



## ORIGINAL ARTICLE

IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY SCREENING OF LACTIC ACID BACTERIA FROM TRADITIONAL CHEESE *PAŠKI SIR*, CROATIANevijo Zdolec<sup>1\*</sup>, Marta Kiš<sup>1</sup>, Snježana Kazazić<sup>2</sup>, Fabijan Oštarić<sup>3</sup>, Ena Horvat<sup>1</sup>, Nikola Čudina<sup>1</sup>, Nataša Mikulec<sup>4</sup><sup>1</sup>University of Zagreb, Faculty of Veterinary Medicine, Zagreb, Croatia; <sup>2</sup>Ruder Bošković Institute, Zagreb, Croatia; <sup>3</sup>Capra Domestica, Perković, Croatia; <sup>4</sup>University of Zagreb, Faculty of Agriculture, Zagreb, Croatia

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**Ethical considerations:** When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: [10.1371/journal.pbio.3000410](https://doi.org/10.1371/journal.pbio.3000410)), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

## ABSTRACT

*Paški sir*, a hard sheep's cheese, has been produced for decades in households on the island of Pag, Croatia, and is now manufactured under controlled conditions in local cheesemaking facilities. Extensive and ecological sheep breeding and cheese production result in low chemical input or hazards and final products with high safety properties. The aim of the study was to investigate the diversity of lactic acid bacteria (LAB) in this type of production and their phenotypic profiles of antimicrobial resistance. MALDI-TOF MS identification revealed a high diversity of indigenous LAB populations, including lactobacilli, enterococci, pediococci, and lactococci, in traditional *Paški sir* cheese. Most isolates representing identified species showed cut-off values below the critical limits required for selecting potential probiotic strains suitable for food production.

**Keywords:** antimicrobial resistance; diversity; lactic acid bacteria; *Paški sir*

## INTRODUCTION

*Paški sir* hard cheese is produced from the milk of the Croatian autochthonous *Paška ovca* sheep, which graze on pastures rich in various types of medicinal and aromatic herbs on the island of Pag, Croatia. This results in a cheese with a characteristic taste and a pleasant aroma. *Paški sir* cheese has been PDO-protected since 2019 and, according to its specification, can be made from raw or pasteurised sheep's milk. The natural microbiota of the milk consists

mainly of heterogeneous species of lactic acid bacteria (LAB), which share a common carbohydrate metabolic pathway with lactic acid as the primary metabolic endpoint. Due to their probiotic properties, LAB have a long history of safe use in traditional and industrial fermented dairy products [1–3]. The dominance of certain LAB species during the production process depends on the processing temperature of the curd and the ripening conditions, which strongly influence the quality of the cheese. LAB initiate complex biochemical reactions that are crucial for

the development of the specific organoleptic properties of this cheese [4, 5].

The indigenous microbiota, i.e. LAB, has not yet been studied in relation to the production chain of traditional *Paški sir* cheese. Identification of lactobacilli has only been carried out in one study by Jenserle [6] using the PCR method and reported three identified species in cheese: formerly *Lactobacillus brevis*, *Lactobacillus plantarum*, and *Lactobacillus paracasei* (now *Levilactobacillus brevis*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei*); [7]. Furthermore, to our knowledge, the antimicrobial resistance profile of LAB within this type of production has also not been studied.

Thus, this study aimed to isolate and identify LAB from the traditional *Paški sir* cheese production chain, i.e., from sheep's milk, lamb abomasum (used to obtain natural rennet), cheese curd, and cheese during ripening. An additional aim was to select strains of the identified species and subject them to antibiotic susceptibility testing to gain an initial insight into the extent of antimicrobial resistance in traditional *Paški sir* cheese production.

## MATERIALS AND METHODS

### Isolation and identification of lactic acid bacteria from the *Paški sir* cheese production chain

*Paški sir* cheese was traditionally produced in a family-owned cheese dairy located in Kolan (Pag), using milk from sheep raised on the farm and following traditional cheesemaking practices. Cheese was produced from raw sheep milk and aged for 120 days. Samples taken for LAB isolation and identification included raw milk from randomly selected lactating sheep in the herd ( $n = 18$ ), abomasum contents of slaughtered lambs, curd, and cheese at different stages of ripening (days 13, 50, 80, and 120).

To isolate LAB and determine the count, 10 g/10 mL of the sample was aseptically taken, diluted in 90 mL of saline peptone water, and homogenised for two minutes (Stomacher 400 Circulator, Seward, UK). From the selected decimal dilutions, 0.1 mL was spread on MRS agar (BioMerieux, Marcy l'Etoile, France) and incubated under anaerobic conditions at 30 °C for 48-72 hours. After incubation, the number of bacteria per gram or millilitre of sample was calculated using an automatic colony counter (Scan 1200, Interscience, Paris, France). Morphologically

different colonies were then cultured in MRS broth, followed by streaking onto MRS agar for identification. A total of 190 colonies of Gram-positive and catalase-negative bacilli, cocci, and coccobacilli were subjected to matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS).

The standard MALDI-TOF MS procedure consisted of plating the colony on the MALDI target plate and adding 1 µL of 70% formic acid (Fisher Chemical, Alcobendas, Spain). After drying at room temperature, each spot was covered with 1 µL of 10 mg/mL alpha-4-cyano-4-hydroxycinnamic acid (CHCA, Bruker Daltonik, Bremen, Germany) in 50% acetonitrile and 2.5% trifluoroacetic acid and allowed to dry. Measurements were performed with a Microflex LT mass spectrometer (Bruker Daltonik, Bremen, Germany), and spectra were recorded in a positive linear mode in the mass range from 2,000 to 20,000 Da. MBT Compass HT version 5.1 software (Bruker Daltonik, Bremen, Germany) was used to compare the spectra with the reference database. External calibration was performed using the Bacterial Test Standard (Bruker Daltonik, Bremen, Germany). The identification criteria were as follows: a logarithmic value of 2.00–3.00 indicated a species identification with high confidence, a value of 1.70–1.99 indicated a species identification with low confidence, while a value of 0–1.69 was considered unreliable [8]. Based on the values of the results representing the highest probability of correct identification, 173 isolates were selected to evaluate the representation of LAB species in the samples.

### Testing the antimicrobial susceptibility of LAB species

Sixteen isolates of lactic acid bacteria, representing bacterial species identified by MALDI-TOF MS, were used for antibiotic susceptibility testing: *Lactococcus lactis*, *Lactiplantibacillus plantarum*, *Carnobacterium maltaromaticum*, *Lactococcus raffinolactis*, *Loigolactobacillus cornyformis*, *Enterococcus faecalis*, *Levilactobacillus brevis*, *Pediococcus pentosaceus*, *Leuconostoc mesenteroides*, *Enterococcus durans*, *Enterococcus faecium*, *Enterococcus italicus*, *Lacticaseibacillus paracasei*, *Companilactobacillus paralimentarius*, *Latilactobacillus sakei*, and *Latilactobacillus curvatus*.

Susceptibility to nine antibiotics was tested using the E-test (BioMerieux, Marcy l'Etoile, France). Antibiotics were selected according to EFSA [9] recommendations in-

tended for the characterisation of microorganisms used as feed additives or as production organisms, as there are no such criteria for isolates from human food. Pure bacterial cultures (density of 0.5 McFarland) were spread in three layers and three directions on MRS agar using a sterile swab. After 15 minutes, E-test strips containing ampicillin (0.016–256 mg/L), vancomycin (0.016–256 mg/L), tetracycline (0.016–256 mg/L), kanamycin (0.016–256 mg/L), streptomycin (0.064–1024 mg/L), clindamycin (0.016–256 mg/L), chloramphenicol (0.016–256 mg/L), gentamicin (0.016–256 mg/L), and erythromycin (0.016–256 mg/L) were applied. After incubation at 30 °C for 24 hours, the minimum inhibitory concentrations (MICs) were determined by reading the lowest point between the inhibition zone and the MIC values on the scale. The results were interpreted according to the EFSA criteria [9]. Multidrug-resistant (MDR) strains were considered as non-susceptible to at least one agent in three or more different classes of antimicrobial drugs.

## RESULTS AND DISCUSSION

The results of the identification of lactic acid bacteria are shown in Table 1. The identified species are listed in order of their proportion within the population of identified colonies. A total of 16 different species were identified from milk, abomasum, curd, and cheese.

In the raw sheep's milk samples, only *Lactococcus lactis* was identified. This species was later displaced in the curd by *Carnobacterium maltaromaticum*, *Leuconostoc mesenteroides*, and *Lactococcus raffinolactis* and was not detected in the cheese during ripening. An increase in species diversity of the population was observed, with the number of identified species rising as the cheese matured. The mixed population consisted mainly of lactobacilli, followed by enterococci, pediococci, leuconostoc, and carnobacteria. Among the lactobacilli, homofermentative species predominated, which is technologically desirable as they do not produce off-flavours, gases, or other unwanted by-products. However, undesirable enterococci (*E. faecalis*) and, in early stages of maturation, *Leuconostoc* species, which are heterofermentative gas-producing bacteria, were also isolated, although to a lesser extent. The presence of enterococci is also common in various hard cheese types (up to 5 log<sub>10</sub> CFU/g), but it can lead to higher levels

of biogenic amines in the cheese, especially tyramine [10]. The final product was dominated by the species *P. pentosaceus* and *L. paracasei*.

Studies of the indigenous microbiota in the production of *Paški sir* cheese have only rarely been carried out, which is probably due to the shift to standardised production from pasteurised milk with the addition of starter cultures. Only Jensterle [6] identified three predominant species of lactobacilli in *Paški sir* cheese using the PCR method: *L. brevis*, *L. plantarum*, and *L. paracasei*. To our knowledge, our research is the first study of the microbiota in the production chain of *Paški sir* cheese in terms of identifying the predominant autochthonous LABs in *Paška ovca* sheep's milk, curd, and cheese during ripening. In addition, *L. plantarum* was isolated from the lamb abomasum, the strain used together with natural rennet in microencapsulated form as an innovation in the production of *Paški sir* cheese [11].

The results of MALDI-TOF MS identification of selected LAB colonies show the predominance of lactobacilli at certain stages of cheese maturation, which is completely different from the microbiota of sheep's milk, in which only the species *Lc. lactis* was present. This species is very important in the dairy industry and has many desirable technological and health properties. In this sense, Pajač et al. [12] confirmed the strong acidification capacity and good proteolytic, lipolytic, and antimicrobial activity of lactococci from the milk of *Paška ovca* sheep. The selected strain *Lc. lactis* was also used experimentally in the production of *Paški sir* cheese in microencapsulated form [11], which did not affect the standard quality of the cheese.

A very high species diversity of the sheep's milk microbiota was found by Quintana et al. [13] using MALDI-TOF MS determination, with lactobacilli predominating. It is also common to find enterococci in sheep's milk, which can persist in the curd and the cheese during ripening [14]. It is known that microbiota composition of raw milk depends on numerous factors, including udder health and production hygiene. Nevertheless, the methodology used for LAB isolation, particularly the method of selecting colonies for identification and the identification method itself, should not be disregarded. Accordingly, the composition of the LAB population determined in our study from different matrices during cheese production should only be compared with studies based on culture-dependent isolation methods. Recently, attention has been drawn to

Table 1. Results of LAB identification in the *Paški sir* cheese production chain

| Sample                       | Number of colonies for identification | Number of identified colonies (MALDI score 2.0–3.0) | Identified LAB species                      | Number of identified species |
|------------------------------|---------------------------------------|-----------------------------------------------------|---------------------------------------------|------------------------------|
| Sheep milk (n=18)            | 18                                    | 18                                                  | <i>Lactococcus lactis</i>                   | 18                           |
| Lamb abomasum (n=3)          | 8                                     | 8                                                   | <i>Enterococcus faecalis</i>                | 7                            |
|                              |                                       |                                                     | <i>Lactiplantibacillus plantarum</i>        | 1                            |
| Curd (n=3)                   | 60                                    | 50                                                  | <i>Carnobacterium maltaromaticum</i>        | 24                           |
|                              |                                       |                                                     | <i>Leuconostoc mesenteroides</i>            | 11                           |
|                              |                                       |                                                     | <i>Lactococcus lactis</i>                   | 9                            |
|                              |                                       |                                                     | <i>Lactococcus raffinolactis</i>            | 3                            |
|                              |                                       |                                                     | <i>Enterococcus faecalis</i>                | 3                            |
| Cheese during ripening (n=4) |                                       |                                                     |                                             |                              |
| Day 13                       | 18                                    | 17                                                  | <i>Leuconostoc mesenteroides</i>            | 13                           |
|                              |                                       |                                                     | <i>Lactocaseibacillus paracasei</i>         | 2                            |
|                              |                                       |                                                     | <i>Latilactobacillus curvatus</i>           | 1                            |
|                              |                                       |                                                     | <i>Enterococcus faecalis</i>                | 1                            |
| Day 50                       | 20                                    | 14                                                  | <i>Loigolactobacillus coryniformis</i>      | 7                            |
|                              |                                       |                                                     | <i>Pediococcus pentosaceus</i>              | 2                            |
|                              |                                       |                                                     | <i>Leuconostoc mesenteroides</i>            | 2                            |
|                              |                                       |                                                     | <i>Latilactobacillus sakei</i>              | 1                            |
|                              |                                       |                                                     | <i>Levilactobacillus brevis</i>             | 1                            |
|                              |                                       |                                                     | <i>Enterococcus faecalis</i>                | 1                            |
| Day 80                       | 24                                    | 24                                                  | <i>Enterococcus faecium</i>                 | 6                            |
|                              |                                       |                                                     | <i>Lactocaseibacillus paracasei</i>         | 4                            |
|                              |                                       |                                                     | <i>Enterococcus durans</i>                  | 3                            |
|                              |                                       |                                                     | <i>Latilactobacillus sakei</i>              | 3                            |
|                              |                                       |                                                     | <i>Pediococcus pentosaceus</i>              | 2                            |
|                              |                                       |                                                     | <i>Leuconostoc mesenteroides</i>            | 2                            |
|                              |                                       |                                                     | <i>Enterococcus faecalis</i>                | 2                            |
|                              |                                       |                                                     | <i>Loigolactobacillus coryniformis</i>      | 1                            |
|                              |                                       |                                                     | <i>Enterococcus italicus</i>                | 1                            |
| Day 120                      | 42                                    | 42                                                  | <i>Lactocaseibacillus paracasei</i>         | 12                           |
|                              |                                       |                                                     | <i>Pediococcus pentosaceus</i>              | 12                           |
|                              |                                       |                                                     | <i>Enterococcus faecalis</i>                | 7                            |
|                              |                                       |                                                     | <i>Enterococcus faecium</i>                 | 5                            |
|                              |                                       |                                                     | <i>Loigolactobacillus coryniformis</i>      | 2                            |
|                              |                                       |                                                     | <i>Latilactobacillus sakei</i>              | 1                            |
|                              |                                       |                                                     | <i>Latilactobacillus curvatus</i>           | 1                            |
|                              |                                       |                                                     | <i>Companilactobacillus paralimentarius</i> | 1                            |
|                              |                                       |                                                     | <i>Leuconostoc mesenteroides</i>            | 1                            |

methods that bypass the classical isolation of live bacteria on microbiological media and instead isolate bacterial DNA from a food sample and subject it for metagenomic sequencing [15]. It is clear that such methods offer a completely new perspective on the composition of the microbiome of milk or cheese, but in the context of product quality and safety, the focus should still be on the microbiota that can be cultured and isolated from the sample (i.e., live cells) so that it can be accurately associated with the observed desirable or undesirable properties of the final

product. Nevertheless, studies on the cultivable microbiota in cheese production can contribute to the selection of viable LAB strains at the specific sampling site and under the process conditions. Thus, the abundance of *Lc. lactis* in raw milk can be explained by the neutral acidity, which is favourable for its growth, compared to the acidic environment, increased salt content, and reduced redox potential in hard cheese, where lactobacilli predominate.

Because of the established diversity of the microbiota, the sensitivity profiles to antibiotics were preliminarily

investigated phenotypically in a selected representative of all 16 isolated LAB species in this study (Table 2). Most LAB species (*Lactococcus lactis*, *Lactiplantibacillus plantarum*, *Carnobacterium maltaromaticum*, *Loigolactobacillus coryniformis*, *Levilactobacillus brevis*, *Leuconostoc mesenteroides*, *Enterococcus durans*, *Enterococcus faecium*, and *Latilactobacillus sakei*) were sensitive to antimicrobial agents relevant in the horizontal transfer of resistance, such as tetracycline or erythromycin, indicating possible lower antibiotic pressure in extensive sheep farming on the island of Pag. However, seven species, namely *Lactococcus raffinolactis*, *Enterococcus faecalis*, *Pediococcus pentosaceus*, *Enterococcus italicus*, *Lacticaeibacillus paracasei*, *Companilactobacillus paralimentarius*, and *Latilactobacillus curvatus*, were resistant to at least one agent according to EFSA criteria [9]. Criteria of multi-drug resistance (MDR; resistance to three or more antibiotic classes) were fulfilled only in two enterococci strains (*E. faecalis*, *E. italicus*) and *L. paracasei*. Resistance was found most frequently to aminoglycosides kanamycin and streptomycin.

Kanamycin and streptomycin, the antibiotics to which resistance was most frequently detected in this study, were closely followed by gentamicin. They belong to the aminoglycoside group and are classified as *critically important*

*antimicrobials* (CIA) according to the WHO MIA List, meaning they are widely used in human medicine and are used for infections where there is evidence of transmission of resistant bacteria or resistance genes from non-human sources. Given the detected resistance in *L. paracasei*, it should be noted that macrolides, such as erythromycin, are also critically important for humans, as they are among the few treatment options for *Campylobacter* infections in humans [16]. This highlights the importance of monitoring resistance to aminoglycosides and macrolides in non-human sources such as fermented products due to the risk of resistance transfer.

It is known that antimicrobial resistance in commensal food bacteria has recently become an important field of research due to the possible horizontal transmission of resistance genes within the food chain [17]. It has been shown that populations of these bacteria in food can carry resistance genes for clinically relevant antibiotics, which may also be transmissible [18]. The role of fermented foods in the transmission of resistance still needs to be evaluated and consumer exposure and potential public health risks assessed [17]. In our study, *L. paracasei* was found to be the most resistant of the species tested, showing resistance to five of the nine antibiotics tested (gentamicin, kanamycin, streptomycin, erythromycin, and tetracycline). These results

Table 2. MIC values (mg/L) of antibiotics in inhibiting the growth of lactic acid bacteria from Paški sir cheese production chain

|                           | Ampicillin | Vancomycin | Gentamicin     | Kanamycin       | Streptomycin      | Erythromycin | Clindamycin   | Tetracycline  | Chloramphenicol |
|---------------------------|------------|------------|----------------|-----------------|-------------------|--------------|---------------|---------------|-----------------|
| <i>Lc. lactis</i>         | 0.19 (2)   | 1 (4)      | 2 (32)         | 6 (64)          | 2 (32)            | 0.047 (1)    | 0.064 (1)     | 0.094 (4)     | 1 (8)           |
| <i>L. plantarum</i>       | 1 (2)      | n.r.       | 0.125 (16)     | 12 (64)         | n.r.              | 0.032 (1)    | 0.125 (4)     | 2 (32)        | 4 (8)           |
| <i>Cb. maltaromaticum</i> | 0.023 (2)  | 0.5 (2)    | 12 (16)        | <b>64</b> (16)  | <b>128</b> (16)   | 0.047 (1)    | 0.125 (4)     | 1 (4)         | 1 (4)           |
| <i>Lc. raffinolactis</i>  | 0.064 (2)  | 1 (4)      | 16 (32)        | <b>96</b> (64)  | <b>128</b> (32)   | 0.047 (1)    | 0.064 (1)     | 0.064 (4)     | 1.5 (8)         |
| <i>L. coryniformis</i>    | 1 (2)      | 1 (2)      | 2 (16)         | 1 (16)          | 4 (16)            | 0.032 (1)    | 0.125 (4)     | 1 (4)         | 1 (4)           |
| <i>E. faecalis</i>        | 0.5 (2)    | 1.5 (4)    | <b>96</b> (32) | 256 (1024)      | <b>1024</b> (128) | 1.5 (4)      | <b>12</b> (4) | <b>16</b> (4) | 1.5 (16)        |
| <i>L. brevis</i>          | 0.5 (2)    | n.r.       | 1.5 (16)       | 24 (64)         | 12 (64)           | 0.094 (1)    | 3 (4)         | 6 (8)         | 1.5 (4)         |
| <i>P. pentosaceus</i>     | 0.5 (4)    | n.r.       | 4 (16)         | <b>64</b> (64)  | 24 (64)           | 0.064 (1)    | 0.125 (1)     | 2 (8)         | 1 (4)           |
| <i>Ln. mesenteroides</i>  | 0.25 (2)   | n.r.       | 1.5 (16)       | 6 (16)          | 8 (64)            | 0.023 (1)    | 0.125 (1)     | 1 (8)         | 1.5 (4)         |
| <i>E. durans</i>          | 0.094 (2)  | 0.38 (4)   | 12 (32)        | 48 (1024)       | 96 (128)          | 0.125 (4)    | 0.016 (4)     | 0.124 (4)     | 1.5 (16)        |
| <i>E. faecium</i>         | 0.094 (2)  | 0.38 (4)   | 8 (32)         | 64 (1024)       | 48 (128)          | 2 (4)        | 0.032 (4)     | 0.094 (4)     | 1.5 (16)        |
| <i>E. italicus</i>        | 0.5 (2)    | 1.5 (4)    | <b>64</b> (32) | 256 (1024)      | <b>768</b> (128)  | 1.5 (4)      | <b>16</b> (4) | <b>8</b> (4)  | 1.5 (16)        |
| <i>L. paracasei</i>       | 0.19 (4)   | n.r.       | <b>64</b> (32) | <b>256</b> (64) | <b>384</b> (64)   | <b>1</b> (1) | 2 (4)         | <b>4</b> (4)  | 0.75 (4)        |
| <i>C. paralimentarius</i> | 1.5 (2)    | n.r.       | 12 (16)        | <b>256</b> (16) | <b>96</b> (16)    | 0.25 (1)     | 0.064 (4)     | 1 (4)         | 1.5 (4)         |
| <i>L. sakei</i>           | 0.023 (2)  | n.r.       | 2 (16)         | 2 (16)          | 4 (16)            | 0.125 (1)    | 1 (4)         | 1 (4)         | 1.5 (4)         |
| <i>L. curvatus</i>        | 0.38 (2)   | n.r.       | <b>16</b> (16) | <b>96</b> (16)  | 0.94 (16)         | 0.94 (1)     | 0.5 (4)       | 0.5 (4)       | 1 (4)           |

n.r. not recommended; in **bold** values are equal to or above MIC cut-off values (in brackets; mg/L)

are consistent with the study by Honi et al. [19], which emphasises the clinical importance of aminoglycoside resistance in the context of probiotics and their potential impact on resistance in the human gut microbiome. Generally, LAB are sensitive to antimicrobial substances that act as cell wall synthesis inhibitors, such as penicillins and beta-lactamase inhibitors, and are mostly resistant to aminoglycosides such as kanamycin, streptomycin, gentamicin, and neomycin [20], which was also observed in this work. Regarding other lactobacilli species, in our study, representatives of *L. cornyformis*, *L. brevis*, *L. sakei*, and *L. plantarum* were sensitive to all antibiotics tested. In other recent studies [21–23], resistance to additional antibiotics was reported in these species, such as oxacillin, fluoroquinolones, or fosfomycin. *Enterococcus faecalis* was the second most resistant species, showing resistance to gentamicin, streptomycin, clindamycin, and tetracycline. Similar results were obtained by Furlaneto-Maia et al. [24], who reported resistance of *E. faecalis* from soft cheese in Brazil to tetracycline, as well as to erythromycin and vancomycin, which was not observed in this study. Chingwaru et al. [25] also recorded a high resistance of *E. faecalis* isolates from milk to ampicillin, vancomycin and a very high resistance to tetracycline. Among the other enterococcal species, *E. italicus* was resistant to streptomycin, clindamycin, and tetracycline, while *E. durans* and *E. faecium* were sensitive to all antibiotics tested. It should be noted that the interpretation of antibiotic resistance in enterococci other than *E. faecium* in this study was performed according to the recommended criteria for *E. faecium* [9]. Teuber et al. [26] reported several decades ago that enterococci are frequently resistant to vancomycin, streptomycin, erythromycin, chloramphenicol, tetracycline, and clindamycin. Similarly, Vesković et al. [27] point out the acquired resistance of enterococci to chloramphenicol, clindamycin, erythromycin, aminoglycosides, beta-lactams, and tetracycline. The concern about enterococcal resistance lies in their ability to horizontally transfer genes for important antibiotics such as vancomycin and methicillin, allowing them to rapidly adapt and spread resistance among microbiota [28, 29]. These findings emphasise the importance of continuous monitoring of enterococcal resistance in the food chain and their ability to transfer resistance to pathogenic bacteria, which poses a serious risk to public health. However, the absence of resistance to vancomycin in our isolates suggests low risk in extensive sheep breeding and traditional cheese production. The use of glycopeptide

antibiotics in animals, such as vancomycin, is associated with the development of vancomycin-resistant enterococci that are transmissible from animals to humans. This poses a serious threat, as glycopeptides are among few treatment options for very severe enterococcal infections in humans [16].

Considering lactococci, in this study *Lc. lactis* was sensitive to all antibiotics, while *Lc. raffinolactis* was resistant to kanamycin and streptomycin. Devirgilis et al. [30] reported that lactococci often exhibit resistance to tetracycline, macrolides, glycopeptides, and polymyxins. In contrast to our results, Chen et al. [20] found diverse resistance profiles among *Lc. lactis* isolated from sheep's milk, including resistance to oxacillin, amikacin, neomycin, gentamicin, tetracycline, norfloxacin, furazolidone, sulfamethoxazole, polymyxin, ceftazidime, kanamycin, vancomycin, and doxycycline. Bulajić et al. [31] also concluded that lactococci from cheese (Serbia) are often resistant to streptomycin, ampicillin, vancomycin, and penicillin. The varying degrees of susceptibility and resistance of lactococci reported in the cited studies are likely due to differences in the extent of antibiotic exposure in animal husbandry and environmental factors, assuming that methodological differences and varying criteria for resistance assessment are disregarded.

The *Pediococcus pentosaceus* isolate from Paški sir cheese tested in this study exhibited resistance only to kanamycin, indicating a lower level of resistance compared to other studies on this dairy-derived species. For instance, Bhukya and Bhukya [32] reported resistance to streptomycin, vancomycin, ciprofloxacin, norfloxacin, and trimethoprim, in addition to kanamycin, in *P. pentosaceus* from buttermilk. Monitoring antibiotic resistance in *P. pentosaceus* is important, since it may carry transmissible resistance genes, particularly for erythromycin and tetracycline, as highlighted by Vesković et al. [27]. *Leuconostoc mesenteroides* was also sensitive to all antibiotics tested in our study, which is a favourable result compared to other reports [27].

## CONCLUSION

The results obtained indicate a moderate prevalence of antimicrobial resistance among selected representatives of LAB species identified from the production chain of

traditionally produced sheep's cheese. Most isolates were susceptible to the tested antibiotics, suggesting possible lower antibiotic pressure in extensive sheep farming on the island of Pag, Croatia. The isolates that showed complete sensitivity to antibiotics were *L. plantarum*, *Lc. lactis*, *E. faecium*, and *E. durans* and are potential candidates for further characterisation in the selection of cheese cultures. The phenotypically observed resistance of individual isolates requires further investigation regarding the resistance transfer to the exposed, non-resistant microbiota.

### Data Availability Statement

The raw data of this article will be made available by the authors, without undue reservation.

### Ethical Statement

This research was approved by the ethical committee of the Faculty of veterinary medicine, University of Zagreb, Croatia; approval number: 640-01/22-02/15; 251-61-01/139-22-28

### Conflict of Interest

The authors declare no conflict of interest.

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### Generative AI Statement

No generative AI and AI-assisted technologies were used in writing the manuscript.

### Authors' Contributions

Nevijo Zdolec – conceptualisation, analysis, writing and editing, resources; Marta Kiš – laboratory analysis;

Snježana Kazazić – MALDI TOF MS identification; Fabijan Oštarić – cheese production and sampling; Ena Horvat – laboratory analysis, writing; Nikola Čudina – writing and editing; Nataša Mikulec – resources, editing.

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