/mL. *L. monocytogenes* was detected in 10 of 15 samples, with an average count of 1.64 $\log_{10}$ cfu/mL. Coagulase-positive staphy-

Table 1. Microorganisms in raw milk [cfu/mL]

Sample	*Salmonella* spp. [cfu/mL]	Aerobic mesophilic bacteria [cfu/mL]	*Escherichia coli* [cfu/mL]	*Enterobacteriaceae* [cfu/mL]	Yeasts [cfu/mL]	Moulds [cfu/mL]	*Listeria monocytogenes* [cfu/mL]	Coagulase-positive staphylococci [cfu/mL]
L1	ND	$3.64x10^5$	$3.73x10^2$	$9.02x10^4$	$5.00x10^3$	$4.55x10^2$	27.3	<10
L2	ND	$2.51x10^5$	$5.45x10^2$	$1.00x10^5$	$7.36x10^3$	$5.45x10^2$	18.2	<10
L3	ND	$4.04x10^5$	$3.73x10^2$	$8.91x10^4$	$1.18x10^3$	$2.73x10^2$	27.3	<10
L4	ND	$3.42x10^5$	$6.27x10^2$	$9.27x10^4$	$1.91x10^3$	$1.82x10^2$	36.4	<10
L5	ND	$3.82x10^5$	$1.38x10^3$	$8.95x10^4$	$4.00x10^3$	$2.73x10^2$	27.3	18.2
L6	ND	$4.42x10^5$	$8.91x10^2$	$1.07x10^5$	$3.73x10^3$	$2.73x10^2$	72.7	<10
L7	ND	$3.82x10^5$	$1.59x10^3$	$1.27x10^5$	$2.45x10^3$	$1.82x10^2$	63.6	<10
L8	ND	$1.96x10^5$	$1.68x10^3$	$1.14x10^5$	$3.00x10^3$	90.9	81.8	9.09
L9	ND	$2.00x10^5$	$8.27x10^2$	$1.05x10^5$	$1.00x10^3$	<10	72.7	<10
L10	ND	$2.87x10^5$	$7.45x10^2$	$9.91x10^4$	$2.36x10^3$	<10	63.6	<10
L11	ND	$1.24x10^5$	$4.73x10^2$	$9.64x10^4$	$1.00x10^3$	$2.73x10^2$	<10	<10
L12	ND	$1.93x10^5$	$1.10x10^3$	$9.20x10^4$	$1.45x10^3$	$1.82x10^2$	<10	<10
L13	ND	$1.35x10^5$	$1.27x10^3$	$8.95x10^4$	$4.91x10^3$	90.9	<10	<10
L14	ND	$2.55x10^5$	$2.65x10^3$	$8.36x10^4$	$4.09x10^3$	$5.45x10^2$	<10	<10
L15	ND	$2.00x10^5$	$1.28x10^3$	$1.10x10^5$	$1.73x10^3$	$6.36x10^2$	<10	<10
MEAN	/	$2.77x10^5$	$1.05x10^3$	$9.91x10^4$	$3.01x10^3$	$2.67x10^2$	32.7	1.82
MEDIAN	/	$2.55x10^5$	$8.91x10^2$	$9.64x10^4$	$2.45x10^3$	$2.73x10^2$	27.3	0
ST DEV	/	$9.92x10^4$	$5.95x10^2$	$1.13x10^4$	$1.76x10^3$	$1.92x10^2$	29.6	4.9

ND – not detected

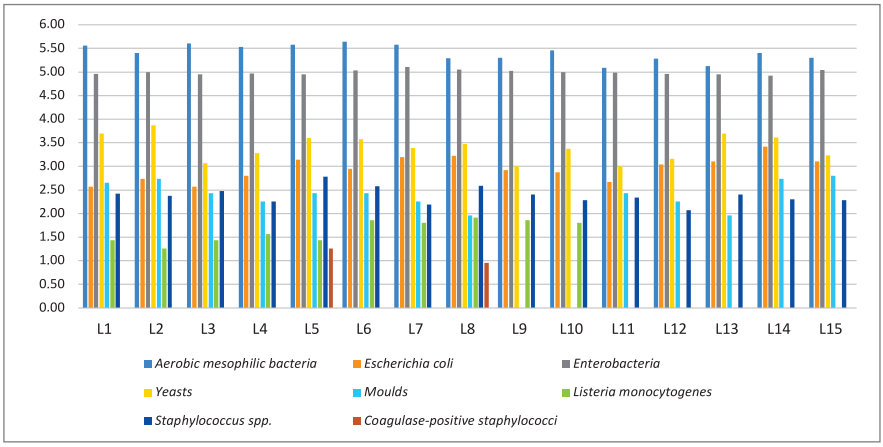

Figure 1. Number of microorganisms (cfu) per mL of milk, in $\log_{10}$

lococci were detected in two of 15 samples, with an average count of 1.11 $\log_{10}$ cfu/mL.

In the total of 15 Livno cheese samples, *Salmonella* spp., *L. monocytogenes*, Sulfite-reducing clostridia, yeasts,

Table 2. Microorganisms in Livno cheese [cfu/g]

Sample	*Salmonella* spp. [cfu/g]	Sulfite-reducing clostridia [cfu/g]	*Escherichia coli* [cfu/g]	*Enterobacteria ceae* [cfu/g]	Yeasts [cfu/g]	Moulds [cfu/g]	*Listeria monocytogenes* [cfu/g]	Coagulase-positive staphylococci [cfu/g]
L1	ND	<10	<10	<10	<10	<10	ND	<10
L2	ND	<10	<10	<10	<10	<10	ND	<10
L3	ND	<10	<10	<10	<10	<10	ND	<10
L4	ND	<10	<10	<10	<10	<10	ND	<10
L5	ND	<10	9	$1.82 \cdot 10^2$	<10	<10	ND	<10
L6	ND	<10	<10	<10	<10	<10	ND	<10
L7	ND	<10	<10	90.9	<10	<10	ND	<10
L8	ND	<10	<10	<10	<10	<10	ND	<10
L9	ND	<10	<10	<10	<10	<10	ND	<10
L10	ND	<10	<10	<10	<10	<10	ND	<10
L11	ND	<10	<10	<10	<10	<10	ND	<10
L12	ND	<10	<10	<10	<10	<10	ND	<10
L13	ND	<10	<10	$2.73 \cdot 10^2$	<10	<10	ND	<10
L14	ND	<10	6.4 x10	$2.73 \cdot 10^2$	<10	<10	ND	<10
L15	ND	<10	<10	<10	<10	<10	ND	<10
MEAN	/	<10	4.85	54.5	<10	<10	/	<10
MEDIAN	/	<10	0	0	<10	<10	/	<10
ST DEV	/	<10	15.86	98.56	<10	<10	/	<10

ND – not detected

moulds and coagulase-positive staphylococci were not detected (<1 $\log_{10}$ cfu/g). *E. coli* was detected in two of 15 samples, and *Enterobacteriaceae* in three of 15 samples (Table 2).

Results of Fisher's exact and Student's t test showed statistically significant *P* values ($P<0.05$) for all microorganisms determined in milk and cheese, except Coagulase-positive staphylococci ($P>0.05$).

Discussion

The absence of *Salmonella* spp. in all samples is encouraging and suggests that appropriate measures were taken during milk production and cheese manufacturing to prevent contamination by this pathogen. However, the presence of other microorganisms, such as *E. coli, Enterobacteriaceae*, yeasts, moulds, *L. monocytogenes*, and coagulase-positive staphylococci emphasises the need for continued monitoring and control strategies to ensure the safety and quality of raw milk and its derived products. According to the national legislation, the criteria for raw cow milk is less or equal to 80,000 cfu/mL (Regulation on Raw Milk, 2011 (Official Gazette of B&H, No. 21/11, 62/14 and 17/19)) as the total number of microorganisms.

Furthermore, according to the same legislation, nine samples tested belong to the third class of raw milk (200,001 – 400,000 cfu/mL), and six samples (L8, L9, L11, L12, L13 and L14) had slightly lower values and were classified in the second class (100,001 – 200,000 cfu/mL). The higher counts of aerobic mesophilic bacteria, *Enterobacteriaceae*, and yeasts in raw milk could indicate insufficient hygiene practices during milking and storage. These findings underscore the importance of implementing good agricultural and manufacturing practices to minimise microbial contamination. In B&H, the criteria for raw milk and Livno cheese are regulated by two legislative acts: the Guidelines for microbiological criteria for food (Food Safety Agency of B&H, 2013), which provides non-binding recommendations for food business operators, and the Regulation on Microbiological Criteria for Foodstuffs, 2013 (Official Gazette of BiH, No. 11/13, 79/16 and 64/18); which establishes mandatory criteria that products must adhere to. The raw milk used in this study underwent pasteurisation and technological processes to produce Livno cheese, which is the final product intended for human consumption. The microbiological analysis of Livno cheese demonstrated compliance with the mandatory criteria for *E. coli* and coagulase-positive staphylococci outlined in the Regulation on microbiological criteria for foodstuffs (2013). Additionally, the microbial results of Livno cheese aligned with the recommended values for *Salmonella* spp., *E. coli*, coagulase-positive staphylococcus/*Staphylococcus aureus*, and sulfite-reducing clostridia, as stipulated in the Guidelines.

The detection of *E. coli* and *Enterobacteriaceae* in some Livno cheese samples suggests that the cheese-making process may not have effectively eliminated these microorganisms. This finding raises concerns about the potential risk of consuming contaminated cheese. Stringent control measures should be implemented during cheese production to mitigate the presence of harmful bacteria. The absence of *Salmonella* spp. and *L. monocytogenes* in cheese samples is a very important finding. Human infections by *L. monocytogenes* have a hospitalisation rate as high as 91%, therefore it poses a great threat to human health (Jemmi and Stephan, 2006). *Salmonella* spp., which was also absent in raw milk samples,

remains a significant health concern in terms of foodborne illnesses. According to the report (EFSA, 2022) from European Food Safety Authority (EFSA), in 2021 there were 60,050 confirmed human cases of salmonellosis with a 14.3% increase compared with the rate in 2020. There were 6,755 cases of illness in foodborne outbreaks, therefore its absence in all samples is noteworthy. Certainly, there is an ongoing need for persistent efforts in prevention and control measures. The absence of coagulase-positive staphylococci, yeasts, moulds and *L. monocytogenes* in cheese, compared to milk, indicates that the cheese-making process has effectively eliminated these microorganisms and reduced the levels of *E. coli* and *Enterobacteriaceae*. These findings were confirmed by the Fisher's test and student's test which showed a significant decrease in the number and presence of microorganisms in cheese compared to milk.

Previous research on Livno cheese was conducted by Dráb et al. (2020) and included the isolation and cultivation of microbial groups, yeast and fungi. This was a valuable study on the microbial diversity of indigenous Livno cheese, but its focus was not on foodborne pathogens. There are several studies regarding quality, physicochemical and sensory characteristics, nutritional value, and production technology of Livno cheese (Sarić and Bijeljac, 2003; Hrković et al., 2011; Marijan et al., 2014; Matić et al., 2014; Kalit et al., 2016). However, according to our knowledge, a study of the microbiological quality of milk, as a raw material, and then Livno cheese, as a final product, has not been conducted before.

Additionally, the reputation and marketability of cheese producers rely heavily on the microbiological quality of their products. Consistently delivering safe and high-quality cheeses builds consumer trust and loyalty. Conversely, any instances of contamination or foodborne outbreaks can have severe consequences for the brand, leading to reputational damage and potential legal implications.

Lastly, prioritising and maintaining high microbiological quality in highly consumed cheeses are of paramount importance. By implementing rigorous quality control measures, cheese producers can ensure the safety, taste, and marketability of their products, safeguarding consumer health and fostering consumer confidence in the industry.

Conclusion

This study highlights the microbial quality of raw milk and Livno cheese, providing insights into the presence and levels of various microorganisms. While the absence of *Salmonella* spp., *L. monocytogenes*, sulfite-reducing clostridia, yeasts, moulds, and coagulase-positive staphylococci in Livno cheese indicates good microbiological quality, the presence of *E. coli* and *Enterobacteriaceae* poses a potential risk. The results underscore the importance of implementing stringent hygiene practices and quality control measures throughout the production chain to ensure the safety of dairy products and to protect public health. Consequently, the study successfully achieved both objectives. Given the limited data from B&H concerning Livno cheese, we believe that any research on this topic is valuable. Future investigations could aim to broaden the scope of the study by including additional dairies in the analysis. Moreover, investigating the antibiotic resistance patterns and molecular characteristics of isolated foodborne pathogens in Livno cheese could be a promising avenue for further research.

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Mikrobiološka kakvoća livanjskog sira

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Cilj je ovog istraživanja bio ocijeniti mikrobiološku kakvoću livanjskog sira i mlijeka kao sirovine za njegovu proizvodnju. Osim toga željeli smo istražiti potencijalne razlike u prisutnosti i količini mikroorganizama između mlijeka i sira u svrhu razumijevanja tehnološkog postupka na mikrobiološku kakvoću konačnog proizvoda. Ukupno 15 uzoraka sirovog mlijeka i 15 uzoraka livanjskog sira analizirano je na prisutnost *Salmonella* spp. i *Listeria monocytogenes*. Detekcija i kvantifikacija izvedene

su za sljedeće mikroorganizme: koagulaza-pozitivni stafilokoki, aerobne mezofilne bakterije (za uzorke mlijeka), *E. coli*, *Enterobacteriaceae*, sulfit-reducirajuće klostridije (za uzorke sira), kvasci i plijesni. *Salmonella* spp. nije ustanovljena niti u jednom uzorku. Mikrobiološka analiza mlijeka otkrila je različite razine aerobnih mezofilnih bakterija, *E. coli*, *Enterobacteriaceae*, kvasaca, plijesni i *L. monocytogenes*, dok su koagulaza-pozitivni stafilokoki bili prisutni samo u dva uzorka. U 15 uzoraka Livanjskog sira, svi testirani mikroorganizmi bili su ispod detektabilnih razina, izuzev *E. coli* (pronađena u dva uzorka) i *Enterobacteriaceae* (pronađeni u tri uzorka). Statistički testovi ukazuju na značajne razlike u prisutnosti i količini mikroorganizama između mlijeka i sira osim za koagulaza-pozitivne stafilokoke. S obzirom na važnost mikrobiološke kakvoće sira za sigurnost hrane i zdravlje potrošača, ovo istraživanje pruža vrijedne uvide u proizvodnju i kontrolu kakvoće ovog tradicionalnog bosansko-hercegovačkog sira.

Ključne riječi: *patogeni prenosivi hranom, javno zdravstvo, livanjski sir, mljekarska industrija, prerada mlijeka*