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Cultivatable Bacteriota of Chronic Wound of Patients with Diabetic Foot Syndrome with Critical Limb Ischemia Based on Wound Biopsy in Peri-Revascularization Period

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Abstract: Diabetic foot syndrome is often associated with inflammation. The aim of this study was to evaluate the impact of improved blood supply on the change in the clinical status and culturable bacteriota of chronic wounds. Patients with diabetic foot and peripheral arterial disease with a Rutherford score of 5 or 6 were included (n = 23). The blood supply to the limb was assessed with laboratory tests and two time-point qualitative cultures using a wound biopsy. The baseline parameters of the blood supply to the limb were Transcutaneous Oxygen Perfusion (TCPO2) of 15.0 mmHg, an Ankle Brachial Index (ABI) of 0.7, and a Toe Brachial Index (TBI) of 0.1, with an average Wound, Infection, Inflammation (WIfI) score of 5.7 (high). The most frequently isolated pathogens were *Staphylococcus aureus* (26.1%), followed by the Enterobacteriaceae family and *Pseudomonas* spp. (13.0%, each). Negative cultures were present in 47.8% (n = 11). The control parameters of blood supply improved; TCPO2 was 38.5 mmHg, the ABI was 0.9, and the TBI was 0.3, with a reduction in the average WIfI score to 3.7 (mild), while total colony-forming units (CFUs) increased by 13.5%. No cases of reocclusion or restenosis were observed during the study; however, small amputations were performed in two patients (8.7%). Five (21.7%) ulcers were significantly reduced and two (8.7%) progressed, while a negative culture at follow-up was obtained in five fewer patients than at baseline and nine patients presented growth despite having an initial negative result. Quantitative reduction was obtained in four (17.4%) cases. Pathogen distribution at follow-up resembled baseline findings. Optimizing clinical environments (enhancing blood flow and controlling inflammation) in general over focusing singularly on microbiota composition or revascularization seems to be crucial and arguably outweighed the impact of microbial change alone; in particular, reperfusion may increase the conditions to bacterial growth at the first stage.

Keywords: chronic wounds; PAD; CLTI; bacteriota; revascularization

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1. Introduction

Diabetic foot syndrome (DF) is a major complication in patients with diabetes mellitus (DM) and is one of the leading causes of amputation at any level [1]. Worldwide DM has been rising rapidly, increasing from 180 million in 1980 to 537 million in 2021, as reported by the International Diabetes Federation Diabetes Atlas. Projections suggest this number will reach 783 million by 2045, equating to one in eight individuals [2] The prevalence of DF among patients with DM is 4–10%, with lifetime incidence reaching a high of 25% [3]. Individuals suffering from DF face a multitude of risks beyond just amputation, including infection, prolonged hospitalization, and, ultimately, a decreased quality of life [4]. Wound healing in DF lies in its multifactorial pathogenesis, where neuropathy, different grades of ischemia or infection are at play, especially in a hyperglycemic environment [5]. Hemostatic disbalance in DF caused by higher activity of fibrinogen, factor VII, thrombin–antithrombin complex, von Willebrand factor, and others, provides hypercoagulation and impairment of blood supply. In addition, hyperglycemia is responsible for oxidative stress upregulation, changing the environment from beneficial to toxic. Finally, high glucose can impair neutrophil chemotaxis and phagocytosis, increasing infection risk [6].

It has been documented that in approximately 50% of patients with DM, diabetic foot ulcers are accompanied by peripheral artery disease (PAD), and it is estimated that 5–10% of individuals with asymptomatic PAD progress to chronic limb-threatening ischemia (CLTI) within five years [7,8]. Hypoxia exacerbates oxidative stress, further impairing wound healing through micro- and macrocirculatory dysfunction. This leads to poor angiogenesis and the retention of unfavorable metabolites in tissues. Thus, revascularization has an impact on microcirculation, decreasing the thickening of the capillary membrane, allowing for a better immunological response and oxidative stress reduction [9]. Additionally, it facilitates nutrient delivery, collagen deposition and epithelialization [10].

The compromised blood flow, along with the decreased healing, may additionally promote the multiplication of microorganisms as a result of local tissue ischemia, which increases the risk of infection [11].

Wounds serve as entry points for skin microbiota and environmental microorganisms, which exploit favorable growth conditions in the wound environment. Different microorganisms are suspected to have a pivotal role in impaired wound healing, an instance in which wounds have failed to progress through the normal stages of healing [9]. In DM, the combination of an infection and ischemia exacerbates complications. Ischemia hampers blood flow, impairing wound healing and making tissues more susceptible to infection. Infections further compromise healing, creating a cycle of worsening tissue damage. However, there are limited data on how ischemia and infection interact in DM wounds. Impaired blood circulation not only decreases the ability of the antibiotics to reach the site of infection but also lowers the essential oxygen levels that are needed for optimal drug metabolism, which, in turn, affects bacterial antibiotic susceptibility and leads to further risk of infection [12]. Additionally, infection may ultimately add to the ischemic pain often felt in these patients [13].

The etiology of diabetic foot infections includes staphylococcus, streptococcus, pseudomonas, and many others, including anaerobes, and in about 15% of cases, osteomyelitis may occur. PAD does not increase anaerobic etiology itself [14]. Infection of DM, initially with one pathogen usually, quickly becomes polyetiological due to good conditions for growth and the multiplication of pathogens in the diabetic foot. The authors of the other studies [15] indicate that the infection itself may be serious enough to be a major factor leading to limb amputation. Thus, diagnosis of infection and its treatment seem to be a continuing challenge in the approach to patients with diabetic foot [16]. But microbiological diagnosis of chronic wound infections according to the Infectious Diseases Society of

America should be based on aspirate, biopsy or a surgical specimen. However, a smear should not be routinely used [17]. The microbiota of DF ulcers is complex, often involving biofilm formation and anaerobic components, which are the most challenging factors in modern wound microbiology. In vivo biofilm assessment and identifying crucial etiological microorganisms still seem to pose problems; some authors emphasize that even less commonly studied microorganisms, such as *Corynebacterium* spp., may play a significant role in deep wound infections, as identified through PCR analysis [18]. Given that up to 50% of patients with clinically significant infections may require amputation, understanding the microbiota is crucial for developing targeted treatment strategies [19].

The mixed events that cause and allow DF to progress are all factors that make ulceration treatment a therapeutic challenge. While wound healing even in healthy individuals is a complex process requiring not only a plethora of cell types, but the creation of capillary beds via angiogenesis [20], the presence of ischemia and infection compounds this complexity. To date, limited research has explored the interaction between ischemia and infection, particularly in relation to microbiological profiles before and after revascularization.

The aim of this study was to assess the relationship between the wound bacteriota in DF and the clinical condition, with revascularization of lower limb arteries as the modifying factor.

2. Materials and Methods

The study included patients admitted to the Department of Angiology between February 2021 and May 2022. The participants met specific inclusion criteria. They were aged 40 years (the lower threshold was set to exclude bias from underdiagnosed diabetes types, and the upper limit excluded very old patients with impaired wound healing); had type 2 diabetes mellitus (DM); presented with ischemic diabetic foot syndrome (DFS); and exhibited critical limb ischemia due to peripheral arterial disease (PAD), classified as Rutherford category 5 or 6. Patients with changes that could suggest neoplastic etiology or different sources of systemic infection were excluded from the study.

During the data collection process, medical history (including chronic conditions, smoking status, and medications) was obtained. Basic laboratory tests, including lipid profiles, blood morphology, kidney function, and C-reactive protein levels, were conducted after a 12-h fasting period.

The severity of chronic limb ischemia was evaluated at admission and at 30-day follow-up using the Rutherford classification system, focusing on categories 5 (minor tissue loss) and 6 (major tissue loss above the transmetatarsal level). The advancement of diabetic foot syndrome and risk of amputation were assessed using two International Working Group on the Diabetic Foot (IWGDF) classification systems: SINBAD (Site, Ischemia, Neuropathy, Bacterial Infection, and Depth) and WIfI (Wound, Infection, Inflammation). According to WIfI, infections were classified as mild (cellulitis < 2 cm), moderate (cellulitis > 2 cm without systemic involvement), or severe (cellulitis with systemic inflammatory response). The status of lower limb ischemia was determined using the ankle-brachial index (ABI) and toe-brachial index (TBI). Microcirculation was evaluated through laser Doppler flowmetry (LDF) and transcutaneous oximetry (tcpO2). Both measurements were taken using the Periflux 6000 system (Perimed AB, Järfälla, Sweden), which includes thermostatic laser Doppler probes and a modified Clark's polarographic oxygen sensor.

Wound biopsies were cultured twice: on the day of admission after qualification for revascularization, and at the 30-day follow-up. Before tissue collection, wounds were debrided to remove superficial fibrin layers. The wound was irrigated with 0.9% sodium chloride solution (lavaseptic), and a sterile scalpel was used to obtain a sample from the deeper layers of the ulcer. Samples were placed in Copan Diagnostics ESwab™ (Murrieta,

CA, USA) tubes for further analysis. After weighing, the sample was homogenized and plated onto Muller–Hinton agar, blood agar, MacConkey agar and Chapman agar using the dilution method (2–5 serial dilutions).

After incubation, the colonies were counted and reported, and the results are presented as colony-forming units (CFU) per mL (CFU/mL). Significant pathogens (*Streptococcus* spp., *Staphylococcus* spp., *Pseudomonas* spp., etc.) were identified using MALDI-TOF MS mass spectrometry (MALDI Biotyper, Bruker, Billerica, MA, USA). The quantitative microbial burden was calculated per gram of tissue using the following formula [21]:

$$\frac{\text{Total number of organisms[CFU]} \times 5(\text{for homogenic dilution}) \times 10^{x}(\text{plate dilution})}{\text{tissue weight [g]}} = [\frac{\text{CFU}}{\text{g}}]$$

Continuous variables were summarized as median and interquartile ranges, while categorical variables were presented as counts and percentages. Comparisons of continuous and nominal variables were performed using the *t*-test and Fisher's exact test, respectively. All statistical analyses were conducted using IBM SPSS Statistics version 26 (IBM Corporation, Armonk, New York, NY, USA). A significance level of $p < 0.05$ was considered statistically significant.

3. Results

The study included 23 patients, with the majority being male (n = 19, 82.6%). The median age was 70 years for males and 61.5 years for females. A large proportion of patients had a smoking history, with 14 (60.9%) being former smokers and 2 (8.7%) being current smokers. The most common chronic comorbidities were hypertension (n = 21, 91.3%), heart failure (n = 10, 43.5%), and coronary artery disease or atrial fibrillation (n = 5, 21.7%) (Table 1). Baseline laboratory findings revealed a median hemoglobin level of 12.6 g/dL (interquartile range (IQR): 11.90; 13.70) and white blood cell count of $9.5 \times 10^3/\mu L$ (IQR: 8.18; 11.25), both within normal ranges. Median LDL cholesterol was 2.0 mmol/L (IQR: 1.47; 2.32). However, C-reactive protein levels were elevated, with a median value of 13.2 mg/L (IQR: 3.34; 50.10; reference <5.0 mg/L). As a glucose control parameter, glycated hemoglobin (HbA1c) was measured, with a median value of 7.8% (IQR: 6.70; 9.50).

Table 1. Study population baseline characteristics.

Studied Factor	N = 23
Gender, male n (%)	19 (82.6%)
Age, median (IQR)	
Males	70.0 (63.0; 75.0)
Females	61.5 (54.5; 68.5)
Smoker n (%)	
Current	2 (8.7%)
Former (non-smoker at day of admission)	14 (60.9%)
Non-smoker	7 (30.4%)
Rutherford ischemia classification n (%)	
5	15 (65.2%)
6	8 (34.8%)
Hypertension n (%)	21 (91.3%)
Coronary Artery Disease n (%)	5 (21.7%)
Heart Failure n (%)	10 (43.5%)
Atrial Fibrillation n (%)	5 (21.7%)
Stroke n (%)	2 (8.7%)
Chronic Kidney Disease n (%)	3 (13.0%)
Chronic Venous Insufficiency n (%)	3 (13.0%)

IQR—interquartile range.

At baseline, the majority of wounds were classified as Rutherford category 5 (minor tissue loss) (n = 15, 65.2%), while eight patients (34.8%) had category 6 wounds (major tissue loss). Taking into consideration the blood supply parameters at follow-up compared to baseline, all measured parameters improved by at least 28.6% (Table 2).

Table 2. Clinical ischemic and diabetic foot assessment parameters.

Factor	Pre-Intervention	Post-Intervention
	Median (IQR)	
Toe-Brachial Index	0.1 (0.06; 0.20)	0.3 (0.18; 0.42)
Ankle-Brachial Index	0.7 (0.36; 0.78)	0.9 (0.78; 1.04)
TCPO2 [mmHg]	15.0 (10.00; 25.00)	38.5 (26.00; 43.00)
SINBAD score	4.0 (3.00; 5.00)	3.00 (2.00; 4.00)

Legend: TCPO2—transcutaneous oxygen pressure, IQR—interquartile range.

By quantitatively analyzing all isolated bacteria in total and comparing the follow-up to the baseline period, an increase in culture density (measured by CFU/g) of approximately 13.5% was observed. We noted the same level or a decrease in the infection parameter (based on the WIfI scale) without correlation to the increasing culture density (by CFU/g, Table 3). There were no major amputations during the study period.

Table 3. Case series with cumulative quantitative change results. In cases of polymicrobial culture, results were summed up.

Age	Sex	Rutherford		Infection (Based on WIfI)		Quantitative Culture [CFU/g]	
		Pre	Post	Pre	Post	Pre	Post
54	F	5	5	mild	no	7×10^7 (*)	5×10^7 (*)
66	M	6	6	mild	mild	0	4×10^4
74	M	5	5	moderate	mild	2×10^7 (*)	1×10^8 (*)
63	M	5	5	moderate	moderate	1×10^7	4×10^4
68	F	6	5	mild	moderate	1×10^5	7×10^4
66	M	5	5	moderate	moderate	0	2×10^5
55	F	6	6	moderate	moderate	6×10^5	8×10^7 (*)
62	M	6	6	moderate	mild	0	4×10^5
70	M	5	5	moderate	moderate	3×10^7 (*)	4×10^6
79	M	5	5	mild	no	2×10^7 (*)	0
81	M	5	5	no	no	0	2×10^8 (*)
73	M	5	5	moderate	no	3×10^5	0
75	M	5	5	no	mild	0	5×10^6 (*)
55	M	5	5	no	no	4×10^7 (*)	3×10^7 (*)
74	M	5	5	mild	no	2×10^8	0
57	M	6	6	moderate	moderate	0	1×10^6
69	F	5	5	no	no	0	0
83	M	5	5	no	mild	2×10^6	0
68	M	6	6	mild	no	0	1×10^5
74	M	5	-	moderate	no data	0	4×10^5
63	M	6	6	moderate	moderate	2×10^7	2×10^5
57	M	5	5	moderate	mild	0	0
84	M	6	6	mild	no	0	6×10^6

Legend: (*)—*S. aureus* as the main etiological factor, M—male; F—female; CFU/g—colony forming units per gram of tissue; WIfI—Wound, Infection, Inflammation scale.

The average WIfI score decreased from 5.7 (high) to 3.7 (mild), with improvement in all wound parameters (Table 4). In eleven cases (47.8%), we obtained an increasing trend in WIfI scale or quantitative measurement, in one case (4.4%) in both, and in eleven cases (47.8%) no change or decrease in both.

Table 4. Wound, Infection, Inflammation scale classification distribution change.

WIfI Classification Parameter	Pre-Intervention	Post-Intervention
Wound		
No ulcer, no gangrene	0 (0.0%)	1 (4.3%)
Small ulcer, no gangrene	8 (34.8%)	10 (43.5%)
Deep ulcer and gangrene limited to toes	13 (56.5%)	9 (39.1%)
Extensive ulcer or gangrene	2 (8.7%)	2 (8.7%)
Ischemia		
Toe pressure\tcpO2 > 60 mmHg	0 (0.0%)	5 (21.7%)
40–59 mmHg	1 (4.3%)	8 (34.8%)
30–39 mmHg	2 (8.7%)	3 (13.0%)
<30 mmHg	20 (87.0%)	6 (26.1%)
Infection		
Lack of infection	5 (21.7%)	9 (39.1%)
Mild < 2 cm cellulitis	7 (30.4%)	6 (26.1%)
Moderate > 2 cm cellulitis/purulence	11 (47.8%)	7 (30.4%)
Severe systemic response	0 (0.0%)	0 (0.0%)

Prior to intervention, 47.8% of wound cultures were negative (n = 11). The most frequently isolated pathogens were *Staphylococcus aureus* (26.1%, n = 6), followed by members of the Enterobacteriaceae family (13.0%, n = 3), and *Pseudomonas* spp. (13.0%, n = 3). In six cases, a single pathogen was identified: *S. agalactiae, P. agglomerans, M. morganii, B. fragilis* and *P. melaninogenica.* In five cases, there was a combination of two strains, and in one case, there were three pathogens.

At follow-up, six cultures (26.1%) remained negative, with qualitative changes and increased CFU/g in 52.2% of cases compared to baseline (Figure 1). Follow-up cultures showed a similar distribution to baseline, with *Staphylococcus aureus* remaining dominant (n = 6), followed by *Klebsiella spp.* (n = 3), *E. faecalis* (n = 3), *S. agalactiae* (n = 2), *S. marcescens* (n = 2) and *E. coli* (n = 2). There was only one case with a single isolated pathogen, i.e., *P. canis*. In ten cases, single-strain etiology was identified, double-strain etiology was found in six samples, and three pathogens were identified in one case.

During phone-call follow-ups, 14 (60.9%) patients declared Fiotic use between discharge and 1-month follow-up and reported one course of antibiotics prescribed at discharge or by a general practitioner. The most commonly used antibiotic was clindamycin (n = 9, 39.1% of all patients) followed by ciprofloxacin (n = 3, 13.0% of all patients).

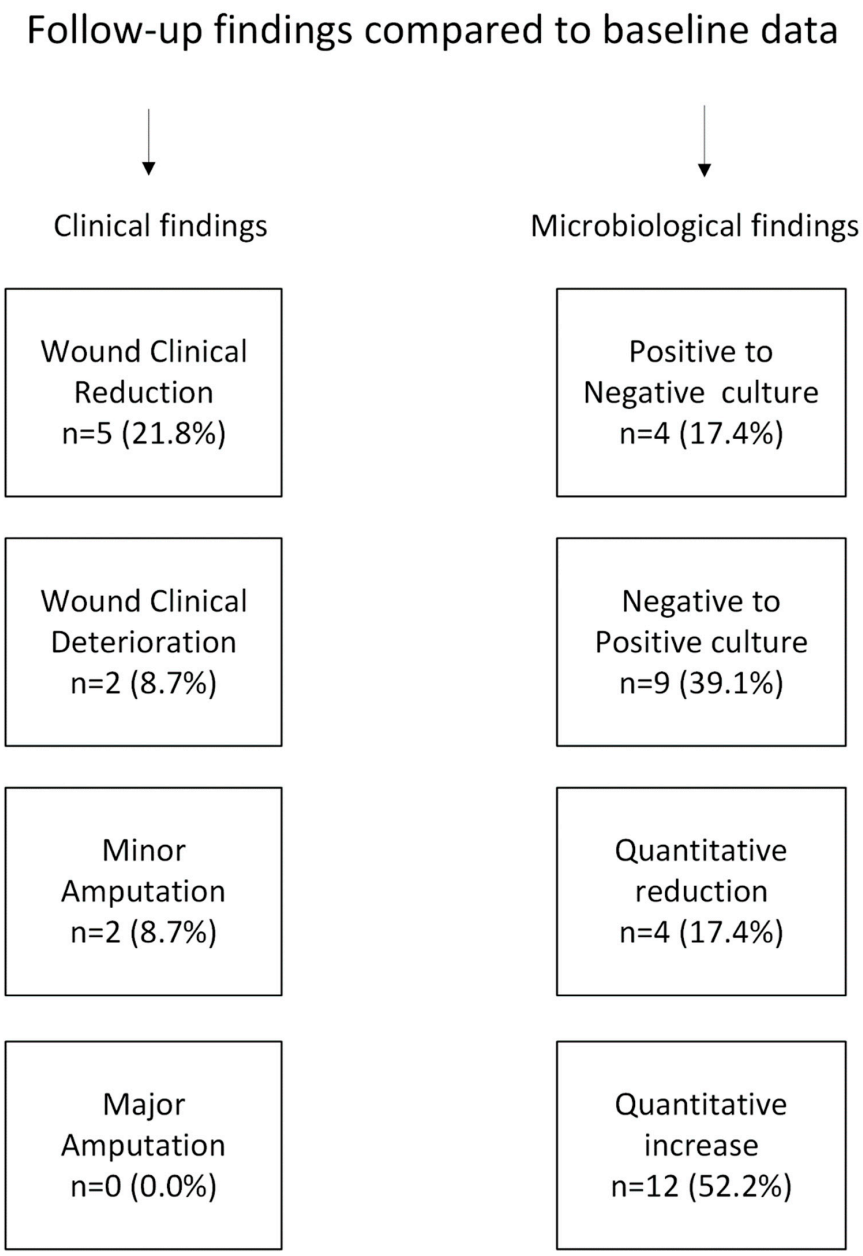

Figure 1. Follow-up clinical and microbiological findings compared to baseline data.

4. Discussion

Our study highlights that the composition of wound bacteriota alone does not play a decisive role in determining the treatment paradigm, including the necessity of antibiotics. Instead, what emerges as paramount is ensuring a favorable clinical environment, which hinges on ensuring adequate blood supply and inflammation control. This may explain why, despite a general 13.5% increase in quantitative bacterial load, the infection component of the WIfI scale and SINBAD scores improved (Table 3). Literature suggests that reperfusion may transiently damage vulnerable tissues, potentially creating conditions conducive to microbial growth and leading to an increase in total CFU/g within one month of follow-up [22,23]. However, chronic wounds often remain in a persistent inflammatory state, which creates a hostile microenvironment that impedes healing [24]. In this context, restoring blood flow not only enhances oxygenation and immune response but also outweighs the potential adverse effects of increased bacterial counts.

The variations in culture results observed in our study largely depend on the sampling technique used—whether a non-invasive, superficial swab or an invasive deep tissue

biopsy [25]. It is important to note that while the overall number of pathogens increased, individual counts may vary, with some showing an increase while others decrease. Conventional swabs may not capture such nuanced changes, as shown in Table 3. Unlike swabs, biopsies allow for both quantitative and qualitative assessments of bacteria, offering a more comprehensive understanding of wound microbiota while reducing the risk of contamination. In contrast, swab collections often have limited clinical value due to poor sensitivity and specificity, leading to the identification of insignificant microorganisms and mistreatment [26]. Huang et al. found that swab cultures may be reliable in guiding antibiotic treatment in grade 2 DFs, but wounds of grade 3 or higher should be assessed with deep tissue biopsies. Relying solely on qualitative assessments may paint an overly grim picture of the situation, while quantitative examinations offer a more balanced perspective and potentially guide more effective treatment strategies. Additionally, swab cultures in higher-grade wounds are associated with a high risk of identifying insignificant pathogens [27], as seen in our study with bacteria from the Enterobacteriaceae family (14.3%, n = 3) and *Pseudomonas* spp. (14.3%, n = 3).

The distribution of pathogens, with a predominance of *Staphylococcus aureus*, is typical for diabetic foot infections and aligns with the findings of a meta-analysis involving over 16,000 patients [28].

Most of the isolated strains were representatives of the skin microbiota, indicating endogenous colonization. However, the presence of strains like *Pantoea agglomerans* or *Pasteurella canis* suggests that proper wound management is crucial to avoid such pathogens becoming potential etiological factors. It is especially crucial for diabetic patients, who are more vulnerable to opportunistic and environmental infections.

Prior to intervention, the median SINBAD score for patients was 4. However, findings show that post-intervention the median score decreased to 3, reflecting a reduced risk of non-healing wounds, infection, and ultimately, amputation [29].

To account for external treatment influences, patients were asked about antibiotic use during the study period (prescribed by any medical doctor). We found that 14 patients (60.7%) reported undergoing one course of antibiotics during the postoperative period up to the 1-month follow-up. Poland has one of the highest antibiotic consumption rates in Europe, which may result in habitual prescriptions even without general symptoms [30]. Between 2006 and 2017, there was a constant increase in the total consumption of systemic antibiotics and the relative consumption of beta-lactamase-sensitive penicillins [31]. Despite the widespread use of antimicrobials in our study group, we observed an increase in CFU/g levels, emphasizing the need for more appropriate use of these agents. Moreover, our study showed a general improvement in all wound parameters post-intervention (Table 2). It is important to note that microbes in chronic wound biofilms are well-adapted to survive challenging conditions, including antibiotics [32]. Biofilms, once thought to develop only in chronic wounds, are now known to evolve in the early stages of wound formation [33]. Excessive focus on eradicating local infections, particularly with systemic antibiotics, may result in more complications than benefits, especially since fluoroquinolones were used despite EMA restrictions calling for reduced consumption [34]. Additionally, only qualitative assessment of wound material is still common, which, as this study showed, does not provide significant clinical information and may falsely suggest a need for antimicrobial treatment. Altmann et al. found that antibiotic therapy, regardless of administration route, had no significant effect on the clinical or microbiological outcomes of treated patients with diabetic foot infections [35].

Identifying patient factors such as chronic diseases and smoking status was critical for this study (Table 1), as these factors significantly affect treatment outcomes [36]. The majority of patients with DF and CLTI were former smokers (60.9%). Smoking, even after

cessation, has long-term effects on vascular health and wound healing [37]. Former smokers often retain some levels of vascular damage incurred during their smoking years, which impairs blood flow and compromises tissue oxygenation [38], both critical factors in wound healing. The vasoconstrictive properties of nicotine and other toxins in cigarette smoke can lead to this reduction, delaying the delivery of oxygen and nutrients essential for wound healing [39]. Moreover, smoking diminishes the body's immune responses and impairs the function of immune cells involved in wound repair, further hindering the healing process. Sorensen et al. found that "former smokers have a higher risk of impaired wound healing compared to non-smokers", with evidence suggesting that even "long-term cessation of smoking does not fully reverse the detrimental effects on wound healing" [40]. This may explain why some wounds in our study showed limited improvement or even enlargement. While the long-term effects of smoking cessation on wound healing remain unclear, optimal infection and inflammation control, alongside microcirculation improvement, must be prioritized in treatment strategies. Further research is needed to explore optimal strategies for former smokers. Nevertheless, maintaining smoking cessation remains vital, as former smokers experience fewer complications than current smokers [40]. Saint-André et al. investigated immunological changes related to smoking, showing that current smokers exhibit increased chemokine CXCL activity and elevated levels of IL-2 and IL-13, while former smokers show only IL-2 and IL-13. These findings suggest that current smokers have impaired innate and adaptive immunity, whereas former smokers primarily have impaired adaptive immunity [41].

In our study, TcPO2 increased post-intervention from 17 mmHg to 30 mmHg, meeting the threshold for diagnosing CLTI according to the latest guidelines of the European Society of Cardiology [42]. Other studies have shown that even a lower cut-off of 25 mmHg predicts diabetic foot ulcer healing [43]. The observed improvement in oxygenation may therefore provide sufficient conditions for progressive wound healing.

5. Conclusions

In conclusion, this study underscores the critical importance of optimizing the overall clinical environment rather than focusing exclusively on microbiota composition or revascularization. Our findings demonstrate that enhancing blood flow and controlling inflammation are paramount for wound healing and often outweigh the impact of microbial changes alone, especially since reperfusion can initially create conditions favorable for bacterial growth. Despite this, revascularization led to significant clinical improvements, even in the context of increased bacterial density. Therefore, medical interventions should avoid reliance on microbial growth fluctuations as the sole determinant of treatment decisions, especially in the context of systemic antibiotic overuse. Excessive antibiotic use, as highlighted in this study and supported by cited literature, often fails to significantly reduce local microbiota levels while unnecessarily exposing patients to potential complications.

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