# ECOLOGIA BALKANICA

2023, Vol. 15, Issue 2

December 2023

pp. 199-207

### A study of the antimicrobial activity of lactic acid bacteria strains isolated from cow's milk and Sharri cheese

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**Abstract.** Lactic acid bacteria due to the safety reason is used in food industry in fermentation and in medicine as a bacteriocins. The aim of this study is to isolate Lactic Acid Bacteria from raw cow's milk and traditional Sharri cheese, and investigate their antimicrobial effects against *Staphylococcus aureus, Listeria monocytogenes, Listeria ivanovii,* and *Listeria innocua.* A total of 110 strains were isolated from milk samples of five farmers and samples of two traditional cheese producers. Among them, 47 strains exhibited inhibitory activity against at least one pathogenic indicator, with 20 isolates originating from milk samples and 27 from cheese samples. Eleven strains could inhibit the growth of all tested pathogenic indicators. Specifically, 68.08% of the isolates showed inhibitory activity against *Staphylococcus aureus,* 51.06% against *Listeria monocytogenes,* 48.94% against *Listeria innocua,* and 36.17% against *Listeria ivanovii.* These findings suggest that LAB cultures isolated from milk and artisanal cheese can serve as potential starter cultures in milk processing technology, which can help promote food security.

Key words: Staphylococcus spp., Listeria spp., milk, Sharri cheese, LAB strains.

### Introduction

The production of milk and artisan dairy products is an important source of income for farmers in Kosovo. Local farmers in Kosovo traditionally use lactic acid bacteria (LAB) to produce fermented milk products, as these bacteria can improve the organoleptic and health properties of the products. Among fermented milk product Sharri cheese is the most well-known and it is produced without the use of starter cultures and has a high sodium salt content (Mestani et al., 2017a; Mestani et al., 2017b; Sakić-Dizdarević et. al., 2023).

In the production of artisanal fermented milk products, there is often a lack of rigorous food safety control and regulations. These aspects are crucial for enhancing the products and ensuring their adherence to acceptable quality standards (Alkić-Subašić et al., 2022; Sakić-Dizdarević et al., 2023). To enhance these standards, LAB can be employed to suppress the activity of harmful microorganisms or food spoilage agents. Besides their potential in enhancing the sensory attributes of food products, LAB also offer health advantages. LAB are microorganisms that can convert energy into lactic acid. They are commonly used as starter cultures in food technology, and some strains possess antimicrobial properties that can combat pathogenic agents.

Numerous previous studies have indicated that LAB possess inhibitory properties (antimicrobial activity) against various pathogenic agents. Furthermore, products fermented with LAB have demonstrated long-term stability without compromising the quality or sensory characteristics of the product (Biolcati et al., 2022; Coelho et al., 2022; Bettera et al., 2023; Sakić-Dizdarević et al., 2023; Ziarno et al., 2023). Of particular interest in this regard are the compounds produced during bacterial development, including lactic and acetic acid, alcohol, hydrogen peroxide, carbon dioxide, an antimicrobial peptide (Mehmeti et al., 1999; Subramanian et al., 2015; Tan et al., 2022; Latif et al., 2023).

In the process of fermenting dairy products, these cultures utilize glucose as a basic energy source and convert it into lactic acid. This acid production significantly reduces the pH, creating an unfavourable environment for pathogenic and spoilage microorganisms. Extensive research has been conducted over the past two decades to investigate the antimicrobial properties of LAB. Given the constant concern regarding foodborne pathogens and the consumer demand for safe and fresh products, LAB has emerged as prominent biopreservative agent (Gálvez et al., 2007; Biliavska et al., 2019; Abdalla et al., 2021). Research has demonstrated that the antimicrobial agents produced by these bacteria possess the ability to inhibit pathogenic microorganisms and prevent food spoilage. As a result, the shelf life of food products can be extended, while their safety is enhanced.

The antibacterial compounds produced by these bacteria, including bacteriocins, can inhibit the pathogenic activity of bacteria such as *Listeria monocytogenes, Staphylococcus aureus*, and *Escherichia coli* (Fröhlich-Wyder et al., 2019; Câmara et al., 2021). Additionally, LAB play a vital role in food preservation and are considered highly effective and safe tools for use in the food industry (Darbandi et al., 2021; Mokoena et al., 2021; Zapaśnik et al., 2021; Nasrollahzadeh et al., 2022).

There is a growing interest in utilizing LAB cultures within the food industry, particularly in the production of traditional cheeses. These cultures are sought after due to their ability to thrive in environments with high salt content and acidity (Silva et al., 2015; Fröhlich-Wyder et al., 2019; Câmara et al., 2021; Ribeiro et al., 2021; Sakić-Dizdarević et al., 2022). Notably, the use of autochthonous starter cultures, which consist of strains capable of producing bacteriocins, can further enhance the safety, quality, and authenticity of traditional cheeses (Alkić-Subašić et al., 2022).

The objective of this study was to extract LAB strains from raw cow's milk and traditional Sharri cheese (produced without starter cultures) and

assess the inhibitory activity of the identified LAB strains against pathogens such as *Staphylococcus aureus, Listeria monocytogenes, Listeria ivanovii*, and *Listeria innocua*. The discovery and isolation of LAB strains with significant inhibitory potential against the specified pathogenic indicators would hold great value both in industrial and scientific contexts. The research aimed to isolate strains from milk and artisanal cheese originating from the Sharr region. LAB strains presumed to be isolated from milk and cheese samples, exhibiting notable inhibitory potential against pathogenic agents, were selected for further investigation.

### Materials and Methods Sampling

Samples of raw cow's milk used for traditional Sharri cheese were obtained from twenty-five dairy farms which all these farmers deliver milk to make cheese in their home in traditional forms. Samples of traditional Sharri cheese were collected from three well-known producers in the Municipality of Sharri, located in the region of the Sharri Mountains in Kosovo and three farmers which are "homemade" producers of traditional Sharri cheese. These cheese producers also source fresh milk from the same twenty-five farmers. For the isolation of lactic acid bacteria strains, nonthermally treated cow's milk was used, and the Sharri cheese samples were collected from farmers who produce traditional Sharri cheese without any thermal treatment or commercial starter cultures. All samples were collected using sterile universal bottles and bags.

## Sample processing and isolation of LAB strains

The isolation of LAB species was carried out using traditional methods as previously described (Mehmeti et al., 2015; Mestani et al., 2017a). In brief, selective nutrient media including De Man-Rogosa-Sharpe (MRS) broth acidified to pH 5.5, and Glucose M17 agar with concentrations of 1.5% and 0.8% (OXOID, UK) anaerobically at 30°C for 72 h in an anaerobic jar (Anaerocult C gas generator, Merck) and mixed with polymyxin B (0.5 g/mL, Sigma-Aldrich, St. Louis, MO, USA), and incubated for 48 h at 30°C under anaerobic conditions. For each milk sample, 1 mL was diluted with 9 mL of physiological solution (0.9% NaCl), while 10 grams of cheese samples were processed with 90 mL of physiological solution (0.9% NaCl). The samples were blended for 5 minutes in a Stomacher (IUL instruments, Denmark) at room temperature.

The samples were subjected to serial dilutions at varying concentrations ranging from 10 to 10<sup>-6</sup>. From each dilution, 100 µl was spread onto M17 agar supplemented with 0.5% w/v glucose (GM17) and MRS agar plates, and then incubated at 30°C for 24, 48, and 72 hours. Pure cultures with distinct morphological structures were selected for evaluation, with 7-10 colonies chosen from each milk sample and 30-40 colonies from each cheese sample. A total of 110 colonies, comprising 40 from milk samples and 70 from cheese samples, were randomly selected for assessing antibacterial activity. Single colonies were re-grown for further analyses. To become in these number of strains we have use several morphological and biochemical characteristics such as: gram colouring, spore formation (Harrigan, W.F. & McCance, M.E. 1976) catalase production, motility, oxidase test, anaerobic growth and salt tolerate under 2%, 4% and 6% NaCl has been analysed. Gas production from glucose in MRS broth containing inverted Durham tubes has been test and growth on different temperature (10, 37, 42°C) for 5 days and resistance in 60°C for 30 minutes (Sherman test). Strains were stored in 13% (v/v) glycerol at  $-80^{\circ}$ C until further analysis.

### Detection and evaluation of the antibacterial effect of LAB

The antimicrobial activity of LAB strains against pathogenic indicators, including Listeria ivanovii, Listeria innocua, Listeria monocytogenes, and Staphylococcus aureus, was discovered and assessed using petri dishes. Initially, petri dishes containing 15 mL of MRS agar medium were prepared. Then, 5 mL of MRS soft agar, mixed with 100 µL of the respective pathogenic indicator, was added to each pre-prepared petri dish with MRS agar. The nutrient medium was allowed to solidify in a sterile environment for approximately 10 to 15 minutes. In each petri dish inoculated with a pathogenic indicator,  $2 \mu L$  of LAB cultures isolated from milk and cheese samples were placed. Following the inoculation of LAB strains alongside the pathogenic indicators, the petri dishes were incubated at 37°C for 24 and 48 hours. Subsequently, they were examined for the presence of inhibitory zones against the test indicators. The spectrum of inhibitory activity of LAB strains against different pathogenic indicators was determined by evaluating and observing the formation of inhibitory zones. Next to the LAB isolates was added dropwise of proteinase K and 1 mg/mL trypsin to distinguish bacteriocins. In same time  $3\mu$ L of the LAB without this enzyme was add to blank control.

Lactic Acid Bacteria are shown to be a significant potential for producing inhibitory substances against all pathogenic indicators were preserved for long-term storage. The cultures were stored in Eppendorf tubes containing BHI broth supplemented with 15% glycerol at a temperature of -20°C. This storage method was employed to facilitate their subsequent characterization.

### **Results and Discussion**

The purpose of collecting samples from various farmers and producers of milk and tradetional Sharri cheese was to assess the antibacterial activity of LAB strains against pathogenic indicators, namely *Staphylococcus aureus*, *Listeria monocytogenes*, *Listeria ivanovii*, and *Listeria innocua*. A total of 110 LAB strains were isolated from cow's milk samples obtained from five farmers, as well as from traditional Sharri cheese samples collected from two prominent cheese producers in the Sharri region and all of them were chosen through a screening for LAB.

The results presented in Table 1 (NOTE: (+) means antibacterial activity of strains against pathogenic indicators: (-) means NON-antibacterial activity of strains against pathogenic indicators) indicate that, out of the 110 presumed LAB strains, 47 exhibited antimicrobial activity against one or more of the tested pathogenic indicators (Staphylococcus aureus, Listeria monocytogenes, Listeria ivanovii and Listeria innocua) a bacterium that already has been isolated from cows' raw milk and higher count of Listeria spp and Staphylococcus aureus and low level of these pathogens in cheese during ripening processing (O'Brien et al., 2009; Gajewska et. al., 2022) while in the end of product this bacterium could not be detected. This fact could be explained by the presence of bacteriocinproducing bacteria in the raw cow's milk used for cheese manufacture. Among these strains, 20 were isolated from milk samples, while 27 were derived from cheese samples (Table 1).

CHARACTERISTICS				PATHOGENIC INDICATOR			
Nr	The code of	nН	Product	Listeria	Listeria	Listeria	Staphylococcus
117.	strains	<i>p</i> 11	Trounct	ivanovii	inoccua	monocytogenes	aureus
1	6123	4.32	Row milk	(+)	(+)	(+)	(+)
2	6223	4.07	Row milk	-	(+)	(+)	-
3	6225	4.37	Row milk	-	-	(+)	(+)
4	6231	3.99	Row milk	-	-	-	(+)
5	6244	4.17	Row milk	-	-	-	(+)
6	6321	4.1	Row milk	-	-	-	(+)
7	6332	3.94	Row milk	(+)	(+)	(+)	(+)
8	6411	4.53	Row milk	(+)	-	(+)	-
9	6541	4.05	Row milk	(+)	-	(+)	(+)
10	6577	4.32	Row milk	-	-	-	(+)
11	6654	4.62	Row milk	-	-	(+)	(+)
12	6677	4.9	Row milk	-	-	(+)	(+)
13	6683	4.08	Row milk	(+)	(+)	(+)	(+)
14	6712	4.04	Row milk	(+)	(+)	-	-
15	6732	4.42	Row milk	-	(+)	(+)	(+)
16	6744	4.76	Row milk	(+)	(+)	(+)	(+)
17	6759	4.19	Row milk	-	(+)	-	-
18	6881	4.07	Row milk	-	(+)	-	(+)
19	6922	4.46	Row milk	-	-	(+)	(+)
20	6955	4.86	Row milk	-	-	(+)	-
21	8408	4.48	Sharri cheese	(+)	(+)	(+)	(+)
22	8538	4.4	Sharri cheese	-	-	-	(+)
23	8561	4.36	Sharri cheese	-	(+)	-	-
24	8629	4.38	Sharri cheese	-	(+)	-	-
25	8637	4.41	Sharri cheese	-	(+)	-	-
26	8692	4.33	Sharri cheese	-	(+)	-	-
27	8743	4.14	Sharri cheese	(+)	-	-	-
28	8753	4.37	Sharri cheese	-	(+)	-	-
29	8984	4.31	Sharri cheese	-	-	(+)	-
30	9267	3.84	Sharri cheese	(+)	(+)	(+)	(+)
31	9235	4.38	Sharri cheese	-	-	-	(+)
32	9274	4.06	Sharri cheese	(+)	(+)	(+)	(+)
33	8168	4.38	Sharri cheese	-	-	-	(+)
34	8170	4.39	Sharri cheese	-	-	-	(+)
35	8171	4.38	Sharri cheese	-	-	-	(+)
36	8172	4.52	Sharri cheese	-	-	-	(+)
37	8173	4.37	Sharri cheese	-	-	-	(+)
38	8174	4.4	Sharri cheese	-	-	-	(+)
39	9352	4.31	Sharri cheese	-	-	(+)	-
40	9412	4.52	Sharri cheese	(+)	(+)	(+)	(+)
41	9356	4.88	Sharri cheese	(+)	(+)	(+)	(+)
42	9416	4.59	Sharri cheese	(+)	(+)	-	(+)
43	9360	4.37	Sharri cheese	-	-	(+)	
44	9424	4.52	Sharri cheese	(+)	(+)	(+)	(+)
45	9440	4.55	Sharri cheese	(+)	(+)	(+)	(+)
46	9445	4.37	Sharri cheese	(+)	(+)	-	(+)
47	9453	4.4	Sharri cheese	-	-	(+)	-
Total LAB strains from raw milk with inhibitory activity				7	9	13	15
Total LAB strains from Sharri cheese with inhibitory activity				10	14	11	17

Table 1. Antibacterial activity of LAB strains isolated from fresh cow's milk and artisanal Sharri cheese.

Based on the findings presented in Table 1, out of the 47 strains exhibiting inhibitory activity, 11 presumed LAB strains demonstrated inhibitory effects against all tested pathogenic indicators. Among these strains, 4 were derived from milk samples, while 7 were isolated from cheese samples. The results of this study highlight the potential antibacterial activity of LAB isolated from milk and traditional Sharri cheese, warranting further investigation. Moreover, selected variants of indigenous LAB have the potential to serve as starter cultures in food technology or for the preservation of fermented milk products. In addition to the production of hydrogen peroxide and various organic acids, the inhibitory activity of LAB may be attributed to the synthesis of other inhibitory substances, particularly bacteriocins, which require special attention and study.

The isolated LAB strains displayed the highest inhibitory activity against the pathogenic indicator Staphylococcus aureus, with 32 isolates (68.08%) exhibiting inhibitory effects. This was followed by 24 strains (51.06%) showing inhibitory activity against Listeria monocytogenes, 23 strains (48.94%) against Listeria innocua, and 17 strains (36.17%) against Listeria ivanovii (Table 1). Listeria ivanovii is well known as non-pathogenic strain while we involved in this study because LAB are used as a starter culture. This importance is to use some of the strains which in same time is used as starter culture and as antimicrobial effect against Listeria species. In same time, previous studies conducted by various researchers have consistently demonstrated that LAB strains Isolated from milk possess inhibitory activity against pathogenic indicators, particularly targeting bacteria such as Staphylococcus aureus, Escherichia coli, Listeria innocua, and Listeria monocytogenes (Savadogo et. al., 2004; Darbandi et al., 2021; Sakić-Dizdarević et al., 2023). The inhibitory effect by indigenous LAB of Sharri cheese is important, since S. aureus have been described as higher contaminants analysed by our group and that with 39.8% (88 out of 221 farmers) were detected in cow's milk samples and Listeria monocytogenes 2.7% (6 out of 221 farmers analysed) (Mehmeti et. al., 2017).

Out of the total isolates, 32 strains (68.08%) exhibited inhibitory activity against the pathogen *Staphylococcus aureus*. Among these isolates, 17

(36.17%) were obtained from cheese samples, while 15 (31.91%) were derived from milk samples. Previous studies have documented the sensitivity of the pathogenic bacterium *Staphylococcus aureus* to LAB strains isolated from milk and its fermented products (Eid et al., 2016; Darbandi et al., 2021; Sakić-Dizdarević et al., 2023).

Our result shown to be a significant potential for producing inhibitory substances against all pathogenic indicators were preserved for longterm storage (Fig. 1). This is an argument more to represent first the LAB as a starter culture and as an antimicrobial agent in cheese and in same time to make more safety dairy products with special focus in cheese.

Out of the total isolates, 24 strains (51.06%) demonstrated inhibitory effects against the pathogen *Listeria monocytogenes*. Among these isolates, 11 (23.40%) were derived from cheese samples, while 13 (27.66%) were obtained from milk samples (Fig. 1 and 2). Previous studies conducted by other researchers have also reported the production of antimicrobial substances by specific strains of LAB with a broad spectrum of activity against pathogenic agents, including *Listeria monocytogenes* (Kumariya et al., 2019; Lee et al., 2020; Wu et al., 2022).

In relation to the pathogenic indicator *Listeria innocua*, a total of 23 isolates (48.94%) exhibited inhibitory effects against it, with 14 isolates (29.79%) originating from cheese samples and 9 isolates (19.15%) from milk samples. The presence of LAB strains demonstrating inhibitory activity against *Listeria innocua* has also been reported previously (Mezaini et al., 2009).

The strains obtained from milk and cheese displayed the lowest occurrence of antibacterial activity against the pathogenic indicator *Listeria ivanovii*. Out of the total 47 strains isolated and tested against this pathogen (as shown in Table 1), 17 strains (36.17%) exhibited inhibitory effects on *Listeria ivanovii*. Among the tested isolates, as indicated in Fig. 1 and 2, 10 isolates (21.28%) were derived from cheese samples, while 7 isolates (14.89%) originated from milk samples. Previous studies have demonstrated that lactic acid bacteria strains possess a broad spectrum of inhibition against various pathogenic bacteria, including *Listeria ivanovii* (Lee et al., 2020).

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Fig. 1. Inhibitory activity of LAB strains against pathogenic indicators:
(A) inhibitory activity of the strain against the pathogen *Staphylococcus aureus*; (B) inhibitory activity of the strain against the pathogen *Listeria monocytogenes*; (C) inhibitory activity against *Listeria ivanovii*; (D) inhibitory activity of the strain against *Listeria innocua*.



**Fig. 2.** Antibacterial activity of LAB strains isolated from fresh cow's milk and artisanal Sharri cheese that have shown inhibitory activity against pathogens in the study.

### Conclusions

Lactic Acid Bacteria play a crucial role in the bio-preservation of diverse food items. Furthermore, the use of multiple LAB strains that produce bacteriocins can have significant applications in enhancing food safety as a bioprotective combination for various food products. In this study, a total of 110 LAB isolates were examined, out of which 47 demonstrated inhibitory activity against at least one pathogenic indicator, including *Staphylococcus aureus*, *Listeria monocytegenes*, *Listeria ivanovii*, and *Listeria innocua*. Among these isolates, 20 strains were derived from milk samples, while 27 strains were obtained from cheese samples.

Eleven of the identified LAB strains exhibitted inhibitory activity against all the tested pathogenic indicators, with four strains originating from milk samples and seven strains isolated from cheese samples. Notably, 68.08% of the isolates (47 in total) displayed inhibitory activity against Staphylococcus aureus, 51.06% showed inhibition against Listeria monocytogenes, 48.94% exhibited inhibitory zones against Listeria innocua, and 36.17% demonstrated inhibitory effects against Listeria ivanovii. The findings of this study support the utilization of indigenous LAB strains as starter cultures or preservative agents to produce artisanal cheese and other fermented dairy products. Moreover, the research highlights the presence of autochthonous LAB cultures in raw milk and traditional products in Kosovo, which possess antimicrobial effects against various pathogenic agents.

Exploring indigenous LAB strains will aid in the selection of optimal cultures to enhance the microbiological safety and extend the shelf life of traditional food products. This collection of LAB strains can be employed to develop specialized starter cultures for Kosovar fermented food products, particularly traditional cheeses.

### Acknowledgments

The authors are grateful to Mrs. Flutra Mestani for the support provided in technical and analytical matters (including the formatting of statistical results).

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> Received: 21.05.2023 Accepted: 08.12.2023