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First Report on the Phenotypic and Genotypic Susceptibility Profiles to Silver Nitrate in Bacterial Strains Isolated from Infected Leg Ulcers in Romanian Patients

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Abstract: Silver-ion-based antiseptics are widely used in treating chronic leg ulcers and, given the emergence of resistance to such compounds, the investigation of silver susceptibility and resistance profiles of pathogenic strains isolated from this type of wound is a topic of great interest. Therefore, in this study, 125 bacterial strains isolated from 103 patients with venous ulcers were investigated to elucidate their susceptibility to silver-nitrate solutions in planktonic and biofilm growth states, and the associated genetic determinants. The isolated strains, both in the planktonic and biofilm growth phases, showed high sensitivity to the standard concentration of 1/6000 silver-nitrate solution. It was noticed that even at concentrations lower than the clinical one (the first 2–3 binary dilutions in the case of planktonic cultures and the first 6–7 binary dilutions in the case of biofilms), the antiseptic solution proved to maintain its antibacterial activity. The phenotypic results were correlated with the genetic analysis, highlighting the presence of silver-resistance genes (sil operon) in only a few of the tested *Staphylococcus* sp. (especially in *S. aureus*) strains, *Escherichia coli* and *Pseudomonas aeruginosa* strains. These results demonstrate that despite its large use, this antiseptic remains a viable treatment alternative for the management of chronic leg wounds.

Keywords: silver nitrate; chronic leg wounds; susceptibility; resistance; sil operon

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1. Introduction

Leg ulcers are lesions due to a lack of skin below the knee that, with chronic evolution for more than six weeks, can occur as a consequence of a primary or secondary circulatory disease [1,2]. This pathology represents a significant health problem, affecting 1–2% of the world's population [3]: between 0.6–3% of individuals over 60 years of age and over 5% of those over 80 years, respectively. The prevalence of leg ulcers varies between 1.9–13.1% and, during lifetime, about 10% of the population will develop such a disease, with a mortality rate of 2.5% [1]. In Romania, there are no statistics related to the prevalence of this disease, excepting the SEPIA study conducted in 2004, which was the first epidemiological survey performed in Romania on the prevalence of chronic venous insufficiency, showing that approximately 32% of patients had signs or symptoms of venous disease, including skin ulcers, more than half of which had not been diagnosed before inclusion in the study and were thus untreated [4]. Regarding the worldwide distribution, the incidence of leg ulcers in different regions of the world varies as follows: in the US, 2–3 million patients are reported annually; in the UK: 3.5 cases per 1000 individuals; Ireland: 0.12 cases per

100 individuals; Switzerland: 0.2 cases per 1000 individuals; New Zealand: 79 cases per 100,000 individuals; China: 1.5–20.3 cases per 100 individuals [1].

The main features involved in leg-ulcer pathology are: increased enzymatic activity of matrix proteases [5], especially metalloproteinases [6], increased local concentration of proinflammatory cytokines that trigger an exaggerated inflammatory response, cell senescence [5], decreased angiogenesis and degradation of the newly formed cell matrix [6], and deficiency of response to growth factors [5], which causes the low mitogenic activity of tissue cells and a delayed repair phase [7]. The evolution of leg ulcers is frequently complicated by the occurrence of chronic-wound-associated infections, the most frequently isolated microbial strains being *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis* and fungi, particularly *Candida* spp. [7]. Although there are numerous international studies on the microbiome's contribution to the evolution of chronic ulcers in Romania, there is very little data on the virulence or resistance phenotypes of microbial strains isolated from these chronic pathologies. Elucidation of resistance profiles, not only to antibiotics, but also to the antiseptics that are used in the treatment of chronic ulcers, is crucial to optimize therapeutic strategies [8,9].

Silver-based antiseptics are among the most used antiseptics for chronic-ulcer therapeutic management. Silver is inactive in its metallic, elementary form, but it becomes bactericidal after ionization, a phenomenon that occurs in the air, but much faster in exudative media, such as exudative wounds [10]. Silver has been described as oligodynamic due to its bactericidal effect at very low concentrations [11]. Silver ions bind to the cell membrane, leading to the alteration of the electric potential [12] by the massive loss of protons across the membrane, resulting in the loss of electrical potential and, ultimately, cell death [11]. Inside the bacterial cell, silver ions interact with nucleophilic groups in proteins, attaching to the sulfhydryl, amino, imidazole, phosphate and carboxyl groups and leading to protein denaturation. Silver interferes with the energy-producing systems and their enzymes [12]. Thus, silver can disrupt respiratory chain enzymes (dehydrogenases) causing premature electron leakage, which when reacting with cytoplasmic molecular oxygen (O_2) will generate a superoxide radical [13]. When used in the form of nanoparticles, ranging in size from 1 to 100 nm and exhibiting different physical and chemical characteristics, the bactericidal effect of silver is amplified, which allows it to be used for the treatment of infections with multi-drug-resistant bacteria [14]. Metal nanoparticles act by changing bacterial cell-membrane permeability [15]. Ag ions released from nanoparticles can bind to thiol (-SH) groups of proteins and enzymes located on the cell surface, disorganize the membrane and disrupt ATP synthesis. In the cytoplasm, silver nanoparticles interact with protein-containing components, such as ribosomes and DNA [16]. Another mechanism of action is the release of oxygen radicals (singlet oxygen). The silver nanoparticles have been proved to exhibit antimicrobial activity against many Gram-negative (*Acinetobacter baumannii*, *Proteus mirabilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *nitrifying bacteria*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Vibrio cholerae*) and Gram-positive (*Enterococcus faecalis*, *Listeria monocytogenes*, *Micrococcus luteus*, *Staphylococcus epidermidis*) bacteria [17].

This broad antimicrobial spectrum of silver and silver nanoparticles has led to the development of silver-containing dressings. The continuous release of silver ions translates into anti-inflammatory effects, stimulation of blood-vessel development, killing of bacterial cells, and hydrating and softening of the necrotic tissue, with the final result being a clean wound [8,18–21]. Silver can be used not only for wound-infection treatment, but also prophylactically, mainly in the case of wounds with an increased risk of infection or re-infection, such as burns, surgical wounds, pressure ulcers adjacent to the anus, exposed bone wounds, and wounds in immunocompromised patients with impaired circulation, such as unbalanced diabetics [22].

Unfortunately, different mechanisms of silver resistance have been described, including silver sequestration in the periplasm and active efflux, particularly in Gram-negative bacteria isolated from chronic wounds. The resistance mechanisms are encoded by multiple

plasmodial genes that could be responsible for the occurrence of multi-drug resistance phenotypes. In this context, the purpose of this paper was to study the phenotypic and genetic profiles of resistance to silver nitrate, a compound currently used in the treatment of leg ulcers, in bacterial strains isolated from Romanian patients with this pathology.

2. Results

In this paper, we tested the efficiency of silver-nitrate solution on 125 strains isolated from patients with leg ulcers. The tested strains were predominantly *Staphylococcus* spp. (47%) (*Staphylococcus aureus*, *S. chromogenes*, *S. xylosus*, *S. warnerii*, *S. haemolyticus*, *S. sciuri*, *S. epidermidis*, *S. hominis*) and *Enterobacteriaceae* (17%) (*Enterobacter aerogenes*, *E. intermedius*, *Serratia marcescens*, *S. odorifera*, *Klebsiella pneumoniae/rhinoscleromatis*), followed by *Enterococcus* spp. (13%) (*E. faecalis*, *E. faecium*), non- *Enterobacteriaceae* (12%) *Chryseomonas luteola*, *Pseudomonas aeruginosa*, *Alcaligenes faecalis*, *Burkholderia cepacia*) and streptococci (11%) (*Lactococcus lactis*, *Aerococccus viridans*, *Streptococcus uberis*).

2.1. Qualitative Screening of the Spectrum of Sensitivity of Different Microbial Strains to Silver-Nitrate Solution

The qualitative screening of antiseptic activity was performed on all isolates and was represented as the average value of the growth-inhibition diameter obtained for the individual strains belonging to *Staphylococcus* spp., *Enterobacteriaceae*, *Enterococcus* spp., non-*Enterobacteriaceae* and streptococci.

Our results showed that the 1/6000 silver-nitrate solution exhibited the best inhibitory effect against the growth of *Staphylococcus* spp. strains (as revealed by the highest growth-inhibition-zone diameters), while the most resistant proved to be the *Enterococcus* spp. strains (Figure 1).

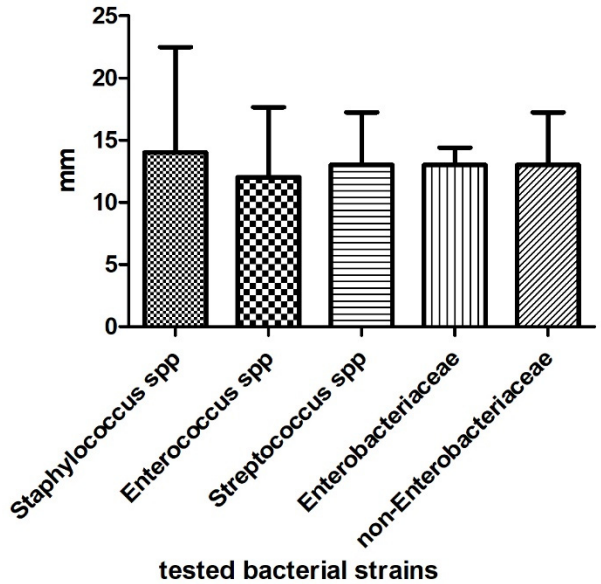

Figure 1. Graphic representation of average growth-inhibition-zone diameters (mm) obtained for the tested strains in the presence of silver-nitrate antiseptic solution of 1/6000 concentration.

2.2. Quantitative Testing of Antiseptic Efficacy of Binary Concentrations of Silver-Nitrate Solution

The quantitative testing of antiseptic efficacy against planktonic cells revealed that the 1/6000 silver-nitrate solution inhibited the growth of the tested bacterial strains at the standard concentration used in medical practice, as well as for the first two (in case of Gram-positive strains) or four (in case of Gram-negative strains) successive binary dilutions, followed by a considerable decrease until the loss of the effect at lower concentrations (Figure 2).

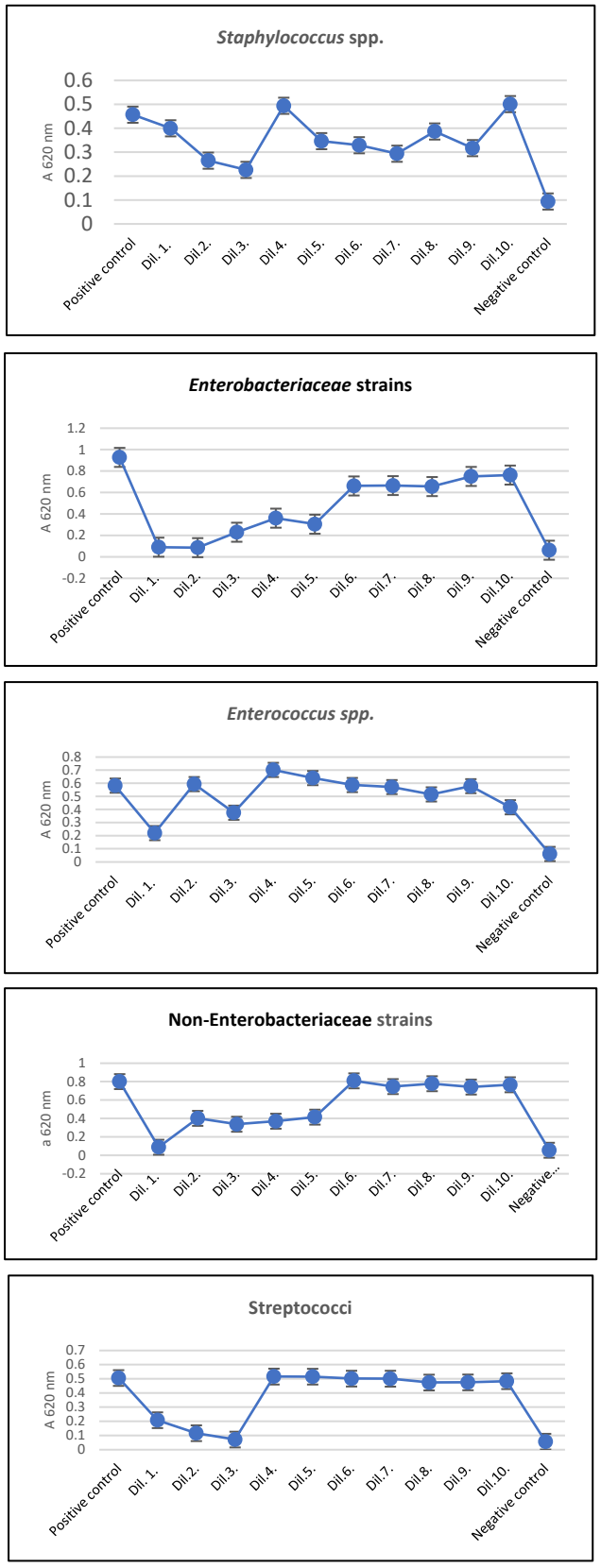

Figure 2. Graphic representation of planktonic growth dynamics (expressed as average value of absorbances obtained for different strains) in the presence of binary concentrations of 1/6000 silver solution for the analyzed strains (Dil.1.–10.-tested binary concentrations) (average value of absorbances obtained for the tested strains are represented).

2.3. Anti-Biofilm-Activity Assay of the Silver-Nitrate Solution

The quantitative testing of antiseptic efficacy against biofilm-embedded cells revealed that the 1/6000 silver-nitrate solution inhibited the growth of the Gram-positive strains (*Staphylococcus* spp., *Enterococcus* spp., *Streptococcus* spp.) and of the *Enterobacteriaceae* tested strains at the standard concentration used in medical practice, as well as at lower concentrations (for the following six to nine serial binary dilutions), while in the case of the *non-Enterobacteriaceae*, which are Gram-negative strains, only the standard clinical concentration and the first binary dilution showed a significant inhibitory effect of biofilm development (Figure 3).

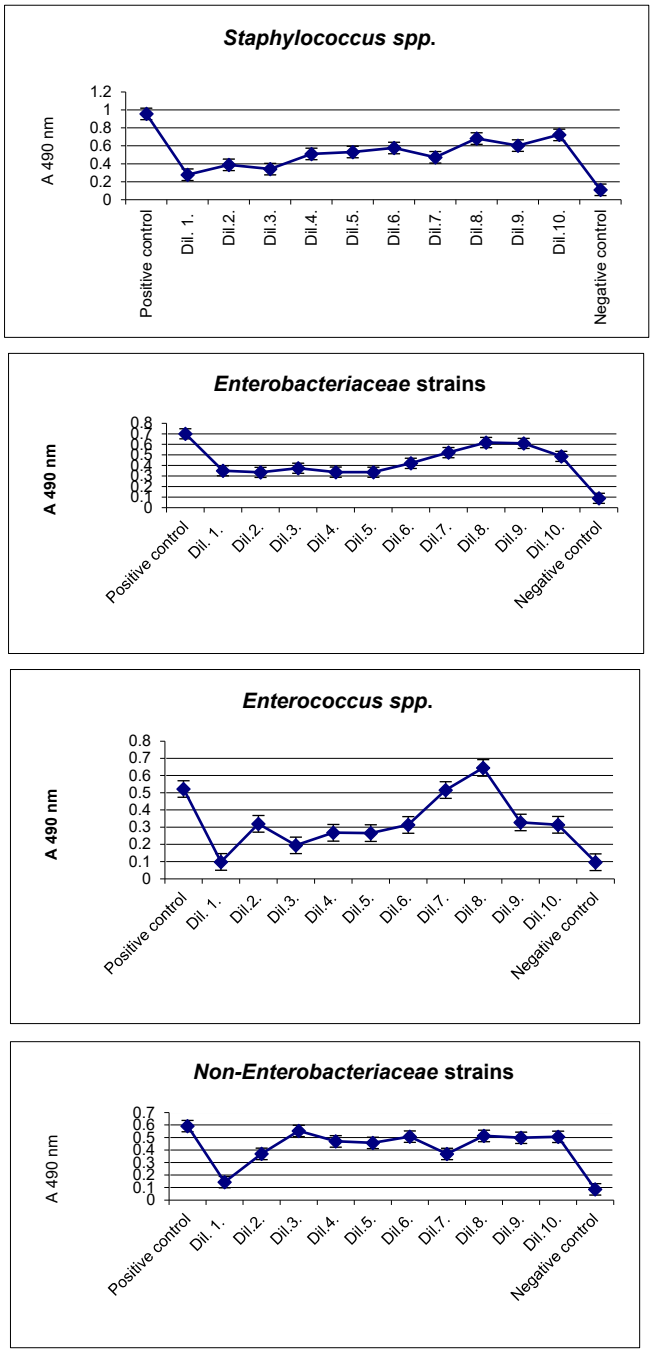

Figure 3. *Cont.*

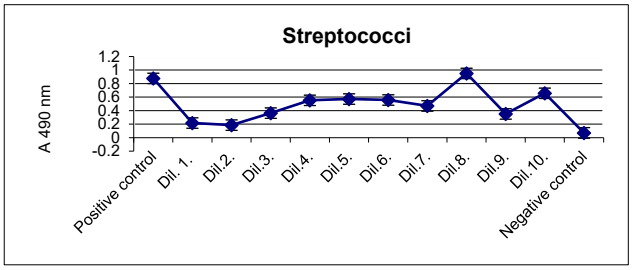

Figure 3. Graphic representation of the inhibition of microbial biofilms developed on the inert substratum in the presence of binary concentrations of the 1/6000 silver-nitrate stock solution for the analyzed strains (Dil.1.–10.-tested binary concentrations) (average value of absorbances obtained for the tested strains are represented).

2.4. Study of the Genetic Determinants of Resistance to Silver Ions

The study of the genetic determinism of the resistance to silver ions allowed us to highlight the presence of the sil genes in only few of the tested strains, i.e., *E. coli*—silE; *S. xylosus*—silS; *S. aureus*; *P. aeruginosa*—silB, *S. xylosus*; methicillin resistant *S. aureus* (MRSA); *E. coli*—silCAB; *S. chromogenes*; MRSA—silRS; MRSA, *S. haemolyticus*—silA (Figures 4–7).

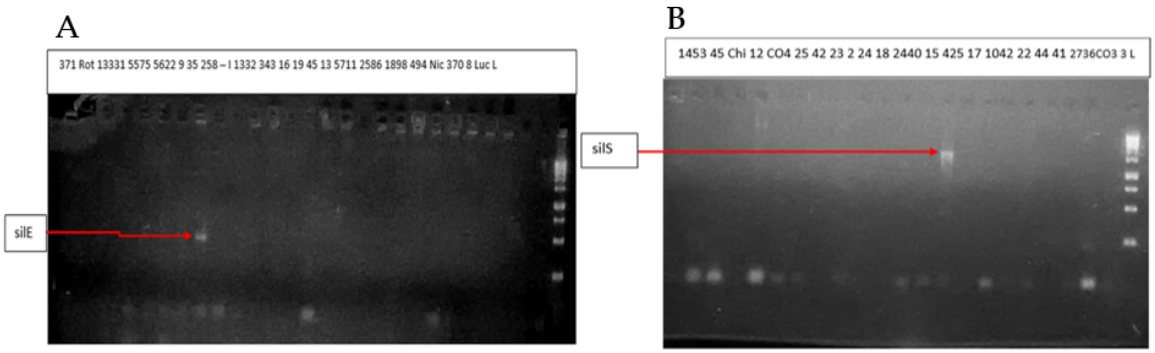

Figure 4. Electropherogram of amplicons obtained by PCR for SilE, SilS, and SilP genes. (**A**) Wells 3–27—amplicons of the analyzed strains; well no. 28, L-GeneRuler molecular weight marker 3000 bp (Mid Range DNA); Positive strains: *E. coli*—258 for the silE gene. (**B**) Wells 1–22—amplicons of the analyzed strains; well no. 23, L-molecular weight marker GeneRuler 3000 bp (Mid Range DNA); Positive strains for the SilS gene: *S. xylosus* (17).

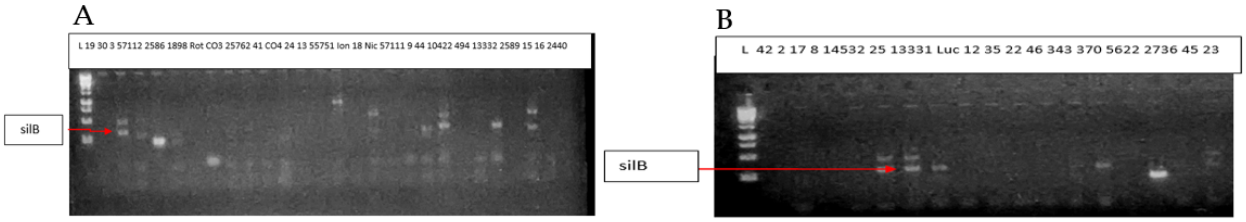

Figure 5. Electropherogram of amplicons obtained by PCR for SilB genes. (**A**) Wells 2–28—amplicons of the analyzed strains; well no. 1, L-Molecular Weight Marker GeneRuler 3000 bp (Mid Range DNA); Positive strains: *S. aureus* number 3015 for the SilB gene. (**B**) Wells 2–19—amplicons of strains 42–23; well no. 1, L-Molecular Weight Marker GeneRuler 3000 bp (Mid Range DNA); Positive strains for SilB gene: number 14532—*P. aeruginosa*; 25—*S. aureus*; 13331—*P. aeruginosa*; 370—*S. aureus*.

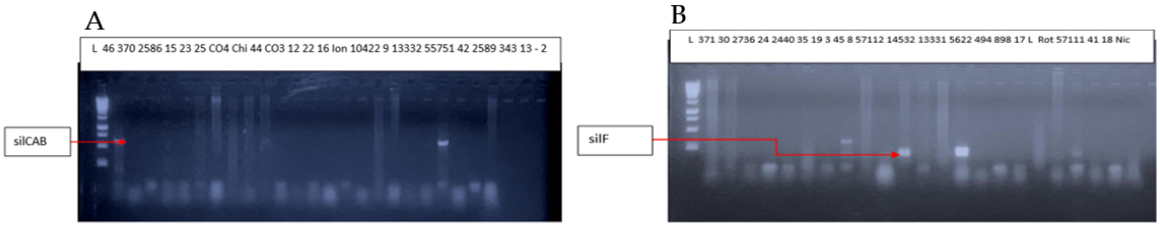

Figure 6. Electropherogram of amplicons obtained by PCR for SilCAB and SilF genes. (**A**) Wells 2–25—amplicons of the analyzed strains; well no. 1, L-Molecular weight marker GeneRuler 10,000 bp (Thermo-Scientific, Waltham, MA, USA); Positive strains for the SilCAB gene: number 46—*S. xylosus*; 55751—MRSA. (**B**) Wells 2–24—amplicons of the analyzed strains; well no. 1, L-Molecular Weight Marker GeneRuler 10,000 bp (ThermoScientific); Positive strains for SilF gene: number 14532—*S. xylosus*; 5622—*E. coli*.

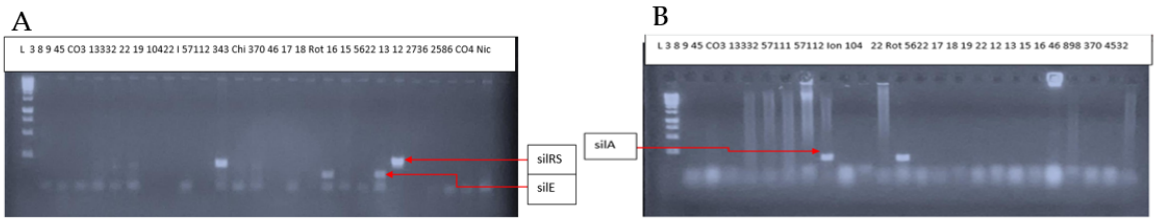

Figure 7. Electropherogram of amplicons obtained by PCR for SilE and SilRS genes. (**A**) Wells 2–28—amplicons of the analyzed strains; well no. 1, L-Molecular Weight Marker GeneRuler 10,000 bp (ThermoScientific); Positive strains for the SilRS gene: number 12—*S. chromogenes*; 343—MRSA; for the SilE gene: 5622—*E. coli*; (**B**) Electropherogram of amplicons obtained by PCR for SilA and SilP genes: wells 2–25—amplicons of the analyzed strains; well no. 1, L-Molecular Weight Marker GeneRuler 10,000 bp (ThermoScientific); Positive strains for the SilA gene: number 112.

3. Discussion

The rising prevalence of diabetes represents a public health and socioeconomic burden [23]. Diabetic foot ulcers occur due to a combination of multiple factors: underlying peripheral vascular disease, impaired leukocyte function and cell proliferation, polyneuropathy and increased plantar pressure, of which unfortunately about 75–85% of cases end in amputation. Because of the angiopathy and nerve damage, the majority of diabetic foot wounds are asymptomatic for a great period of time, leading to complications [23–26]. The neuropathy allows ulceration to develop after unrecognized trauma, whereas poor blood supply (ischemia) inhibits wound healing [27].

The evolution of the diabetic foot ulcer is divided into four stages: black (necrosis), yellow (inflammatory exudate), red (granulation formation), and pink (epithelial formation) and, regarding them, the treatment principles refer to T = tissue; I = infection/inflammation; M = moisture balance and E = wound edges [28–30].

Silver dressings are used to treat wounds in order to lower the microorganism load in acute or chronic ulcers and aid in creating an antibacterial barrier [23]. The most important bactericidal mechanism exhibited by silver-based antiseptics is the binding to the bacterial surface, which affects the membrane structure and function as well as the release of reactive oxygen species, which are active in both planktonic and biofilm-embedded cells [11,13].

Although the VULCAN study highlighted the lack of therapeutic superiority of silver dressings in the treatment of venous ulcers compared to other dressings, and that they are also more expensive, other studies have demonstrate the cost-effectiveness of silver-containing dressings because by using them: the healing time of wounds is reduced [31,32] and thus the duration of hospitalization drops [33,34]; the frequency of changing dressings and the need for analgesics, as well as bacteremia secondary to MRSA-infected wounds are decreased [8,35–37]. The most commonly used modern dressings in clinical practice are hydrogels, hydrocolloid, alginates, foams, and films [38].

Different studies have evaluated the efficiency of silver dressings. Huang C et al. designed a silver-ion dressing that is composed of a grid structure of sodium carboxymethyl cellulose and 1.2% silver ions, which has broad-spectrum antibacterial properties [8]. Due to these highly optimized properties, nano-silver dressings have more advantages in diabetic foot therapy [21]. Adding epidermal growth factor to the nano-silver material can lead to wound healing [39]. Another clinical study evaluated the combination of alginate fibers, prontosan gel, and silver ions. Alginate fibers can form a gel in combination with the wound moisture, thus ensuring a microacidic environment to promote wound healing. Prontosan gel is compatible with silver ions. The use of combination therapy is more effective at removing necrotic tissue, preventing biofilm formation, and removing exudate [40]. The silver-releasing foam dressing has proven to be more effective than silver sulfadiazine in the wound healing of diabetic foot ulcers [41].

Although silver is usually considered a benign material, it can also produce secondary effects such as: the formation of ulcers in burns treated with silver, the staining or destruction of skin cells when directly applied to the treatment of vulgar warts, and sometimes increased serum levels of silver, even argiria and argirosis in self-medicated patients with colloidal silver solutions. There is also a concern about the toxicity of silver nanoparticles to other organisms (especially aquatic organisms) [42].

One of the problems raised by the large use of silver-based antiseptics is the emergence of silver resistance. The endogenous resistance has been described mostly in Gram-negative bacteria and involves multiple mechanisms, such as: derepression of the chromosomal Cus system or of the silver-resistance genes (sil genes) [43]; loss of outer-membrane porins (OmpF or OmpF/C), leading to reduced outer-membrane permeability; active efflux of silver out of the cell (transporter CusCFBA) [44]. CHASRI is a copper-homeostasis and silver-resistance island that is involved in silver resistance after mutation in CusS and/or silS genes of members of *Enterobacteriaceae* family [45]. Components of the cus operon (CusF and CusB) are overexpressed in *E. coli* silver-resistant strains [45]. In 1975, an outbreak of *Salmonella enterica serovar Typhimurium* that was resistant to silver was recorded in a burn unit. The epidemic strain proved to have an exogenous silver resistance due to the acquisition of a plasmid harboring the sil system [43,44]. Later, it was shown that plasmid gene-mediated resistance to silver can be conferred by sil and the related gene operon present in the copper-resistant operon (pco) [37]. The plasmid pMG101 is 180 kb and provides resistance to silver, mercury, tellurite, and antibiotics (ampicillin, chloramphenicol, tetracycline, streptomycin and sulfonamides). The silver-resistance gene cluster contains nine genes, out of which seven are named, and two are lesser known with open reading frames (ORFs): silP, ORF105, silA, silB, ORF96, silC, silS, silR and silE. Silver-resistance gene box encodes two efflux pumps (silP-ATPase and silCBA-chemiosmotic), and two periplasmic proteins that bind Ag^{+} (silE), regulatory genes (silS-membrane kinase sensor and silR) [45]. These proteins are responsible for silver resistance through a combination of mechanisms involving silver sequestration in the periplasm (via SilE and SilF binding) and active efflux (via the resistance-nodulation-division (RND)-type efflux transporter SilCBA and the putative P-type ATPase transporter SilP) [45].

As silver ions are widely used in treating leg ulcers, burns, plantar ulcers, the investigation of silver susceptibility and resistance profiles of pathogenic strains isolated from this type of wound is a topic of great interest. Therefore, in this study, 125 bacterial strains isolated from 103 patients with venous ulcers were investigated to elucidate the susceptibility of the skin microbiome to silver-nitrate solutions and study the genetic determinants for this resistance. These bacterial strains were previously characterized for their virulence and antibiotic-resistance features at the phenotypic and genotypic level [46,47].

The isolated strains, both in the planktonic and biofilm growth phase, showed sensitivity to the standard concentrations of 1/6000 silver-nitrate solution that is currently used as an antiseptic substance in treating leg ulcers.

Regarding the genetic support of silver resistance, Sütterlin et al. studied silE, silP, and silS genes in strains isolated from leg ulcers, observing their presence in strains of

Enterobacter aerogenes, *E. cloacae*, *Klebsiella pneumoniae*, *K. oxytoca*, *E. coli*, *Citrobacter*, *Proteus*, *Providencia*, *Salmonella* and *P. aeruginosa* [35,47]. Finley et al. analyzed 859 strains belonging to *Staphylococcus, Escherichia*, *Pseudomonas*, *Klebsiella*, *Enterococcus*, and *Enterobacter* genera. They concluded that 32 of the strains were positive for the tested sil genes (silA, silB, silCBA, silE, silF, silP, silRS), 14 being positive for all 7 genes [48]. Woods et al. isolated 60 strains from human and horse ulcers and tested them for the presence of sil genes (silA, silB, silCBA, silE, silRS, silF, sil F), identifying 10 *E. cloacae* strains that were positive for all the tested sil genes [49]. Other strains reported to be resistant to silver were *A. baumannii*, *S. typhimurium*, and *P. stutzeri* [12].

In our study, the investigation of the genetic determinism of silver-ion resistance allowed the identification of the following plasmid resistance markers from the sil operon: the presence of silA, silB, silC genes encoding for efflux pumps, the silE gene encoding periplasmic proteins that bind Ag^+ and silR, and silS regulatory genes in *Staphylococcus* spp. (MRSA, *S. chromogenes*, *S. haemolyticus*) strains, followed by *E. coli* and *P. aeruginosa*. However, all these strains harboring silver-resistance genetic determinants remained susceptible to the silver-nitrate concentration currently used for treating leg ulcers.

4. Materials and Methods

4.1. Chronic Leg Wound Bacterial Strains Collection

The tested strains were isolated at the Dermatovenerology Department of the Central Military University Emergency Hospital "Carol Davila" in Bucharest, between October 2014 and September 2015, from 103 hospitalized patients with skin ulcers (secondary to chronic venous insufficiency, arterial insufficiency, type 1 and 2 diabetes, necrotizing vasculitis, Kaposi's disease, squamous-cell carcinoma, bone necrosis). The study protocol complied with the ethical prerogatives of the 1975 Helsinki Declaration and the standards of Good Clinical Practice (GCP). The Dermatovenerology Department obtained the approval of the Ethics Commission of the Central Military University Emergency Hospital "Carol Davila" from Bucharest. The strains were isolated from wound secretion. Harvesting was performed before any antibiotic therapy or within the first 24 h using exudate swabs. The cotton swab was collected from the most representative area of the ulcer, either the purulent or exudative area. In the absence of representative areas, it was harvested from the edge of the ulcer. One sample was taken from each ulcer if there were multiple ulcers. Two exudate swabs were used for this study: an exudate swab was used to make a direct smear of the pathological product "at the patient's bed", while the second swab was used to transfer the pathological product (pp) in the storage and transport environment. Containers with sterile culture medium (thioglycolate broth) were used to store and transport the pathological product. To obtain isolated colonies, sowing was practiced in the "open pentagon" of the solid-blood agar medium distributed in a Petri dish by depleting the inoculum with the bacteriological loop. After sowing, the Petri dish was incubated at 35–37 °C, for 18–24 h. The representative isolated colonies were further purified and identified by conventional biochemical tests, Vitek-2 and MALDI-TOF.

4.2. Qualitative Screening of the Spectrum of Sensitivity of Different Microbial Strains to Antiseptic Substances by the Adapted Version of the Diffusion Method

With the help of sterile forceps, sterilized filter-paper disks were distributed in solid-blood agar medium distributed in Petri dishes previously seeded with the bacterial inoculum of the test strain. Using the micropipette, 10 µL of pharmaceutical antiseptic solution of silver nitrate 1% diluted 1/6000 in distilled water were distributed on the filter-paper disk. The plates were left at room temperature for 20–30 min to ensure the diffusion of the substance. Then the plates were incubated for 16–18 h at 37 °C, with the lid down. The reading of the results involved observing and measuring the area of inhibition of microbial growth around the discs impregnated with the compound.

4.3. Quantitative Assay of the Antimicrobial Activity of Silver-Nitrate Solution on Planktonic Cultures

It was performed by the binary-microdilution technique in a liquid medium (Mueller Hinton), made in 96-well plates, to determine the minimum inhibitory concentration (MIC). For this purpose, ten binary serial dilutions of the antiseptic stock solution were performed in a volume of 150 µL of Mueller Hinton medium, and then the wells were seeded with 50 µL of microbial suspension with a MacFarland density of 0.5. Each test was performed with a positive control (a series of wells exclusively containing culture medium inoculated with microbial suspension) and a negative, sterility control.

After incubating the plates at 37 °C for 24 h, the absorbance of the obtained liquid cultures was measured at 620 nm.

4.4. Quantitative Assay of the Antimicrobial Activity of Silver-Nitrate Solution on Biofilms Developed on Plastic Wells

It was performed by the purple-crystal-microtitration technique. After reading the absorbance of the liquid content of the well, the 96-well plates were emptied, washed with saline phosphate buffer to remove non-adherent bacteria, fixed for 5 min with cold methanol, stained for 15 min with violet-crystal alcohol solution, and washed to remove the excess dye. The fixed and stained biofilms were subsequently resuspended with 33% acetic-acid solution, and the absorbance of the stained suspension was measured spectrophotometrically at 490 nm.

4.5. Genotypic Highlighting of Silver Ion Resistance Markers in Isolated Strains

Genomic DNA was extracted from 45 strains of *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp., *Enterobacteriaceae* and non-enteric Gram-negative bacilli, showing susceptibility only to the highest tested concentration of silver-nitrate solution, using the Wizard®® SV Genomic DNA Purification System kit (Promega, Woods Hollow Road, Madison, WI, USA) according to the manufacturer's recommendations. The obtained DNA was used as a template in 4 multiplex PCR reactions and one simplex PCR to identify SilE, SilS, and SilP carrier strains; SilB; SilCAB and SilF; SilE and SilRS; SilA and SilP.

The sequences of the primers used, their specificity, and the amplification programs used are shown in Tables 1 and 2, and the components used in these reactions are shown below.

Table 1. Sequences of primers used and their specificity.

Gene	Primers	Primer Sequences	Bp No.
silE	silE-F silE-R	5′-GTACTCCCCCGGACATCACTAATT-3′ 5′-GGCCAGACTGACCGTTATT-3′	400
silP	silP-F silP-R	5′-CATGACATATCCTGAAGACAGAAAATGC-3′ 5′-CGGGCAGACCAGCAATAACAGATA-3′	24
silS	silS-F silS-R	5′-GGAGATCCCGGATGCATAGCAA-3′ 5′-GTTTGCTGCATGACAGGCTAAAGACATC-3′	1500
silRS	silRS-F silRS-R	5′-GGCAATCGCAATCAGATTTT-3′ 5′-GTGGAGGATACTGCGAGAGC-3′	125
silCBA	silCBA-F silCBA-R	5′-CGGGAAACGCTGAAAAATTA-3 5′-GTACGTTCCCAGCACCAGTT-3′	600
silF	silF-F silF-R	5′-CGATATGAATGCTGCCAGTG-3′ 5′-ATTGCCCTGCTGAATAAACG-3′	20
silB	silB-F silB-R	5′-CAAAGAACAGCGCGTGATTA-3′ 5′-GCTCAGACATTGCTGGCATA-3′	300
silA	silA-F silA-R	5′-CTTGAGCATGCCAACAAGAA-3′ 5′-CCTGCCAGTACAGGAACCAT-3′	20

Table 2. Components of the PCR reactions.

Gene/Genes	Concentration				
	Primer	**Mix**	**Water**	**DNA**	**Final Volume**
silE, *silS*, *silP*	0.5 µM	10 µL	6 µL	1 µL	20 µL
silB	0.5 µM	10 µL	8 µL	1 µL	20 µL
silE, *sil RS* *silA*, *silP*	0.5 µM	10 µL	7 µL	1 µL	20 µL
silCBA, *silF*	0.5 µM	10 µL	7 µL	1 µL	20 µL

The amplification program was carried out according to the conditions shown in Figure 8:

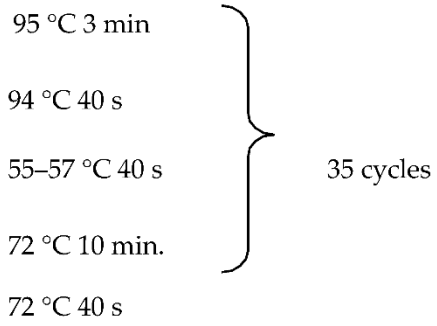

Figure 8. The parameters used for the PCR detection of silver resistance genes.

5. Conclusions

The isolated strains, both in the planktonic and biofilm growth phase showed sensitivity to the standard concentration of 1/6000 silver-nitrate solution). It was noticed that even at concentrations lower than the clinical one (first two to four binary dilutions in the case of planktonic cultures and first six to nine binary dilutions in the case of biofilms), the antiseptic solution proved to maintain its antibacterial activity. This demonstrates that despite its large use, this antiseptic remains a viable treatment alternative for the management of chronic leg wounds. The phenotypic results were correlated with the genetic analysis, highlighting the presence of resistance genes in only a few of the tested strains. However, the strains harboring genetic resistance markers proved susceptible to the silver-nitrate-solution concentrations that are used in the clinical settings. In conclusion, by the phenotypic and genotypic characterization of silver-nitrate susceptibility and resistance in a high number of bacterial strains isolated from leg ulcers, the results of this study contributed significantly to the completion of the little-investigated local epidemiological picture of this pathology, thus providing additional means of guiding clinicians in selecting the appropriate therapy.

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