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Green synthesis of silver nanoparticles and their inhibitory effects on resistant udder pathogens

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ABSTRACT
The study covers a new process for synthesizing silver nanoparticles using dry lavender leaves (Lav-AgNPs) as a reducing and stabilizing agent, and evaluation of their inhibitory effect on the resistant udder pathogens. Of all 480 investigated dairy cows, 402 cows (83.8%) had a negative CMT score, and 78 cows (16.2%) had a positive CMT score (5.6% quarters), of which 94 (4.9%) represented quarters infected with a bacterial pathogen. The most frequent udder pathogens were non-aureus staphylococci (NAS), isolated from 46.7% of all positive samples. Other important udder pathogens were *Staphylococcus aureus* (*S. aureus*) (19.1%) and *Streptococcus uberis* (*Str. uberis*) (10.7%). Generally, the isolated staphylococci and *Str. uberis* showed high resistance to β-lactams and aminoglycosides. In particular, isolates that showed resistance to penicillin and gentamycin were tested for the inhibitory effect of pure AgNPs and synthesized Lav-AgNPs. Lav-AgNPs at a concentration of 200 ppm showed strong resistance to the tested pathogens, and an inhibition zone of 13±0.73 mm was formed for *S. aureus*, 16±1.15 mm for *Staphylococcus chromogenes* (*S. chromogenes*), 14±0.52 mm for *Staphylococcus haemolyticus* (*S. haemolyticus*), and 14±0.56 mm for *Str. uberis*. In conclusion, green synthesis provides an anti-bacterial product that represents an alternative to antibiotic substances.

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Green synthesis; silver nanoparticles; antibacterial activity; lavender leaves; *S. aureus*; *Str. uberis*

1. Introduction

Bovine mastitis affects all dairy herds, influences herd management, and ranks among the most frequently treated diseases. It is an inflammatory response of the mammary gland to invasion by microorganisms. It is considered a multifactorial disease, influenced by the sick animal (cow), pathogens, and the environment (Cobirka et al. 2020). The economic losses are a result of both direct and indirect damages caused by mastitis. The direct losses encompass expenses related to treatment, wasted milk, time spent on labour, deaths, and the costs associated with recurring instances of mastitis. The indirect losses encompass several negative consequences, such as heightened culling, diminished milk production, compromised milk quality, loss of financial benefits, premature cessation of lactation, animal welfare concerns, and other related health issues (Hassan et al. 2023). The most common causes of bovine mastitis are primarily bacteria, which have been confirmed in up to 90% of intramammary infections. The most common bacterial pathogens responsible for bovine mastitis are *S. aureus*, non-aureus staphylococci (NAS), *S. agalactiae, Str. uberis*, or other streptococcus species and coliforms (El-Diasty et al. 2019; Taponen and Pyorala 2009). These bacteria are commonly found in intramammary infections because they can be transmitted through various means, including contaminated milk from diseased cows, improper hygiene during milking, as well as through bedding, urine, faeces, and other sources of contamination (Haxhiaj et al. 2022).

In the veterinary therapy of mastitis, antibiotics have been used for over 40 years, ranging from narrow-spectrum to broad-spectrum. Mammary gland infections in dairy cows are traditionally treated either by systemic or intramammary use of antimicrobials, depending on the type of mastitis and the overall health status of the cow. Their routine utilization in cattle herds resulted in an increased resistance of the mammary gland pathogens, most likely associated with an excessive use or inadequate concentration of antibiotics (ATBs) (Holko et al. 2019). The increase in resistance of udder pathogens to antimicrobials was also supported by the fact that on most farms the cows were dried off with intramammary antibiotics to create a protective barrier in the udder during the

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mammary gland involution (Haveri et al. 2007). According to the study by Ferroni et al. (2020), management strategies contribute to the elevated antibiotic usage, particularly on intensive dairy farms. Researchers examined 101 cattle farms, both beef and dairy, in central Italy and compared the annual antibiotic consumption. Antibiotic administration was notably greater in dairy operations, with a threefold increase compared to beef farms. This surge was primarily linked to the use of antibiotics in lactating and drying cows.

The result of such therapeutic procedures is an increase in chronic intramammary infections with the presence of pathogens exhibiting persistent antibiotic resistance (Tiwari et al. 2013). The global spread of multi-drug resistant (MDR) microorganisms caused the researchers to focus on nanotechnology-based approaches to use the benefits of nanomaterials to combat antimicrobial resistance. The antibacterial properties of nanoparticles have been used in medicine, food industry, and cosmetics (Beyene and Ghosh 2022; Sumaiya et al. 2022).

Nanoparticles can be manufactured through a variety of techniques, primarily categorized as top-down (involving physical and physicochemical methods) and bottom-up (involving syntheses and reactions) methods. The top-down approaches encompass techniques like mill grinding, laser ablation, chemical vapor deposition (CVD), among others, which aim to break down the larger material structures into nanoparticles. In contrast, bottom-up approaches, which can be either chemical or biological, entail the synthesis and reactions that enable the step-by-step formation of nanoparticles using molecular or atomic constituents. Biomaterial-based synthesis is more environmentally friendly than chemical-based synthesis (Marambio and Hoek 2010). The biological (green) approach involves utilizing natural resources and physiologically active chemicals found in plants to create nanoparticles through synthesis. Nevertheless, biosynthesis is not without drawbacks, such as the potential for selecting an unsuitable biomaterial, resulting in increased costs and delays in the overall production process of nanoparticles. Therefore, it is necessary to choose a biomaterial suitable for the effective synthesis of nanoparticles with properties such as seasonal and geographical availability without time limitation, easy handling, rapid growth, and amenability to a simple preparation of an extract (Balážová et al. 2023; Malik and Sehrawat 2013).

Nevertheless, nanotechnology is an expanding discipline that presents the opportunity to create novel materials on a nanoscale, with significant potential to transform the dairy industry by providing innovative alternatives. There is an urgent need for new therapeutic alternatives to address prevalent and expensive diseases, like bovine mastitis, in order to decrease the reliance on antibiotics. The utilization of nanotechnology is progressively being incorporated into the development of novel treatments for bovine mastitis (Abd EL-Tawab et al. 2018; Baláž et al. 2017).

Silver nanoparticles, extensively examined with respect to their effect on bovine mastitis strains, exhibit a wide-ranging antimicrobial capacity at a nanoscale level. The liberation of silver ions and the integration of antibiotics with silver nanoparticles have demonstrated efficacy in eradicating bacteria, including those displaying antibiotic resistance (Abd El-Aziz et al. 2021; Bruna et al. 2021). These nanoparticles possess potent antifungal and antibacterial properties even at minimal concentrations, suggesting their potential in curbing bacterial proliferation (Siddiqi et al. 2018).

The main objectives of this study include biosynthesis of silver nanoparticles de-rived from dry lavender leaves (Lav-AgNPs) and evaluation of their antimicrobial activity against bacterial agents of bovine mastitis. This research contributes to the search for alternatives to antibiotics used for the treatment of mastitis in dairy cows.

2. Materials and methods

2.1. Extract and AgNPs colloid preparation

Lavender leaves were collected during the flowering period in the botanical garden in Košice. Silver nitrate ($AgNO_3$, 99.8%) purchased from Penta Chemical Unlimited was used as a silver precursor. Deionized water was used for the preparation of solutions and for dilution purposes. Extract preparation: Lavender leaves were washed twice with deionized water and dried at 45°C. The dried leaves were crushed in a mortar and mixed with 100 ml of deionized water. The mixture was then heated in a water bath at a temperature of 70–80°C for 10 min. This temperature range was chosen to achieve a good transfer of natural substances (contained in plant cells) into the extract. The mixture was then cooled to room temperature and the solid component was separated using filter paper. The filtered solution was centrifuged at 9000 rpm for 30 min. Figure 1 shows dried lavender leaves, $AgNO_3$ stock solution (Ag concentration 500 mg/l) and extract prepared from dried lavender leaves (Ex-L). Preparation of AgNPs: For the green synthesis of AgNPs, 200 ml of extract was added gradually to 1000 ml of $AgNO_3$ solution heated to 80°C with constant stirring over 10 min. After adding the extract solution was mixed for 20 min. The reduction of Ag^+ ions to Ag^0 and the formation of AgNPs was indicated by the color change of the solution and confirmed by UV-vis analysis.

2.2. Characterization of AgNPs

The prepared AgNP colloid solutions were analyzed by a UV-Vis spectrophotometer (UNICAM UV-Vis spectrophotometer UV4). The samples were measured in the wavelength range of 350–700 nm. A solution of H_2O:extract at the ratio of 5:1 was used as a control. The size and morphology of the nanoparticles were studied using transmission electron microscopy (TEM, JEOL model JEM-2000FX, an accelerating voltage of 200 kV). The examination of Ag nanoparticle size distribution was conducted using image analysis software (ImageJ, version 1.53t). Scanning electron microscopy (SEM/FIB ZEISS-AURIGA Compact) was used to observe the existence and morphology of AgNPs. The materials were analyzed for their phase composition using X-ray diffraction analysis (XRD) conducted with a ZEISS instrument from Oberkochen, Germany.

2.3. Dairy cow farms, housing and milking

For the practical purposes of the study, two dairy farms were monitored in eastern Slovakia. All monitored herds were housed in

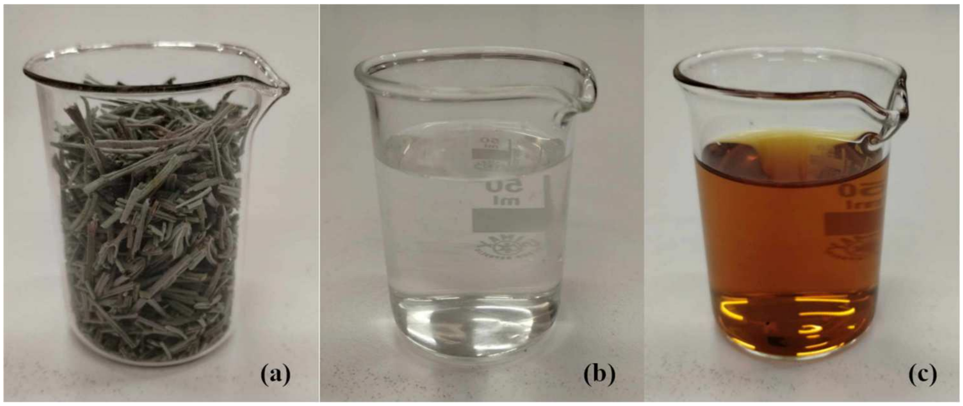

Figure 1. Dry lavender leaves (a), $AgNO_3$ solution (b) and extract of dried lavender leaves (c).

modern buildings with high-air ventilation and automated waste removal. In the stables, the cows were divided into two sections, each provided with a feeding table. Each section was further divided into four separate subsections.

During the study, 480 dairy cows from both farms were milked twice a day in a parallel or herringbone milking parlour (BouMatic). Prior to the milking process, the milk obtained from each section of the udder was subjected to sensory examination using a cup equipped with a double bottom. The teat disinfection procedure involved the application of a foaming solution, followed by drying with a disposable paper towel. Following the completion of automated milking, the last teat disinfection was carried out using a lactic acid-based product (Agromont Nitra, SR) when the milk flow rate dropped below 0.2 L/min.

2.4. Examination of cows and milk sampling

Before milk sampling, all 480 cows were subjected to clinical examination and udder palpation. After sensory assessment of milk forestrips from each quarter, they were evaluated using the CMT, according to Tančin (2013). For the laboratory diagnosis of bacterial pathogens according to National Mastitis Council (2001) guidelines, on the basis of positive CMT scores ranging from 1 to 3, 108 positive quarter milk samples were transported in coolers with ice packs to the Laboratory of the University of Veterinary Medicine and Pharmacy in Košice. The samples were collected from cows that did not receive any antibiotics during at least 7 previous days.

2.5. Cultivation of samples and detection of udder pathogens

Each milk sample (0.2 ml) was inoculated onto Columbia agar plates supplemented with 5% sheep blood (Oxoid Ltd, UK). These plates were then incubated at 37°C and assessed after 24 h. In order to more accurately determine the bacterial pathogens that cause mastitis, colonies from the blood agar plates were transferred to different selective culture media, including Edwards Medium, Staphylococcal Medium No. 110, and MacConkey Agar (Oxoid, Ltd., Basingstoke, Hants, UK). The subcultures were then incubated for 24 h at a temperature of 37°C. Subsequently, tests for catalase activity, hemolysis, pigment formation, coagulase, and gram staining were performed using the methods published by Malinowski et al. (2006). Species identification was performed using biochemical tests, including STAPHYtest 24, STREPTOtest 24, or ENTEROtest 24, and evaluated with the TNW Pro-Auto 7.0 software (Erba-Lachema, Brno, CZ).

All presumptive staphylococci and streptococci from the biochemical tests were confirmed by a matrix-assisted laser desorption/ionization (MALDI-TOF) biotyper (Bruker Daltonics, Leipzig, Germany). Mass spectrometry measurements were carried out on bacterial extracts prepared according to Ozbey et al. (2022). A single colony was selected with a sterile wooden stick and streaked onto a well in a 96-well special micro steel plate (MSP; Bruker Daltonics, Bremen, Germany) in a film-like manner. After drying, 1 µL of HCCA matrix solution was added and allowed to dry completely at room temperature. The MALDI 96 MSP was placed into the MALDI-TOF MS device and operated using an optimized method in linear positive ion mode in the mass range of 2000–20000 Da for the identification of microorganisms. A nitrogen laser operating at 337 nm with a frequency of 60 Hz was used as the ion source. Laser pulses consisting of 240 bursts of 40 packets were applied for each column measurement to obtain the spectra. Each sample was run in triplicate, and only isolates that showed a log score $\geq$ 2, indicating specific species identification, were included in further testing.

2.6. Detection of sensitivity to antimicrobials in selected udder pathogens

The susceptibility of the isolated udder pathogens was tested in vitro against 10 antimicrobial agents. The susceptibility tests were conducted on 22 isolates of *S. chromogenes*, 14 isolates of *S. aureus*, 9 isolates of *S. haemolyticus*, 8 isolates of *S. uberis*, and a reference strain of *S. aureus* CCM 4750 from the Czech Collection of Microorganisms in Brno, Czech Republic. The tests were performed on Mueller Hinton agar, with the addition of 5% sheep blood for the *Str. uberis* isolate, using the standard disc diffusion procedure. Before spreading on Mueller-Hinton agar, the suspensions of strains were prepared in physiological saline at a density of 0.5 McFarland from fresh cultures on agar (CLSI 2018). The antibiotic discs used in the current study were penicillin (PEN;10 µg), amoxicillin (AMC; 10 µg), amoxicillin+clavulanic acid (AXC; 20/10 µg), erythromycin (ERY; 15 µg), lincomycin (LNC; 10 µg), cefoxitin (CFX; 30 µg), ciprofloxacin (CPR; 5 µg), gentamycin (LNC; 10 µg), neomycin (NMC; 10 µg), streptomycin (STR; 10 µg), and tetracycline (TET; 30 µg). The diameter of the zone of inhibition was measured in millimeters and the findings were interpreted according to the previously established method. The measured sizes of the inhibitory zones were assessed (as susceptible, moderate, or resistant) based on CLSI breakpoints (CLSI 2021). The control used in the assays was the reference strain *S. aureus* CCM 4750 from the Czech Collection of Microorganisms in Brno, Czech Republic. The choice of antimicrobials reflects the range of intramammary products used for the treatment of mastitis available in Slovakia.

2.7. Antimicrobial activity of Lav-AgNPs against bovine mastitis in vitro

Testing of the antibacterial activity of Lav-AgNPs against selected udder pathogens which are the main cause of bovine mastitis, using the reference strain *S. aureus* CCM 4750 as the control, was conducted by means of the diffusion method. Based on the phenotypic resistance to β-lactams and aminoglycosides, antibiotics were selected for 4 isolates of *S. aureus* and 2 isolates from each mammary gland pathogens (*S. chromogenes, S. haemolyticus* and *Str. uberis*), together with the reference strain *S. aureus* CCM 4750, for further antibacterial activity testing of Lav-AgNPs and pure AgNPs. A sterilized cork borer was used to produce 3-mm-diameter bores. The plates were incubated at 37°C for 20 h. The antibacterial activity was tested at concentrations of 50, 100, 150, and 200 µg/mL of Lav-AgNPs and pure AgNPs (Figure 2). As the concentration of Lav-AgNPs increased, the bactericidal ability also increased, resulting in a larger diameter of the antibacterial ring. Distilled water was used as a negative control, and gentamicin sulfate and penicillin (Biosera, Nuaille, France) with a concentration of 10 µg/mL were used as positive control. The diameters of the inhibition zones were assessed using ImageJ 1.48 software (NIH Image, Bethesda, MD, USA) after incubation. The antibacterial effects of the samples were recalibrated using the following equation:

$$\%RIZD = [(IZ_{Sample}XIZ_{Negativecontrol})/IZD_{gentamicinorpenicillin}]X100$$

where RIZD is the percentage of the average relative inhibition zone and IZD is the diameter of the inhibition zone in mm (Balážová et al. 2023).

2.8. Statistical analysis

GraphPad Prism 5 (GraphPad, San Diego, CA, USA) was used for statistical analysis. The inhibition zones of antibacterial activity testing of Lav-AgNPs and pure AgNPs were compared using the Dunnett test, where the control values were the inhibition zones of the reference strain (*S. aureus* CCM 4750). The results were expressed as means (M) ± standard deviation SD, and a value of $P < 0.05$ (*) was considered significant.

3. Results

3.1. Characterization of AgNPs

Using a UV-vis spectrophotometer, the spectrum of a colloidal solution of AgNPs prepared using an extract from dried lavender leaves was measured, Figure 3. The spectrum shows an absorption maximum at 424 nm, which is typical of spherical AgNPs.

The size and the morphology of the AgNPs were determined using TEM analysis. The TEM microphotograph of AgNPs is shown in Figure 4(a) and confirms the presence of spherical nanoparticles. The size distribution histogram, Figure 4(b), shows that ~80% of AgNPs are within 20 nm in size.

The spherical silver nanoparticles (AgNPs) were subjected to detailed analysis using scanning electron microscopy (SEM), as depicted in Figure 5. The SEM analysis not only confirmed the presence of silver within the nanoparticles but also revealed the presence of additional elemental signals, including carbon (C), chlorine (Cl), oxygen (O), and calcium (Ca). These elements likely originated from the biological components present in the extract used during the synthesis of the nanoparticles. Specifically, the presence of carbon may have resulted from the carbon sputtering utilized during the sample preparation for SEM analysis.

3.2. Occurrence of mastitis and resistance of udder pathogens

An examination of two dairy herds showed that of 480 dairy cows, the 402 (83.8%) had a negative CMT score and 78 (16.2%) had a positive CMT score. A positive CMT score (1–3) for one or more quarters was detected in 108 (5.6%) out of 1920 examined quarters. Out of all 78 positive dairy cows, a subclinical form of mastitis was detected in 67 cows (14.0%) and manifested without visible clinical symptoms. The udder and milk appeared normal, but an infection was still present with a positive CMT score and an increased SCC. Clinical mastitis was detected in 11 (2.3%) dairy cows, manifested by local symptoms of pain in the affected quarters with altered milk secretion. Based on the anamnesis, a CMT score of trace or 1–3 was identified as bacterial agents causing clinical or subclinical mastitis in 94 (4.9%) quarter samples, and 14 samples were identified as negative or contaminated. Out of all culture-positive samples, 91.5% samples yielded a single culture and 8.5 samples yielded a mixture consisting of *E. coli* + NAS (n = 5) and *E. coli* + *Enterococcus* spp. (n = 3). NAS were the most frequent udder pathogens (46.8%), followed by *S. aureus* (19.1%), *Str. uberis* (10.6%) and *E. coli* (9.6%). Environmental pathogens accounted for 80.9% of positive samples. In particular, *S. aureus*, *S. haemolyticus, S. chromogenes* and *Str. uberis* were isolated from 21 infected quarters with clinical signs of mastitis (Table 1).

Fourteen (17.7%) isolates of *S. aureus,* 22 isolates of *S. chromogenes (*27.8%), 9 isolates of *S. haemolyticus* (11.4%) and 8 isolates of *Str. uberis* (10.1%) were confirmed by MALDI TOF with log score ≥ 2 indicating specific species identification and included in further testing of *in vitro* resistance to 10 antimicrobials (Table 2).

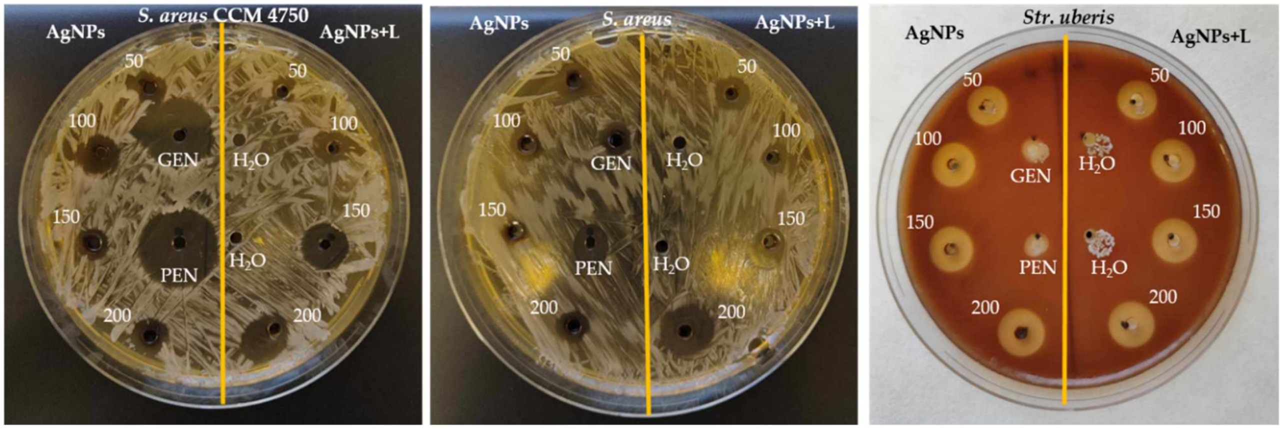

Figure 2. *In-vitro* bactericidal potential of Lav-AgNPs and pure AgNPs against selected resistant udder pathogens (µg/mL); PEN (penicillin; 10 µg); GEN (gentamicin; 10 µg); Lav-AgNPs – silver nanoparticles derived from dry lavender leaves; AgNPs – pure silver nanoparticles.

High resistance to β-lactams and aminoglycosides was noted in the tested staphylococci. Tetracycline, amoxicillin with clavulanic acid, rifaximin, and ciprofloxacin had a generally modest resistance. Intermediate sensitivity to aminoglycoside and β-lactams antimicrobials was detected in one and two isolates of *S. aureus*, respectively. The intermediate sensitivity to aminoglycoside antimicrobials was observed in two isolates of *S. chromogenes* and one isolate of *S. haemolyticus*. In the tested isolates of *Str. uberis* the highest resistance to aminoglycosides (50%) and β-lactams antimicrobials (25%) was recorded. In one isolate of *Str. uberis,* intermediate sensitivity to β-lactams antimicrobials was observed. The obtained results presented in Table 2 show that the resistance of *S. aureus*, NAS and *Str. uberis*, in particular to β-lactams and aminoglycosides, indicates the trend of increasing multi-antimicrobial-resistance of pathogens on the monitored dairy farms. Mainly, high resistance to streptomycin, gentamycin, ampicillin, penicillin and amoxicillin in the tested strains of *S. aureus* and NAS was observed.

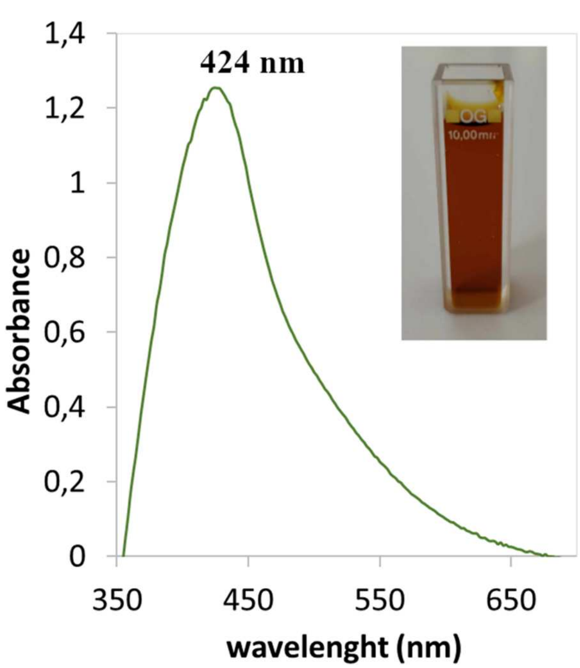

Figure 3. UV-vis analysis of AgNPs.

3.3. Antibacterial effects of pure AgNPs and Lav-AgNPs

The crystallographic phases present in the sample were determined using X-ray diffraction (XRD) analysis. By comparing the XRD of the sample with the Powder Diffraction File (PDF-2) database, the presence of silver was confirmed. The silver exhibited characteristic diffraction peaks at 20 angles of 38.10°, 44.37°, 64.18°, and 77.55°, corresponding to the (111), (200), (220), and (311) crystal planes of silver. The analysis revealed that the sample contained 91.6% silver. Additionally, the presence of silver chloride (AgCl) was identified, with its diffraction pattern matching PDF 00-031-1238, accounting for 8.4% of the sample.

According to CLSI breakpoints, the concentration for PEN (penicillin; 10 µg) and gentamicin (10 µg) with an inhibition zone of ≥ 29 and ≥ 15 mm for tested bacteria was only demonstrated by the reference strain *S. aureus* CCM 4750. Other isolates were resistant to the tested antibiotics with resulting inhibition zones below the recommended value. In addition to the disc diffusion method with antibiotic discs, resistant isolates were tested at concentrations of 50, 100, 150, and 200 µg/mL of Lav-AgNPs and pure colloidal solution of AgNPs. Table 3 shows that pure colloidal particles of AgNPs did not have such an inhibitory effect as the colloidal solution of Lav-AgNPs prepared using an extract from dried lavender leaves in the same concentrations. As the concentration of Lav-AgNPs increased, the bactericidal ability also increased, resulting in a larger diameter of the antibacterial ring. The maximum inhibition zone of Lav-AgNPs was 13 ± 0.48 mm for *S. aureus*, 14 ± 0.52 mm for *S. haemolyticus*, 16±1.05 mm for *S. chromogenes* and 14 ±0.56 mm for *Str. uberis* (Table 3).

Figures 6 and 7 illustrate the calculation of the relative antibacterial effect of AgNPs and Lav-AgNPs with respect to the tested antimicrobials. In the first case (Figure 6), inhibition zones were recalculated for penicillin, and their percentage effect at different concentrations of AgNPs and Lav-AgNPs against all tested pathogens. The calculation showed that concentrations of 150 and 200 µg/ml of Lav-AgNPs exhibited

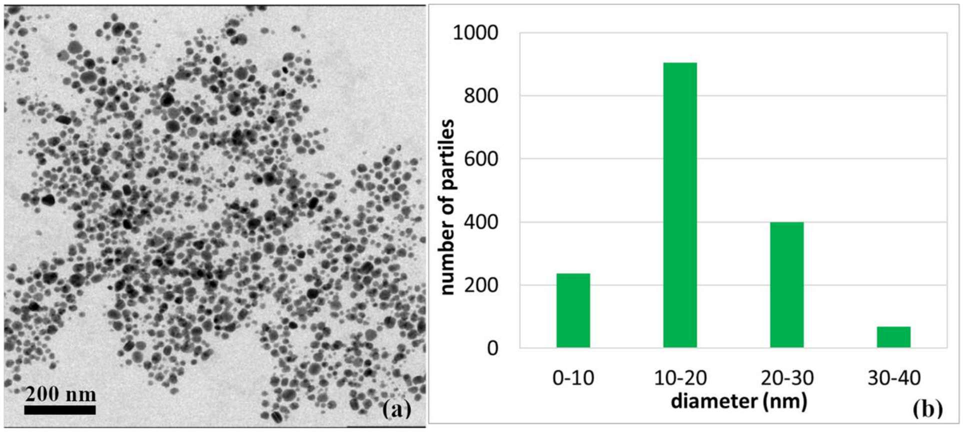

Figure 4. TEM microstructure of AgNPs (a), and size distribution of nanoparticles (b).

a 100% or higher effect against *S. aureus* and *Str. uberis*. For the other tested NAS, the relative effectiveness was below 100%.

In the second case (Figure 7), the relative antibacterial effectiveness of AgNPs and Lav-AgNPs was calculated relative to gentamicin. By comparing the concentrations and inhibition zones, a 100% or higher effect of Lav-AgNPs at concentrations of 150 and 200 µg/ml was confirmed against all tested pathogens. This indicated that a colloidal solution of silver nanoparticles prepared using an extract from dried lavender leaves is a complete substitute for antimicrobials from the class of aminoglycosides.

Values in rows labelled with different letters (Aa,Bb,Cc,) exhibit statistical significance ($p < 0.05$) according to Tukey's test of ANOVA; [1]PEN 10 µg/mL, [2]GNT 10 µg/ml; AgNPs – pure colloidal silver nanoparticles; AgNPs+Lav – colloidal solution of silver nanoparticles prepared using an extract from dried lavender leaves.

SA ref – reference strain *S. aureus* CCM 4750; SA – *S. aureus* isolated from mastitic samples; SH – *S. haemolyticus* isolated from mastitic samples; SCH – *S. chromogenes* isolated from mastitic samples; Str. U – *Str. uberis* isolated from mastitic samples; AgNPs – pure colloidal silver nanoparticles; AgNPs+L colloidal solution of silver nanoparticles prepared using an extract from dried lavender leaves.

4. Discussion

4.1. Occurrence of mastitis and antibiotic resistance of udder pathogens in dairy farms

Despite improvements in zootechnical and animal hygiene conditions in dairy farming over the past decade, the biggest challenge remains the rapid identification of 'problem cows' and determining the mastitis causing pathogens. Currently, there are numerous direct and indirect tests available on the market for mastitis detection, but only a small number of them are used directly in practice (Neculai-Valeanu and Ariton 2022).

Among the most common indirect methods for detecting mastitis on production farms is the monitoring of somatic cell count (SCC) using the California Mastitis Test (CMT). For suspicious cows that do not exhibit clinical signs of mastitis but

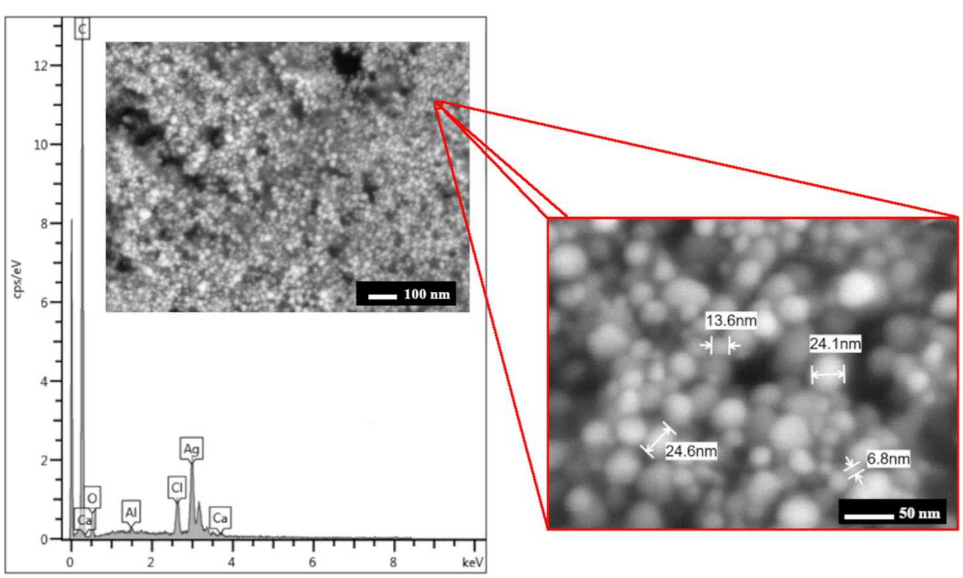

Figure 5. Energy Dispersive X-ray Spectroscopy (EDX) analysis of AgNPs; the inner picture shows SEM microstructure of AgNPs prepared using Ex-L.

Table 1. CMT evaluation and representation of udder pathogens after MALDI-TOF identification.

Status	Number of tested cows	%	
Cows with negative CMT	402	83.8	
Cows with positive CMT	78	16.2	
SM[1]	67	14.0	
CM[2]	11	2.3	
	Number of tested quarters	%	
Total tested quarters	1920	100.0	
Negative	1812	94.4	
CMT score 1 - 3	108	5.6	
Infected	94	4.9	
Udder pathogens	**Number of isolates**	% (out of 94 infected quarter milk samples)	
		SM[1]	**CM[2]**
S. chromogenes	24	23.4	2.1
S. aureus	18	8.5	10.6
S. haemolyticus	10	5.3	5.3
S. warneri	5	5.3	-
S. epidermidis	5	5.3	-
Str. uberis	10	6.4	4.3
E. coli	9	9.6	-
Enterococcus spp.	5	5.3	-
Mixed infection[3]	8	8.5	-

SM[1]: Subclinical mastitis is a condition when no visible indications of infection are noticed in the udder or milk, but there is still an underlying infection present. This can be determined by a positive California Mastitis Test (CMT) score and an elevated Somatic Cell Count (SCC). CM[2] refers to clinical mastitis, which can present with a range of symptoms varying in severity. These symptoms include a positive CMT score, bacteriological culture, a high level of SCC, changes in the consistency of the milk such as the presence of flakes, clots, or pus, and a decrease or complete loss of milk output. Mixed infection[3] – involve a combination of infection with two bacterial infections.

show visual changes in milk during udder preparation and cleaning for milking, it is advisable to perform CMT on all quarters of the udder with sampling for pathogen culture (Tančin 2013).

Through examination of the udders of 480 cows from two farms, we found positive CMT scores in 78 cows for one or more quarters. Out of the 108 quarter milk samples collected, bacterial pathogens causing clinical or subclinical mastitis were confirmed in 94 (4.9%) samples.

Based on the anamnesis, CMT score and laboratory examination, a subclinical form of mastitis was confirmed in 67 cows (13.9%) and in 11 cows (2.3%) clinical manifestations of IMI were visible with swelling of the affected quarters and a change in milk secretion. Our results indicate that the prevalence of CM on monitored farms was in contrast with the studies by Rahman et al. (2010) and Silva et al. (2021), who reported the prevalence of clinical forms from 2.0% to 4.1% in lactating cows.

According to Singha et al. (2021), subclinical cases of mastitis are more common than the clinical cases of IMI, which was confirmed in our study in 13.9% positive dairy cows. On average, in the herd there are 15–40 undetected cases of SM for every CM in cows. Olivares-Pérez et al. (2015) determined the rate of subclinical mastitis as 20.5% in 259 dairy animals in a study conducted in Mexico. There is an increase in SCC that leads to reduced milk production to the level of 60–140 L per cow per year in animals with subclinical mastitis.

Holko et al. (2019) identified *S. aureus*, *Str. agalactiae*, *Str. uberis*, NAS, and gram-negative bacteria as the primary bacterial pathogens responsible for mastitis in dairy cows. Primarily, intramammary infections (IMI) caused by *S. aureus* and *Str. uberis* can range in severity from asymptomatic to clinical mastitis. Subclinical infection is frequently asymptomatic. The persistent subclinical infection may occasionally advance to clinical mastitis, characterized by significant alterations in milk (clotting, haemorrhage) and the udder (pain, swelling), along with systemic symptoms (fever, loss of appetite). In this investigation, we found that *S. aureus*, *S. haemolyticus*, *Str. uberis*, and *S. chromogenes* were the predominant pathogens responsible for CM.

Recently, non-aureus staphylococci (NAS) have raised concern among dairy farmers due to their observed potential as mastitis-causing pathogens, often being the most commonly isolated pathogens. The prevalence of NAS can be attributed to their ability to thrive in cows, residing in the mammary gland of both diseased and healthy animals. Additionally, some NAS species exhibit greater antibiotic resistance compared to *S. aureus* (Hosseinzadeh and Saei 2014; Supre et al. 2011).

Among all NAS found in cattle, *S. haemolyticus, S. chromogenes, S. epidermidis, S. warneri, S. cohnii, S. simulans, S. hominis, S. capitis,* and *S. xylosus* are the most prevalent species (Hamel et al. 2020; Supre et al. 2011).

A similar occurrence as in the studies mentioned above was observed in our study, showing an increase in NAS from mastitis samples on the monitored farms. Out of the 94 infectious

Table 2. Antimicrobial resistance of selected udder pathogens isolated from mastitic milk samples.

Bacterial strains ($n = 53$)	*S. aureus* ($n = 14$)		*S. chromogenes* ($n = 22$)		*S. haemolyticus* ($n = 9$)		*Str. Uberis* ($n = 8$)	
Antibiotic/resistance	%	n	%	n	%	n	%	n
PEN	*50.0*	7	*40.9*	9	*44.4*	4	*25*	2
AMC	*50.0*	7	*36.4*	8	*33.3*	3	*25*	2
AXC	*7.1*	1	*9.1*	2	*0.0*	0	*0.0*	0
ERT	*21.4*	3	*9.1*	2	*11.1*	1	*25.0*	2
RFX	*14.2*	2	*9.1*	2	*0.0*	0	*12.5*	1
LNC	*28.6*	4	*13.6*	3	*11.1*	1	*25.0*	2
CPR	*14.2*	2	*13.6*	3	*0.0*	0	*12.5*	1
GNT	*28.6*	4	*9.1*	2	*22.2*	2	*50.0*	4
STR	*28.6*	4	*22.7*	5	*22.2*	2	*50.0*	4
TET	*14.2*	2	*4.5*	1	*0*	0	*25.0*	2

PEN – penicillin; AMC – amoxicillin; AXC – amoxicillin+clavulanic acid; ERT – erythromycin; RFX – rifaximin; LNC – lincomycin ; CPR – ciprofloxacin; GNT – gentamycin; STR – streptomycin; TET – tetracycline.

Table 3. Inhibition zone of AgNPs and Lav-AgNPs against udder pathogens causing bovine mastitis (mean ± SD).

Concentration	Zone of inhibition (M ± SD, mm) *S. aureus* CCM 4750		*S. aureus*		*S. haemolyticus*		*S. chromogenes*		*Str. uberis*	
[1]PEN	30 ± 0.91		5 ± 0.30		14 ± 0.65		18 ± 0.74		5 ± 0.45	
[2]GNT	23 ± 1.05		8 ± 0.55		10 ± 0.70		10 ± 0.95		5 ± 0.30	
	AgNPs	**AgNPs + Lav**	**AgNPs**	**AgNPs + Lav**	**AgNPs**	**AgNPs + Lav**	**AgNPs**	**AgNPs + Lav**	**AgNPs**	**AgNPs + Lav**
50 µg/ml	5 ± 0.40^{A}	4 ± 0.54^{a}	8 ± 070^{B}	6 ± 0.72^{b}	5 ± 0.42^{A}	6 ± 0.20^{b}	8 ± 0.65^{B}	9 ± 0.30^{c}	8 ± 0.84^{B}	8 ± 0.75^{c}
100 µgm/l	8 ± 0.46	6 ± 0.36^{a}	6 ± 0.55	4 ± 0.41	5 ± 0.35	7 ± 0.46	6 ± 0.70	10 ± 0.54^{b}	9 ± 0.60	9 ± 0.58^{b}
150 µg/ml	6 ± 0.35^{A}	10 ± 0.46^{a}	2 ± 0.48^{B}	9 ± 0.66	7 ± 0.65^{A}	12 ± 1.1	6 ± 0.82^{A}	12 ± 0.70^{b}	12 ± 0.54^{C}	12 ± 0.60^{b}
200 µgm/l	8 ± 0.65^{A}	13 ± 0.73^{a}	7 ± 0.74	13 ± 0.48	7 ± 0.60	14 ± 0.52	8 ± 0.60	16 ± 1.05^{b}	14 ± 0.80^{B}	14 ± 0.56

Values in rows labeled with different letters (Aa,Bb,Cc,) exhibit statistical significance ($p < 0.05$) according to Tukey's test of ANOVA; 1PEN 10 µg/mL, 2GNT 10 µg/ml; AgNPs - pure colloidal silver nanoparticles; AgNPs+Lav - colloidal solution of silver nanoparticles prepared using an extract from dried lavender leaves.

samples, NAS was isolated in 46.8%, including *S. chromogenes*, *S. haemolyticus*, *S. warneri*, and *S. epidermidis*. Particularly, *S. chromogenes* and *S. haemolyticus* were confirmed in both subclinical and clinical mastitis cases, indicating they may possess a similar aggressive potential as *S. aureus*.

In a Finnish study investigating the identification and cause of mastitis, Taponen and Pyorala (2009) highlighted a much greater likelihood of clinical mastitis (CM) resulting from *S. aureus* and NAS. This finding was also validated in both dairy herds observed in the study. Out of the 94 samples that were contaminated, NAS (46.8% of positive findings) and *S. aureus* (19.1%) were the most commonly discovered, accounting for 62 cases or 65.9% of the total. Our study found that the occurrence of mastitis on the Slovak dairy farms we evaluated was higher for *S. aureus* and certain NAS compared to other pathogens affecting the udder.

The main complications associated with the treatment of intramammary staphylococcal or streptococcal infection include the fact that many strains can exhibit multiple virulence factors. Their ability to form biofilms and resistance to antimicrobials are among the key virulence factors that facilitate the adherence and colonization of these pathogens in the mammary gland epithelium with ineffective antimicrobial treatment (Vasiľ et al. 2012).

The resistance to one or more antimicrobials detected in our study involved 20 isolates (44.4%) of staphylococci and 4 isolates (50%) of *Str. uberis*. The tested staphylococci phenotypically showed high resistance to a combination of antimicrobial classes, such as β-lactams and aminoglycosides. Similarly, the tested *Str. uberis* showed the highest resistance to aminoglycosides and β-lactam antimicrobials (Table 2).

The rise in resistance is also attributed to the practice of administering antimicrobials to dairy cows during the drying-off process, in addition to treating clinical cases of IMI during lactation. The largest proportion of antimicrobials is administered for the intramammary treatment of CM and dry cow therapy. According to the studies by Ferroni et al. (2020) and Crispie et al. (2004), dairy management practice is associated with increased use of antimicrobials in comparison with farms without market milk production. This is also the reason why almost all drugs commonly used in veterinary practice are no longer effective against the pathogens isolated from SM and CM.

The research conducted by Holko et al. (2019), Idriss et al. (2013), and Zigo et al. (2022) on dairy farms in Slovakia has confirmed that udder pathogens (*S. aureus*, *Str. uberis*, and *Str. agalactiae*) as well as NAS have shown increased resistance to antibiotics commonly used for intramammary treatment of dry cows. These findings have raised concern about the future emergence of beta-lactam resistance in *S. aureus*, NAS, and *Str. uberis*. A study conducted in 2016 revealed that over half of dairy farmers in Chiang Mai province typically administered penicillin/streptomycin (52.31%) for systemic treatment of cattle diseases (14). Furthermore, the majority of dairy farmers in the mentioned area utilized ampicillin/cloxacillin (87.7%), followed by cephapirin (82.3%), for intramammary treatment of bovine mastitis (Udin 2016). Given that β-lactam antimicrobials are commonly employed for mastitis treatment,

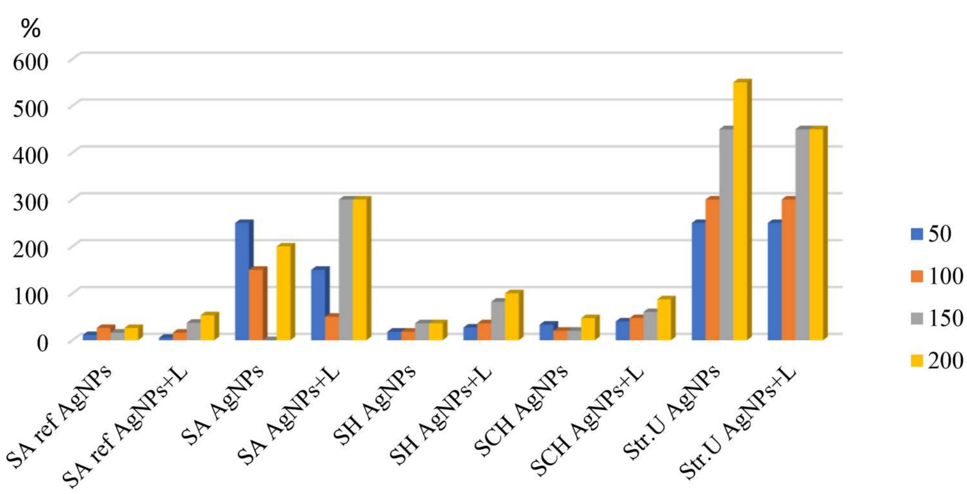

Figure 6. Relative efficiency of AgNPs and Lav-AgNPs with respect to penicillin (%).

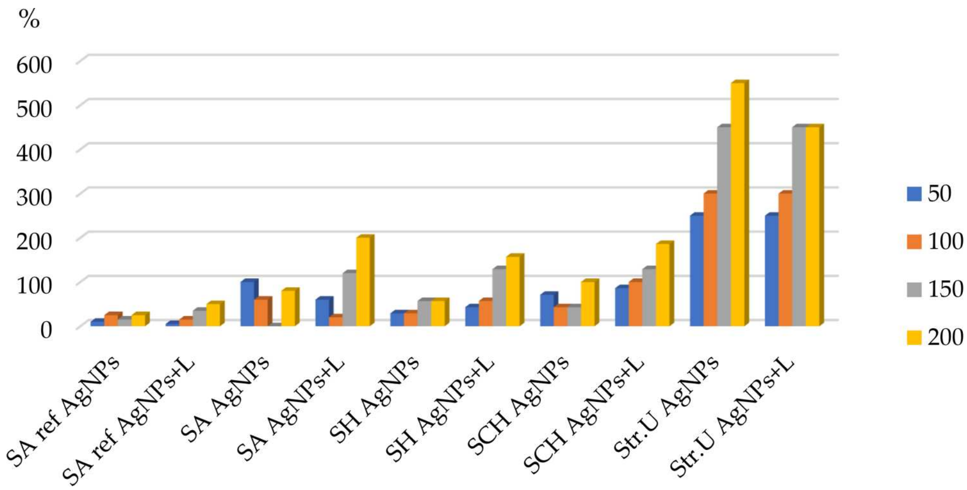

Figure 7. Relative efficiency of AgNPs and Lav-AgNPs with respect to gentamicin (%).

Note: SA ref – reference strain *S.* aureus CCM 4750; SA – *S.* aureus isolated from mastitic samples; SH – *S.* haemolyticus isolated from mastitic samples; SCH – *S.* chromogenes isolated from mastitic samples; Str. U – *Str. ube*ris isolated from mastitic samples; AgNPs – pure colloidal silver nanoparticles; AgNPs+L colloidal solution of silver nanoparticles prepared using an extract from dried lavender leaves.

the escalating usage of these drugs may serve as crucial evidence regarding the efficacy of treating bovine mastitis caused by staphylococci and streptococci in the future. Continuous monitoring of antibiotic usage and antimicrobial resistance (AMR) among udder pathogens on dairy farms is essential to adapt treatment protocols for bovine mastitis accordingly.

4.2. Effect of green synthesis of silver nanoparticles on the reduction of udder pathogens

In response to the growing resistance of bacterial infections and the presence of Methicillin-Resistant Staphylococcus (MRS) in veterinary medicine, the European Union intends to decrease the sales of antimicrobial drugs for livestock by 50% by the year 2030. This initiative is part of the '*Farm to Fork*' strategy (EU 2020). One of the promising options for replacement and reduced use of antibiotics is the green synthesis of silver nanoparticles which demonstrated a significant inhibitory effect on pathogens in human and veterinary medicine (Siddiqi et al. 2018).

Our results confirmed that the use of biological synthesis of silver nanoparticles prepared using an extract from dried lavender leaves opens a new perspective in the field of green and sustainable technologies. They allowed us to conclude that Lav-AgNPs act as antibacterial agents effective against resistant udder pathogens. Similar results were obtained in the study by Zhongbing et al. (2008) in which gram-positive membrane disorganization was confirmed by SEM of bacteria in ultrathin sections.

The advantage of using silver nanoparticles lies in their action, which is free of toxic impact on cells while being capable of antimicrobial effects and inhibiting bacterial growth even at low concentrations. The most common mechanism of antibacterial activity of Ag nanomaterials is based on the principle of surface interaction of nanoparticles with bacterial cells or the action of Ag+ ions generated during the dissolution of Ag0 nanostructures. Increasing the concentration of free radicals in the environment is another potential mechanism for disrupting microbial cell membranes and internal structures (Baláž et al. 2017).

Shalabayev et al. (2019) demonstrated that nanoparticles exhibit an affinity for phosphorus or sulfur, making proteins within the cell membrane or inside the cell, which contain sulfur such as DNA, the prime targets for nanoparticle binding. The surface area and dimensions of silver nanoparticles enable them to access and attach to the DNA within the cells. As they bind to the constituents present in the cell membrane, they prompt the release of silver ions, thereby enhancing cell membrane permeability. Nanoparticles with larger surface areas facilitate optimal contact and interaction with microbial cells.

Another proposed mechanism for explaining the effect of AgNPs on the cell wall is the relationship between the thickness and composition of the cell wall, as well as the presence of peptidoglycan and the charge of lipopolysaccharide (Siddiqi et al. 2018). Balážová et al. (2022) documented that AgNPs have antibacterial and antifungal properties. The antibacterial efficacy of AgNPs can be influenced by the morphology, dimensions, and concentration of the nanoparticles. The size has been shown to have a strong effect on activity. Spherical, rod-shaped or truncated triangular nanoparticles showed various biocidal activities against *E. coli* in the study by Pal et al. (2007). We synthesized spherical nanoparticles, with 81% of them having an average size of around 20 nm. Malik and Sehrawat (2013) established the predominance of spherical silver nanosized crystallites (16–30 nm) after a short incubation period using an ethanolic extract of *A. maurorum*. Authors Vo-Van et al. (2023) confirmed that the spherical shape of AgNPs and a size distribution of 15–47 nm have been effectively encapsulated by the plant extract used for their surface interaction with bacterial cells of *S. aureus* and *Pseudomonas aeruginosa* at concentrations ranging from 50 to 200 µg/mL.

The inhibitory effect of AgNPs was tested against *S. aureus*, NAS, and *Str. uberis*. To differentiate the antimicrobial effect of nanoparticles prepared from the extract, the activity of pure nanoparticles was also monitored.

In our case, pure AgNPs at concentrations of 50, 100, 150 and 200 μgm/l showed the same lower antibacterial activity against *S. aureus* and NAS compared with the nanoparticles prepared via green synthesis. On the other hand, the antibacterial activity of pure silver nanoparticles at concentrations of 150 and 200 μg/ml against *Str. uberis* was comparable to silver nanoparticles prepared via green synthesis (Lav-AgNPs). For the tested staphylococci it was demonstrated that pure AgNPs at various concentrations without antibacterial support from the plant material were not able to sufficiently inhibit the bacterial growth. Opinions on the use of pure silver nanoparticles or their encapsulated form produced by green synthesis for antibacterial effects vary. Dehkordi et al. (2011) observed the antibacterial effectiveness of pure silver nanoparticles against *S. aureus* strains obtained from subclinical mastitis-afflicted cows. Using a broth dilution method, they determined the minimum inhibitory concentrations (MIC) for the tested *S. aureus* field isolates. Their findings revealed that MIC values ranging from 1.25 to 10 μg/ml, and specifically MIC values of 5 and 10 μg/ml for *S. aureus* inhibited 50% and 90% of the tested isolate population, respectively. This in vitro outcome strongly suggests that pure silver nanoparticles exhibit considerable activity against *S. aureus* originating from mastitis.

A similar study on the antibacterial effect of silver nanoparticles was conducted by Abd El-Aziz et al. (2021). The udder pathogen *Str. agalactiae* isolated from infected cows with clinical mastitis was tested against AgNPs, which ranged from 15 to 50 nm, had a spherical shape, and a 50% and 90% minimum inhibitory concentration of 8 and 16 μg mL^{-1}, respectively; also, the combination of AgNPs and cinnamon oil showed reasonable antimicrobial activity against the tested pathogen.

Unlike the use of pure AgNPs for the inhibition of bacterial pathogens, many studies emphasized the effectiveness of AgNPs prepared via green synthesis using various plant extracts (Gupta et al. 2020; Marslin et al. 2015; Salayová et al. 2021; Vo-Van et al. 2023). The findings of our investigation show that Lav-AgNPs, produced using an extract derived from dehydrated lavender leaves, displayed remarkable antibacterial efficacy against *Staphylococcus aureus* and *Str. uberis* in a controlled laboratory setting. This is important because intramammary infections produced by bacteria that are resistant to antibiotics are challenging to treat with antibiotics (Holko et al. 2019). Our findings correspond to the conclusions of Marslin et al. (2015) who also showed that AgNPs prepared via green synthesis strongly inhibited the growth of *Staphylococcus aureus* and *Escherichia coli* isolated from mastitic milk samples.

Gupta et al. (2020) demonstrated excellent antibacterial activity of AgNPs synthesized using curcumin as a reducing and stabilizing agent (Cur-AgNPs) against *S. aureus, Pseudomonas aeruginosa*, and *Candida auris.* Thus, the obtained results showed that using AgNPs encapsulated by a suitable plant extract appears promising for further research.

The above results indicate that AgNPs showed better antimicrobial activity against resistant *S. aureus* and *Str. uberis* when used in the form of Lav-AgNPs after green synthesis. Incorporation of AgNPs into an extract from dried lavender leaves changed the surface charge, as determined by our measurement. The negatively charged tested udder pathogens were resistant to AgNPs alone but, in combination with the extract from dried lavender leaves, the activity was higher than that of the antibiotics penicillin and gentamicin.

Toxicity is a significant concern when using silver nanoparticles, as they can induce oxidative stress, inflammation, and cellular damage in mammalian cells (Elbehiry et al. 2019). The antibacterial activity of silver nanoparticles can be optimized through modifications, such as altering their size, shape, and surface properties. Understanding these pathways can enhance their efficacy against bacteria while minimizing potential toxic effects, aiding in the development of more effective and safer silver-based antibacterial agents. For the treatment of mastitis, the route of administration is crucial for ensuring that silver nanoparticles effectively reach the site of infection within the mammary gland. Currently, topical or intramammary routes are considered, but these methods must be refined to maximize retention time and minimize systemic absorption that could lead to toxicity. Additionally, the effectiveness of Lav-AgNPs might be compromised by the presence of biofilms or the complex structure of mammary tissue, which can hinder their antibacterial action (Menichetti et al. 2023).

In our study, we attempted to highlight the possibility of synthesizing AgNPs using a method that is both time-and cost-effective and, importantly, does not pose an environmental burden. Our results indicate that AgNPs prepared in this way have wide applicability and represent a relevant contribution to the issue of the biosynthesis of nanoparticles.

5. Conclusions

The results of the study indicated that, in addition to the major udder pathogens *S. aureus* and *Str. uberis*, NAS is another major risk to dairy cows. Causal udder pathogens and NAS, such as *S. chromogenes*, and *S. haemolyticus* showed high resistance to β-lactam and aminoglycoside antibiotics, which are often used for the treatment of dairy cows during lactation and drying. This study points to new possibilities for the elimination of resistant udder pathogens without the use of antibiotics involving green synthesis. In this study, lavender leaves were utilized as both a reducing agent and a stabilizer for synthesizing AgNPs. The UV-visible spectroscopy analysis of the samples indicated that the dispersion of silver nanoparticles exhibited a localized surface plasmon resonance absorbance at a wavelength of 430 nm. The Lav-AgNPs that were prepared have a spherical morphology, with an average diameter of 20 nanometers. Furthermore, the Lav-AgNPs exhibited antimicrobial activity at concentrations of 150–200 μg/ml against the resistant *S. aureus* and *Str. uberis* known to cause bovine mastitis.

Due to their relatively rapid, less expensive, and widespread applications, the plant materials mediating the synthesis of silver nanoparticles appear promising for antimicrobial therapy in the dairy sector. Because of the rising incidence of antibiotic resistance of the causative isolates, more research on alternatives to antibiotics needs to be considered.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Authors' contributions

Conceptualization, SO and FZ; methodology, LM; software, FZ; validation, OV; formal analysis, ZF; investigation, SO and FZ; resources, IFR; data curation, SO; writing-original draft preparation, FZ; writing-review and editing, JZ and EP; visualization, JO; supervision, EP; project administration, ZL. and MV; funding acquisition, FZ. FZ. All the authors revised and edited the manuscript. All authors have read and approved the final manuscript.

Institutional review board statement

The clinical examination of the cows and the collection of milk samples for this study were authorized by the Ethics Committee at the University of Veterinary Medicine and Pharmacy in Košice under the reference number EKVP 2022/05, in accordance with EU legislation 2010/63/EU, specifically article 1:5 which pertains to practices that are not expected to cause pain, suffering, distress, or lasting harm equivalent to or greater than that caused by the implementation of good veterinary practice.

Data availability statement

The data that supports their conclusions is available from the authors of this manuscript upon request.

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