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Original article

CHRONIC WOUNDS - MICROBIOLOGY AND BIOFILM FORMATION

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Abstract

Introduction: Chronic wounds are wounds that do not heal for more than 4 weeks. Among the most important causes of wound chronicity are wound infection, biofilm formation with microbial agents that are resistant to antimicrobial agents.

Aim: To investigate microbial agents in chronic wounds, their potential to form biofilm.

Material and methods: This was a prospective study involving 24 patients from the University Clinic for in Skopje. Microbiological swabs were taken from patients with chronic wounds on the lower extremities, and were analyzed at the Institute of Microbiology and Parasitology, Faculty of Medicine in Skopje. The biofilm formation potential in isolates was determined using the tissue culture plate (TCP) biofilm detection method.

Results: In 17 patients, at least one microorganism was isolated from 23 wounds, a total of 36, and in 7 patients there was no positive isolate from 7 wounds. Eighteen wounds (60%) had an isolate with the potential to form a biofilm. The following microorganisms from the wound samples were recorded: 17 (47%) isolates of the genus *Staphylococcus*, 4 (11%) isolates each from the genera *Proteus* and *Pseudomonas*, 3 (8%) isolates each from *Enterococcus* and *Escherichia*, 2 (6%) isolates from *Streptococcus*, 1 (3%) each isolate from the genera *Acinetobacter*, *Enterobacter* and *Candida albicans*.

Conclusion: In 60% of the isolates, a biofilm formation with different degrees was confirmed. A statistically significant association was found between the degree of wound healing and isolates with low potential for biofilm formation, as well as the type of isolate.

Keywords: chronic wounds, microbial agents, biofilm, microbial swab, TCP

Introduction

Chronic wounds are wounds that do not heal for more than 4 weeks[1]. Among the most important causes of wound chronicity are wound infection, biofilm formation by microorganisms that are resistant to antibiotic therapy. Wound classification is based on etiology according to the Rank-Wakefield system, duration of wound healing, skin integrity, degree of contamination, and morphological characteristics[2]. Chronic wounds can be

classified as pressure wounds (decubitus and neuropathic ulcers), inflammatory wounds (autoimmune and primary skin disorders), vascular wounds (venous, arterial and mixed ulcers), malignant wounds (primary and secondary skin malignancies), wounds of other etiology (burns, radiation injuries, frostbite, vascular ulcers, insect bites)[2]. The wound healing process consists of four overlapping phases of hemostasis, inflammation, tissue proliferation, and tissue remodelling[3]. In most cases, healing restores the skin barrier function, but sometimes wounds do not heal, and the stages of the healing process are prolonged. This leads to a permanent, non-healing condition defined as chronic wounds[4]. Infection, as a leading cause of delayed wound healing, with pathogenic microbes, bacteria and fungi, invades the wound bed and forms a biofilm. Factors that cause the proliferation of microbes in chronic wounds are devitalized tissue, moist and inflammatory processes that are unregulated[5].

A biofilm is defined as a community of surface-attached or self-attached microorganisms embedded in a hydrated matrix of extracellular polymeric substances that provides protection from antimicrobial agents and host defences[6].

Bacteria can colonize and infect wounds, resulting in a prolonged wound healing process. Most chronic wounds are polymicrobial. The structure of biofilms is made of microbial aggregates, which are packed in an extracellular matrix (ECM). The ECM consists of polysaccharides, proteins and glycoproteins, called extracellular polymeric substance (EPS)[7,8]. The bacteria that form a biofilm are: *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Streptococcus pyogenes*, *Bacillus subtilis*, *Staphylococcus aureus*, and from the fungi *Candida albicans*[9,10].

Biofilms are more resistant to antimicrobial therapy than the bacteria themselves. It is the result of an impaired host inflammatory response. Leukocytes lack the ability to produce reactive oxygen species and have difficulty penetrating the biofilm[11]. This results in impaired phagocytosis of bacteria. The biofilm exopolymer blocks complement activation, suppresses the lymphoproliferative response, and impairs the ability of phagocytes to detect opsonins attached to the bacterial cell wall[12].

Bacteria in biofilms have reduced metabolic activity. Metabolically active cells are usually the targets of antimicrobial agents, so bacteria are resistant to these agents[13]. The exopolysaccharide in the biofilm is a mechanical protector of the bacteria from antimicrobial agents and immune cells[14].

In biofilms, bacteria can transfer plasmid-mediated antimicrobial resistance genes between them, again leading to treatment resistance[12].

This study aimed to investigate the microbiological agents of chronic wounds in 24 patients treated at the University Clinic for Dermatovenerology in Skopje, and to determine the potential of microorganisms to form a biofilm.

Materials and methods

The study was conducted at the University Clinic for Dermatovenerology and Institute of Microbiology and Parasitology, Faculty of Medicine, Ss. Cyril and Methodius University in Skopje. It was a prospective study involving 24 patients who received outpatient and inpatient treatment at the University Clinic for Dermatovenerology in Skopje. Criteria for inclusion in the study: patients of both genders aged over 18 years, with lower extremity wounds persisting for more than 4 weeks, wounds larger than 1 cm^2, who signed consent for participating in the study.

Exclusion criteria: chronic wounds caused by malignant tumors, inflammatory dermatoses, patients with burns, patients receiving oral antibiotic therapy in the last 7 days, patients receiving local antibiotic therapy for the wound in the last 7 days, patients with no

possibility of communication and cooperation, without given consent to participate in the study, with acute phlebothrombosis or deep vein thrombosis of the lower extremities, and patients with acute soft tissue infection (cellulitis, erysipelas).

In each patient, a complete history, dermatological status and medical documentation for the presence of a chronic wound on the lower extremities were taken. All wounds were digitally photographed, and their surface was calculated with SketchAndCalc. Elliott M Dobbs, 2011.Web[15]. A sample was taken from the wound of each patient for further microbiological investigation for isolation and identification of strains of microorganisms as well as identification of the biofilm. This sample was taken using a microbiological swab. Treatment in all patients was according to the standard wound care protocol. In the healing process of a wound that was diagnosed as chronic, a time-related assessment was considered. If during 4 weeks of standard care, the surface of the wound is reduced by 50%, it is likely that healing will occur with the same treatment in 12 weeks. If less than 50% reduction occurs, it is unlikely to heal with this treatment and a change in treatment and reassessment is necessary[16]. Data on the sensitivity of microorganisms to antimicrobial agents were also used in the treatment. Patients were monitored in a period of one month, with regular examination once a week, according to the protocol for wound treatment described above. After completion of the antibiotic therapy, a control microbiological swab was made.

Microbiological methods

Isolation and identification of strains

Wound specimens were cultivated on standard microbiological nutrition media (Columbia agar for aerobic and facultative anaerobic bacteria, Schedler agar for anaerobic bacteria, Saburo agar for yeasts, and glucose broth). Planted substrates were incubated for 24 to 48 hours for the detection of aerobic and facultatively anaerobic bacteria, 48 hours for anaerobic bacteria in anaerobic pots using the Gas Pack system method, and up to 7 days for the detection of yeasts[17]. Additionally, microscopic Gram stain examination and standard biochemical test were done where needed.

Specimen storage

Following identification, the selected strains were numerated and subcultivated on solid nutrition media to get pure bacterial culture. To avoid multiple subcultivation and genetic modification of the original isolate from each species, one well-isolated colony was selected for long preservation (frozen at -80◦C in trypticase soya broth supplemented with 20% glycerol).

Biofilm assay

Biofilm detection

All selected strains were screened for mucus-producing strains by the adhesion method in a glass tube and safranin staining. The first step was to make a suspension of the strains to be tested in borosilicate glass tubes containing brain broth. Next step, the test tubes were incubated for 48 hours, in aerobic conditions, at a temperature of 35°C. Then, the supernatant was discarded, and the glass tubes were stained with 0.1% safranin solution, washed with distilled water three times and dried. Appearance of a layer of stained material on the inner wall of the test tube means the result is positive. The appearance of a coloured ring at the place of the liquid-air contact surface means the result is negative.

The strains that were defined as "mucus-producing" during the screening were further processed according to:

Tissue culture plate method

For the cultivation of the biofilm, a modification of the TCP method set by Christensen *et al*. was used. [18]. When performing this method, one colony of the previously mentioned trypticase soy salt agar was cultured in 5 ml of TSB, for 18 hours at 37°C. Then, a suspension of a bacterial culture in stationary growth phase was diluted with the appropriate medium at a ratio of 1:100. Then 100 μl of each bacterial suspension was inoculated into the wells of a sterile polystyrene flat bottom microtiter plate. To obtain a mean value of each strain, three samples were placed (in three wells). The penultimate or last well of the row contained nutrient medium with a suitable reference strain (positive control) or only medium (negative control). The microtiter plates were incubated for 24 hours at a temperature of 37°C without mixing, in order to create the biofilm. After incubation, the supernatant was carefully removed with a pipettor, and the wells were washed three times with 200 μL of 85% NaCl to remove planktonic microorganisms.

Biofilm biomass quantification

The mass of the biofilm was determined by measuring the absorbance of crystal violet[18]. After removing the planktonic microorganisms, the wells were thoroughly dried (30 minutes at 60°C); the biofilm formed in them was fixed with 2% sodium acetate and then 0.1% solution of crystal violet (120 μl) was added to each well to stand for 10-15 minutes at room temperature. Then the excess dye was removed by rinsing with deionized water, and the bound dye was dissolved by adding 120 μl of 75% ethanol. The absorbance of each well was measured at 570 nm using a microplate spectrophotometer (ELISA microplate reader). The optical density (OD) of each strain and the negative control was calculated as the arithmetic mean of the absorbance of the three wells. This value was compared to the mean absorbance of the negative control wells. The degree of absorption is proportional to the amount of biofilm present. At that point a “cut-off "value (ODc), which is known as three standard deviations (SD) above the mean OD of the negative control, was determined:

ODc=means OD of the negative control+(3x SD of the negative control). Interpretation of the results for biofilm production was as follows: absence of biofilm formation (OD strain <ODc), weak biofilm formation (ODc<ODstrain <2x ODc), moderate biofilm formation (2x ODc)<OD strain<(4xODc) and strong biofilm formation (4xOD c) <OD strain[19].

Quality control

International reference strains were used as a positive control for the ability to produce biofilm: for Gram-negative bacteria - biofilm producing strain *E. coli* ATCC 25922, for Gram-positive bacteria - biofilm producing strain *Staphylococcus aureus* ATCC 29213 proposed for the control of quality by the National Committee for Clinical Laboratory Standards - NCCLS.

Results

The average age of patients (n=24) who participated in the study was 65.5±13.5 years, 12 men (50%) and 12 women (50%). According to national distribution, there were: Macedonians 79.17% (n=19), Roma 8.33% (n=2), Serbs 8.33% (n=2) and Vlachs 4.17% (n=1). According to family status, 16.67% (n=4) lived alone, and 83.33% (20) lived with a family. According to profession, there were: soldiers 4.17% (n=1), constractors 4.17% (n=1), craftsmen 4.17% (n=1), masseurs 4.17% (n=1), unemployed 16.67 % (n=4), security guards 8.33% (n=2), retired 58.33% (n=14) (Table 1).

Table 1. Basic data about patients included

Parameters		n-number	%
Age	Average age ± SD		
Gender	men	12	50
	women	12	50
Nationality	Macedonians	19	79.17
	Roma	2	8.33
	Vlachs	1	4.17
	Serbs	2	8.33
Marital status	single	4	16.67
	married	20	83.33
Profession	soldier	1	4.17
	constractor	1	4.17
	craftsmen	1	4.17
	masseur	1	4.17
	unemployed	4	16.67
	security guard	2	8.33
	retired	14	58.33

Out of a total of 24 patients, 7 (29.16%) patients had wounds without an isolated microorganism, and 17 (70.84%) had wounds with an isolated microorganism (Table 2).

Table 2. Grouping of patients based on wound isolates

	n-број	%
Patients with wounds without isolated strains	7	29.16
Patients with wounds with isolated strains	17	70.84

A total number of 30 wounds were included in the analysis, with or without the appropriate isolate, considering patients with different numbers of wounds of interest.

Microbiology of wounds

Microbiological swabs taken before the start of treatment showed the absence of microorganisms in 7 wounds (23.3%), 1 isolated microorganism was present in 12 wounds (40.0%), 2 isolates in 9 wounds (30.0%), 3 isolates in 2 wounds (6.7%) (Figures 1, 2).

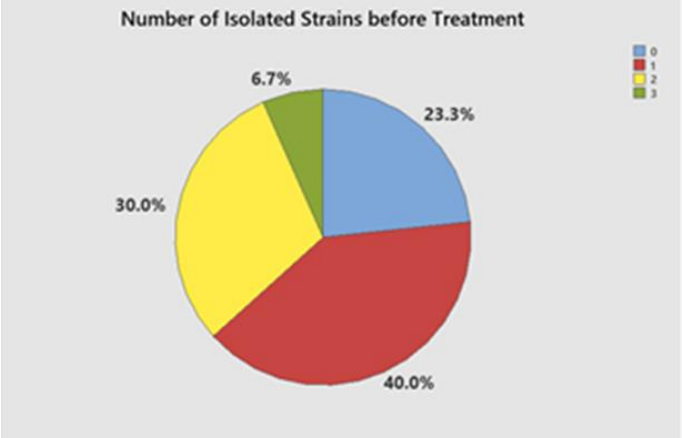

Fig. 1. Percentage representation of the number of isolates in wounds before starting treatment

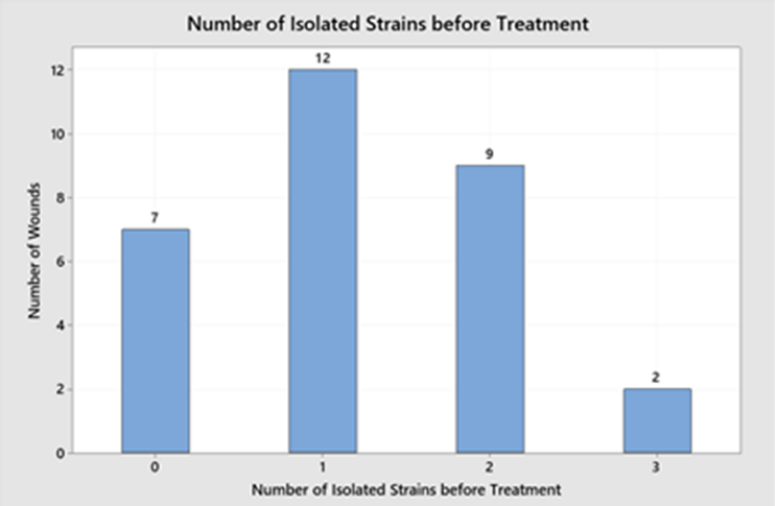

Fig. 2. Number of isolates before starting treatment

After the end of treatment, a control swab was taken and it showed 16 wounds without isolate or 53.3%, 8 wounds with 1 isolate or 26.7%, 4 wounds with 2 isolates or 13.3%, 1 wound with 3 isolates or 3.3% and 1 wound with 4 isolates 3.3% (Figures 3, 4).

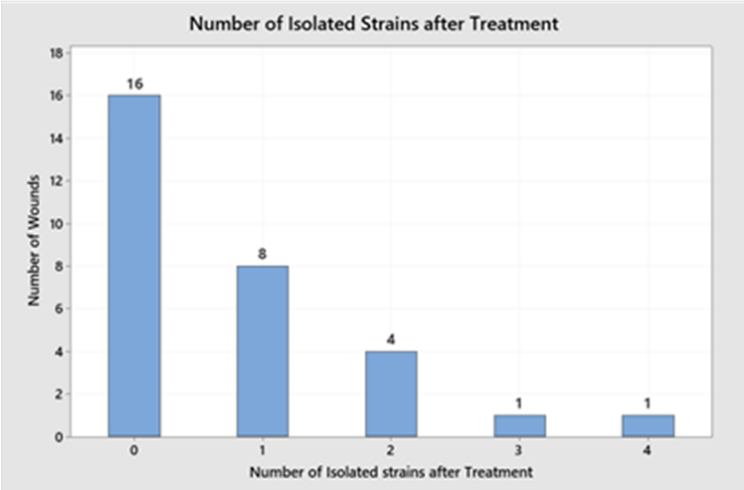

Fig. 3. Number of isolates after completion of treatment

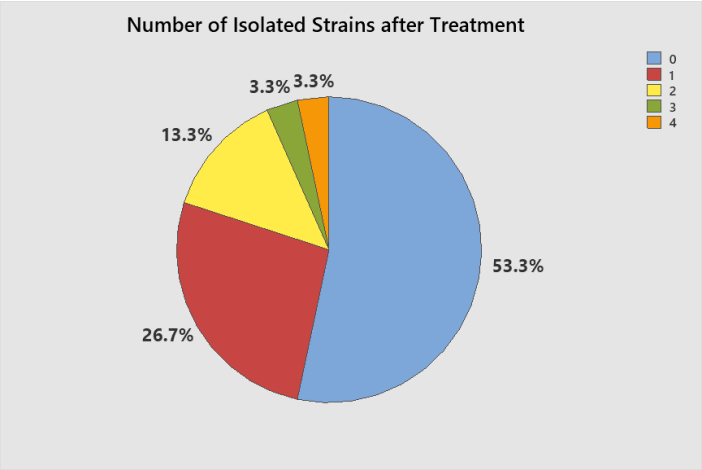

Fig. 4. Percentage of isolates in wounds after completion of treatment

The impact of the number of isolates on the degree of wound healing was analyzed. Spearman's coefficient of r=0.043 indicated a very weak positive correlation, which means the number of isolates before treatment had no influence on the degree of wound healing (Figure 5).

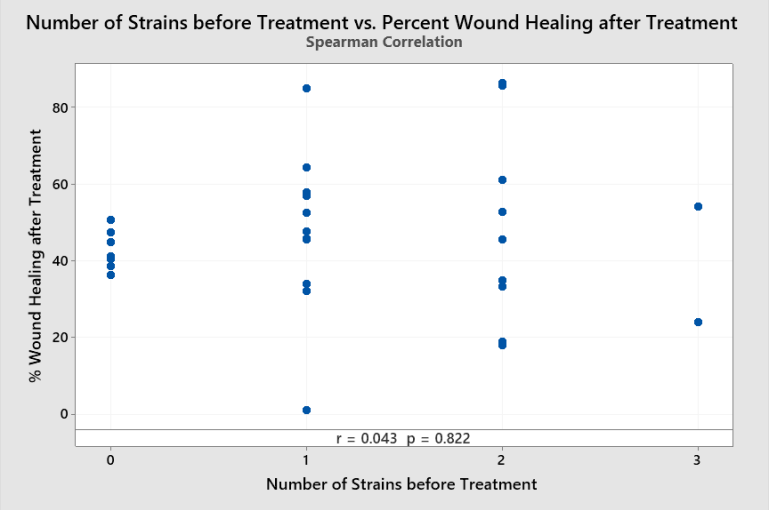

Fig. 5. Spearman's rank correlation coefficient of the number of isolates before treatment and the degree of wound healing after treatment

A correlation was made between the degree of wound healing in a group of patients without isolates compared to a group of patients with isolates. The two sample Student t-test statistical analysis showed no statistical difference in the degree of healing of wounds without isolate (42.702±5.10) compared to wounds with isolate (48.62 ±20.7). p=0.460 (Figure 6).

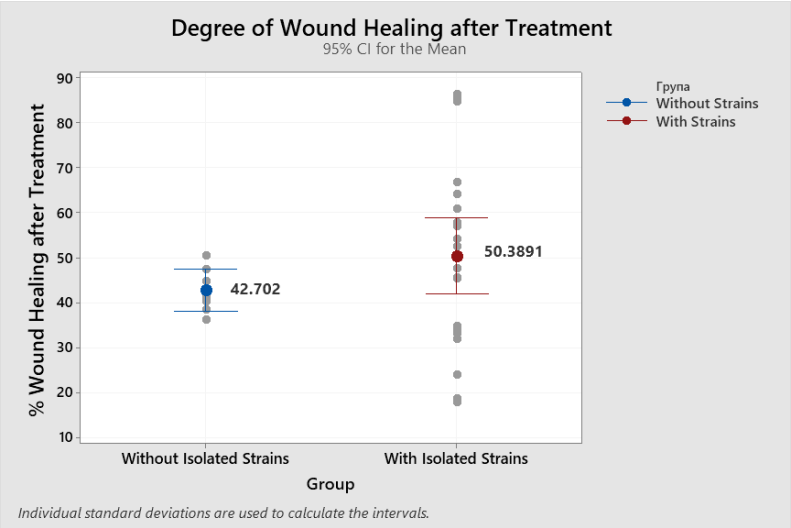

Fig. 6. Wound healing rate after treatment in wounds with isolate and wounds without isolate

Regarding the heterogeneity of species isolated from wound samples, the following was recorded: 17(47%) isolates of the genus *Staphylococcus*, 4 isolates each or 11% of the genera *Proteus* and *Pseudomonas*, 3 isolates each or 8% of *Enterococcus* and *Escherichia*, 2 isolates each or 6% of the genus *Streptococcus* and 1 isolate each or 3% of the genus *Acinetobacter*, *Enterobacter* and *Candida albicans* (Table 3).

Table 3. Percentage of individual species isolated from wound samples

Type of isolate	N	Percentage	Average value	SD	Minimum	Median	Maximum
Acinetobacter	1	3%	23.936	*	23.936	23.936	23.936
Candida albicans	1	3%	23.936	*	23.936	23.936	23.936
Enterobacter spp.	1	3%	54.087	*	54.087	54.087	54.087
Enterococcus	**3**	8%	31.5	18.6	17.9	23.9	52.6
Escherichia coli	2	6%	34.426	0.602	34.000	34.426	34.851
****Escherichia Coli ESBL+*	1	3%	45.567	*	45.567	45.567	45.567
MRSA	**4**	11%	36.90	17.56	18.75	33.98	60.92
Proteus mirabilis	**4**	11%	41.12	19.40	17.86	42.85	60.92
Pseudomonas aeruginosa	**4**	11%	37.59	15.52	18.75	38.75	54.09
****MSSA***	**10**	28%	63.52	16.05	45.70	57.21	86.36
*****MRSCN***	**3**	8%	56.73	10.77	45.45	57.83	66.90
Streptococcus pyogenes	2	6%	85.917	0.632	85.470	85.917	86.364

**MRSA*- Methicillin resistant *S. aureus*, ***MSSA*- Methicillin sensitive *S. aureus*, *****MRCNS*- Methicillin resistant coagulase negative *staphylococcus*, ******Escherichia Coli ESBL+ -Escherichia Coli* Extended Spectrum Beta Lactamase +

In 5(29.41%) out of a total of 17 patients with an isolate from a wound, the isolates were the same before and after the treatment. Two patients (11.76%) hade initially isolated MSSA, which in both cases was associated with one or two more isolates (*Streptococcus pyogenes*, i.e., *P. aeruginosa*, *Enterobacter*), the latter with a high potential for biofilm formation. In 2(11.76%) patients, *P. aeruginosa* with a high potential for biofilm formation was isolated, before and after treatment, associated with one more isolate of *E. coli* before, and MRSA after treatment with a moderate/weak potential for biofilm formation. High-potential *Enterococcus* was isolated before and after treatment in only 1(5.88%) patient, associated with *C. albicans*.

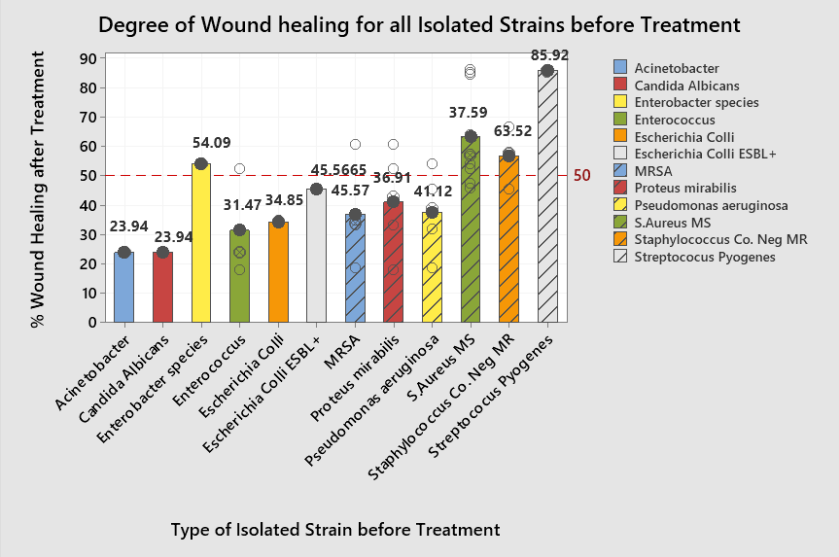

Fig. 7. Influence of the type of microorganism on the degree of wound healing

Isolates represented more than 3-fold (bold isolates in Table 3) in wounds were analyzed for statistical significance using the Analysis of Variance (One-Way Anova) method. Kolmogorov-Smirnov distribution test showed a normal distribution ($p>0.150$).

Bartlett method of variance test showed (p=0.980) equivalent variances. One-Way Anova (assuming equivalent variances and Fisher Pairwise comparison) statistical test showed that MSSA had a statistical significance in relation to *Proteus mirabilis* (p=0.032), *Pseudomonas aeruginosa* (p=0.015), MRSA (p=0.012) and *Enterococcus* (p=0.07). The degree of wound healing in MSSA was the highest and statistically higher than the other isolates that had a low degree of healing (Figure 7, Table 4).

Table 4. Fisher pairwise comparisons: grouping information using the Fisher LSD method and 95% Confidence

Type of isolated strain before treatment	N	Mean	Grouping	
MSSA	10	63.52	A	
MSRCN	3	56.73	A	B
Proteus mirabilis	4	41.12		B
Pseudomonas aeruginosa	4	37.59		B
MRSA	4	36.90		B
Enterococcus	3	31.5		B

Among isolates with the potential to create a biofilm depending on the degree of its production, a correlation was analyzed between the degree of wound healing and the degree of biofilm production (weak, moderate, strong). For this purpose, the Variance method (One-Way ANOVA) was used again. Kolmogorov-Smirnov distribution test showed a normal distribution (p>0.150). Bartlett method of variance test showed (p=0.992) equivalent variances. One-Way ANOVA (assuming equivalent variances and Fisher pairwise comparison) statistical test showed that isolates with low biofilm production were statistically significant compared to isolates with moderate biofilm production (p=0.041). So far, there are insufficient data to determine the correlation between isolates with a strong potential for biofilm production and the degree of wound healing (Figures 8 and 9). Insufficient data have been available to assess the rate of wound healing in patients with biofilm-forming isolates compared to non-biofilm-forming isolates because only one isolate sample (MRSA) yielded a non-biofilm-producing result.

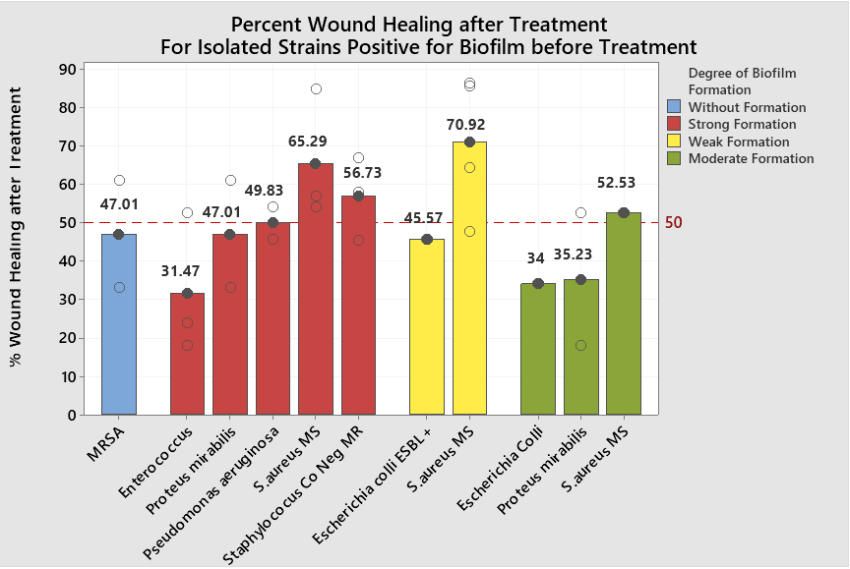

Fig. 8. The influence of the presence and degree of biofilm production in wound samples with isolates before treatment in relation to the degree of wound healing

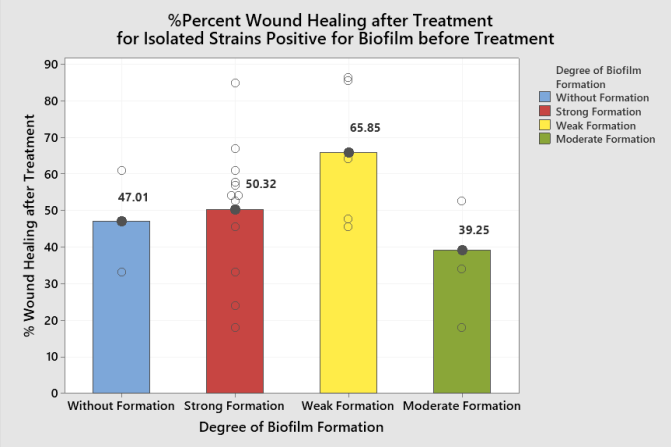

Fig. 9. The influence of the presence and degree of biofilm production in wound samples with isolates before treatment in relation to the degree of wound healing

Classification of isolates based on their biofilm-forming capacity

A total of 18 strains isolated from wound patients were examined for biofilm-forming ability *in vitro*. The analyses were performed using the TCP method. When interpreting the results, we used the "cut-off" value (ODc) calculated based on the received optical density of the negative samples (ODn): ODn=0.1187, 0.1152, 0.1173; ODc=0.4682, Standard Deviation (SD) – 0.00176; "cut-off" value (ODs) defined as three standard deviations (SD) above the OD of the negative control: ODc = mean OD of negative control+(3xSD of negative control); ODc = 0.1170 + 3x0.00176ODc = 0.1170 + 0.3512 = 0.4682

Biofilm production results were interpreted as follows: absence of biofilm formation (ODc < ODc) (ODstrain <0.468); weak biofilm formation (ODc<ODstrain <2xODc) (0.468<ODstrain < 0.936); moderate biofilm formation (2xODc <ODstrain<4xODc) (0.936 <ODstrain< 1.872); strong biofilm formation (4xODc <ODstrain) (1.872 <ODstrain)

Table 5. Potential for biofilm formation of the isolates

Isolated strain from wound		
Type	OD 570 nm	Biofilm formation degree
**MRCNS*	1.956	Strong
Escherichia coli	1.801	Moderate
***MSSA*	0.777	Weak
Proteus mirabilis	1.552	Moderate
Enterococcus	2.313	Strong
MSSA	1.736	Moderate
Proteus mirabilis	2.471	Strong
****MRSA*	0.366	No biofilm
Pseudomonas aeruginosa	2.442	Strong
*****Escherichia coli ESBL*	0.596	Weak
Pseudomonas aeruginosa	2.501	Strong
MSSA	1.897	Strong
MSSA	0.749	Weak
Enterococcus	2.003	Strong
MSSA	0.831	Weak
MSSA	0.973	Moderate
Enterococcus	1.989	Strong
MSSA	2.127	Strong

**MRCNS* - Methicillin resistant coagulase negative *staphylococcus*, ***MSSA*- Methicillin sensitive *S. aureus*, ****MRSA*- Methicillin resistant *S. aureus*, *****Escherichia Coli ESBL*+ -*Escherichia Coli* Extended Spectrum Beta Lactamase +

According to the results obtained, all isolates were classified as: microorganisms with strong capacity (9 strains or 50%), with moderate capacity (4 strains or 22.22%), with weak capacity (4 strains or 22.22%) and without the ability to create biofilm (1 strain or 5.55%) (Table 5). Regarding the heterogeneity of the biofilm producing species isolated from wound samples, there were: 9 (50%), 2(11.11%), 2(11.11%) 3(16.66%) and 2(11.11%) members of the genera *Staphylococcus*, *Pseudomonas*, *Proteus*, *Enterococcus* and *E. coli*, respectively.

Discussion

In our study involving 24 patients with chronic lower extremity wounds, an analysis was made, and results were obtained for the microbiology of chronic wounds, the biofilm-forming potential of the isolates, as well as a correlation between the degree of biofilm production and the degree of wound healing. So far, in the literature there are no data from studies on the correlation of the degree of biofilm production and the degree of healing of chronic wounds.

Our results showed that the number of isolates before initiation of treatment had no influence on the degree of wound healing. There was also no statistical significance regarding the degree of wound healing between the group of patients who had wounds without an isolate compared to the group of patients who had wounds with a microorganism isolate. The correlation between the degree of biofilm production and the degree of wound healing was analyzed. The results showed that isolates with low potential for biofilm production had a higher degree of wound healing compared to those with moderate potential for biofilm production. There were no sufficient data to correlate isolates with strong potential for biofilm production and degree of wound healing.

It is estimated that in 60% of chronic wounds, the biofilm is the cause of their chronicity[20]. A total of 24 patients were included in our study, 7 patients with chronic wounds without isolate and 17 patients with chronic wounds with isolate. They were analyzed according to the number of wounds, considering the fact that some patients had more than 1 chronic wound that was of our interest. Isolates from a total of 30 wounds were analyzed, of which 23 were with isolates, and 18(60%) were with the potential to create a biofilm.

Bowler *et al*., as well as Davies *et al*., in their studies detected that biofilm forming bacteria in chronic wounds were: *P. aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Streptococcus pyogenes*, *Bacillus subtilis*, *Staphylococcus aureus*, and from the fungi *Candida albicans*[9,10]. Summarizing the available literature, the most common bacteria in chronic wound biofilms are ESKAPE pathogens (*Enterococcus faecalis*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter spp*). Other bacteria, such as coagulase-negative *staphylococci* and *Proteus spp.* are also involved in biofilm formation[21,22]. While the focus until now has largely been on the various bacterial pathogens in chronic wounds, the role of fungi, especially Candida species in wound biofilms is of increasing importance[23,24]. However, it should be emphasized that most of this literature is the result of studies of bacteria isolated from wounds rather than direct study of the wound biofilm.

The results in our study showed the potential for biofilm formation in isolates of the following genera: *Staphylococcus* (MRCNS - methicillin resistant coagulase negative *staphylococcus*, MSSA - methicillin sensitive *staphylococcus aureus*, MRSA - methicillin resistant *staphylococcus aureus*), *Pseudomonas aeruginosa*, *Escherichia* (*Escherichia coli*, *Escherichia coli* ESBL+), *Proteus* (*Proteus vulgaris*, *Proteus mirabilis*), *Enterococcus*.

Regarding the heterogeneity of biofilm producing species isolated from wound samples, there were: 9 or 50%, 3 or 16.66% isolates of the genera *Staphylococcus, Enterococcus,* and 2 isolates or 11.11% were of the genera *Pseudomonas, Proteus,* and *E. coli*.

Dowd *et al*. introduced the concept of functional equivalent pathogroups (FEP) in which the individual members of the biofilm community do not cause disease individually, but their co-aggregation in the FEP is what provides the synergistic effect. This provides the biofilm community with favorable factors necessary to maintain sustained inflammation and infection in the wound[25]. In samples taken from wounds before the start of treatment in patients included in our study, out of a total of 30 wounds 1 isolated microorganism was present in 12 wounds (40.0%), 2 isolates in 9 wounds (30.0%), 3 isolates in 2 wounds (6.7%), or more precisely a total of 11(36.7%) wounds showed a polymicrobial nature and in those same wounds the potential for creating a biofilm of the isolates was in the group of strong and moderate production.

The polymicrobial nature in terms of the presence of *Candida albicans* as a poor prognostic sign for biofilm formation was discussed in a study by Allison *et al*.[26]. One of the two wounds with 3 isolates in our study was with an isolate of *Candida albicans*, *Fusarium*, *Enterococcus*, where the last mentioned had a strong potential for biofilm formation that confirmed the previously mentioned fact.

Gardner *et al.* indicate that considering the entire wound microbiome, wound duration positively correlates with bacterial diversity and species richness with relative abundance of *Proteobacteria* and negatively correlates with relative abundance of *Staphylococcus*[27]. Of all the isolates in our study, the degree of wound healing in MSSA is the highest and statistically higher compared to the other isolates that have a low degree of healing, more precisely, it was shown that MSSA had a statistical significance in relation to *Proteus mirabilis* (p=0.032), *Pseudomonas aeruginosa* (p=0.015), MRSA (p=0.012) and *Enterococcus* (p=0.07).

Quantitative assessment of bacterial aggregates from different depths on wound surfaces revealed the localization of *S. aureus* biofilms superficially compared to those of *P. aeruginosa*, which were found much deeper. Knowledge of this spatial organization of biofilm microflora further emphasizes the benefit of debridement in its clinical practice[28]. This is another confirmation that the wound healing rate of MSSA is higher compared to *Pseudomonas aeruginosa* (p=0.015), which may be the result of debridement treatment.

Conclusion

A biofilm is one of the leading causes of wound chronicity. In 60% of isolates, the potential to create a biofilm with different degrees (weak, moderate, strong) was confirmed. A statistically significant association was found between the degree of wound healing and isolates with low potential for biofilm formation, as well as the type of isolate. This study included a small sample of participants, and in the future a larger number of participants and isolates are necessary that will offer more detailed information about the microbiology of chronic wounds, the degree of biofilm formation and its association with the degree of wound healing.

Conflict of interest statement. None declared.

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