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Research Article

Wound Grade–Associated Microbiome Diversity in Diabetic Ulcers: A Metagenomic Analysis

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Abstract
The wound's microbiome in patients with diabetes plays a vital role for developing of diabetic ulcers. Molecular sequencing technologies have enhanced our understanding of microbial diversity in diabetic wounds, making microbiome profiling analysis essential for supporting effective clinical management. A metagenomic approach enables the more accurate identification of pathogenic bacteria to support rational antibiotic use and the development of more targeted therapies. This study aimed to identify the bacterial diversity in diabetic ulcers before and after antibiotic therapy using a metagenomic approach to analyze changes in the microbiome profile and detect potentially antibiotic-resistant pathogenic bacteria. Wound swabs from each patient were genomically extracted for metagenomic analysis using nanopore sequencing. The samples from individuals administered antibiotics were coded 4, 7AB, and 8, whereas the samples from patients not administered antibiotics were coded 11. Limitations of this study include its relatively small sample size (n = 4). This study shows that the species with the highest abundance in sample 11 was *Acinetobacter junii*, in sample 4 was *Proteus mirabilis*, in sample 7AB were *Alcaligenes faecalis* and *Pseudomonas aeruginosa,* and in sample 8 was *Acinetobacter baumannii*. Both groups who did not receive antibiotic treatment and those who received antibiotic therapy showed the presence of diverse microorganisms, including potentially pathogenic bacteria. Future treatment regimens should be based on the type of bacterial infection in wounds of diabetic patients.

Keywords: diabetic ulcer, grade, infection, metagenomic, nanopore sequencing, pathogenic, wagner

1. INTRODUCTION

Diabetic ulcers are among the most severe complications of diabetes mellitus, posing substantial therapeutic challenges and frequently leading to non-traumatic amputations that may result in mortality [1]. Poorly controlled blood glucose levels are commonly observed in patients with diabetic ulcers, and diabetic foot ulcers (DFUs) represent the most prevalent form [2]. Globally, the prevalence of DFUs is estimated to be approximately 6.3% among individuals with diabetes, with marked geographical variations. North America reports the highest prevalence (13 %), followed by Africa (7.2%), Asia (5.5%), and Europe (5.1%), whereas Oceania has the lowest prevalence at 3%. The lifetime risk of developing DFUs ranges from 15% to 34%, with an annual incidence between 2% and 6% [3][4]. In Indonesia, the prevalence of DFUs varies depending on the study setting, ranging from 7.3% to 12% among hospitalized patients with type 2 diabetes and reaching up to 24% in community-based estimates [2][5][6].

Diabetic ulcers frequently progress to chronic wounds and are associated with an increased risk of infection. These wounds are often colonized by multidrug-resistant (MDR) bacteria, complicating treatment and delaying wound healing. Common microorganisms identified in infected diabetic wounds include *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* [7]-[10]. Persistent infections in DFUs may result in severe complications, including sepsis and amputation, both of which are associated with a markedly increased mortality rate. Several studies have reported high long-term mortality rates among patients with DFUs, with mortality rates approaching 30% within 5 years and increasing substantially following major amputation [11].

Although antibiotics remain the primary treatment for diabetic wound infections, increasing reports of bacterial resistance and multidrug-resistant infections have been noted. Antibiotic resistance has been identified in bacterial isolates from diabetic wounds, including *S. aureus* resistant to benzylpenicillin, amoxicillin, dicloxacillin,

Table 1. Patient characteristics based on wound duration, antibiotic type, and wound grade.

Code Patient	Gender	Age (years)	Duration of Diabetes (years)	Random Blood Glucose (mg/dL)	Fasting Blood Glucose (mg/dL)	Length of wound	Antibiotic amount	Antibiotic type (mg)	Wound grade	Cause of wound
4	Female	58	5		124	24 month	1	Azithromycin 500	II	blistering
7AB	Female	72	5	277		4 week	1	Cefixim 200	II	friction
8	Male	75	20	-		4 week	1	Cefixim 200	IV	-
11	Male	52	5	167		2 week	0	-	I	exposure to motorcycle exhaust

oxacillin, ertapenem, imipenem, and tetracycline; *P. aeruginosa* resistant to tigecycline, nitrofurantoin, trimethoprim, cefazolin, ampicillin, ceftriaxone, and ertapenem; and *E. coli* resistant to nitrofurantoin, aztreonam, ampicillin, ceftriaxone, and cefazolin [12][13]. Previous studies have also reported the presence of hemolytic bacterial strains isolated from diabetic ulcer swabs, including β-, α-, and γ-hemolysin-producing bacteria. In addition, multidrug-resistant and biofilm-forming strains of *P. aeruginosa* and *S. aureus* carrying blaTEM and sul1 resistance genes have been identified in diabetic ulcer specimens [14][15].

Conventional culture-based diagnostic methods may not fully capture the complexity of microbial communities in diabetic wounds. In this context, metagenomic sequencing has emerged as a valuable complementary approach for the etiological diagnosis of diabetic foot ulcers [16]. Metagenomic approaches have substantially improved the characterization of complex polymicrobial communities associated with DFUs. Both 16S rRNA gene sequencing and shotgun metagenomic analyses have consistently demonstrated that the DFU microbiota are predominantly composed of members of the *Firmicutes, Proteobacteria, Actinobacteria*, and *Bacteroidetes* phyla, and have revealed the important contribution of anaerobic microorganisms that are frequently undetected by conventional culture-based methods [17]. Frequently detected genera and species include *S. aureus, P. aeruginosa, Corynebacterium striatum, Streptococcus, Enterococcus, Prevotella, Porphyromonas, Bacteroides*, and other anaerobic bacteria [16][18]. Severe or chronic ulcers with higher Wagner grades are commonly associated with obligate anaerobes and gram-negative bacteria, including *Bacteroides, Prevotella, Morganella, and Gammaproteobacteria* [18].

Metagenomic sequencing enables the rapid and comprehensive identification of diverse pathogenic microorganisms with high sensitivity and accuracy, thereby reducing diagnostic delays and supporting timely antimicrobial intervention. Furthermore, metagenomic approaches facilitate the simultaneous detection of microbial diversity and potentially antibiotic-resistant pathogenic bacteria in diabetic wounds [16]. However, information on the metagenomic profiles of diabetic ulcer microbiota

in Indonesian patients remains limited. Therefore, this study aimed to identify the bacterial diversity in diabetic ulcers before and after antibiotic therapy using a metagenomic approach to analyze changes in the microbiome profile and detect potentially antibiotic-resistant pathogenic bacteria.

2. MATERIALS AND METHODS

2.1. Materials

Diabetic ulcer swab samples were collected from the Balutan Luka Terkini '@nan' clinic located in the Tambun Selatan District, Bekasi Regency, West Java, Indonesia. The patients selected for sampling were diabetic patients who had consumed one or more antibiotics (patients with samples 4, 7AB, and 8). One sample from a patient who was not treated with antibiotics (code 11) was used for the comparison. Healthcare professionals obtained wound specimens using sterile swabs saturated with 10 mL of phosphate-buffered saline. Metagenomic analysis employing next-generation sequencing (NGS) methods (unculturable bacteria) was performed at PT. Genetic Science in Indonesia [19]. Ethical approval was obtained from the Human Research Ethics Committee of Bogor Agricultural University (1172/IT3.KEPMSM-IPB/SK/2024).

2.2. Methods

2.2.1. Isolation of the DNA for Metagenomic Analysis

Genomic DNA was isolated from diabetic ulcer swab specimens using the ZymoBIOMICS DNA Miniprep kit (Zymo Research, USA). Briefly, 250 µL of the swab sample was mixed with 750 µL of lysis buffer in a microcentrifuge tube, followed by thorough homogenization and centrifugation at 10,000 × g for 1 min. Subsequently, 400 µL of the clarified lysate was loaded onto a Zymo-Spin IIIF filter and centrifuged at 8,000 × g for 1 min. The resulting flow-through was combined with 1,200 µL of DNA binding buffer and processed through an IICR column by centrifugation at 10,000 × g for 1 min. The column was sequentially washed using 400 µL, 700 µL, and 200 µL of DNA wash buffer, with each washing step followed by centrifugation at 10,000 × g for 1 min. Finally, the column was transferred to a clean microtube, and DNA was eluted with 35 µL of DNase/RNase-free water after a 2-min incubation, followed by centrifugation at 10,000 × g for 1 min.

2.2.2. Metagenomic Sequencing Analysis

The DNA concentration was quantified using a Qubit fluorometer and a Nanodrop spectrophotometer. Oxford Nanopore Technologies kits were used for library preparation. MinKNOW software (version 22.05.7) was used for the nanopore sequencing. Guppy (version 6.1.5) was used for base calling [20]. NanoPlot was used to assess FASTQ file quality, and NanoFilt was used for quality filtering [21][22]. A centrifuge was used to classify the filtered readings [23]. The 16S RefSeq database was used to determine bacterial indices. Krona Tools, Pavian, and RStudio (Posit

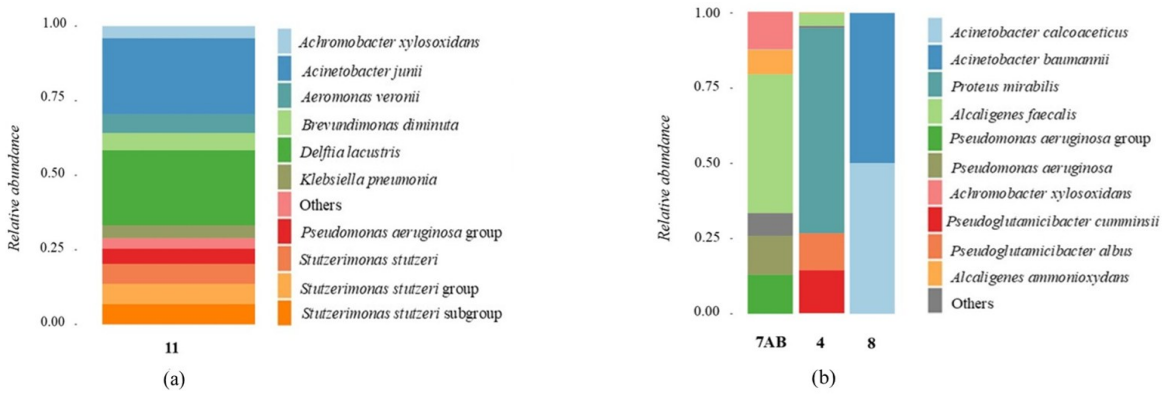

Figure 1. Relative abundance for bacterial species in diabetic wounds as determined by metagenomic analysis. (a) Relative abundance for bacterial species in diabetic wounds (sample 11, absent antibiotic treatment). (b) Relative abundance based on bacterial species isolated from diabetic wounds (samples 7AB, 4, and 8 with antibiotic therapy).

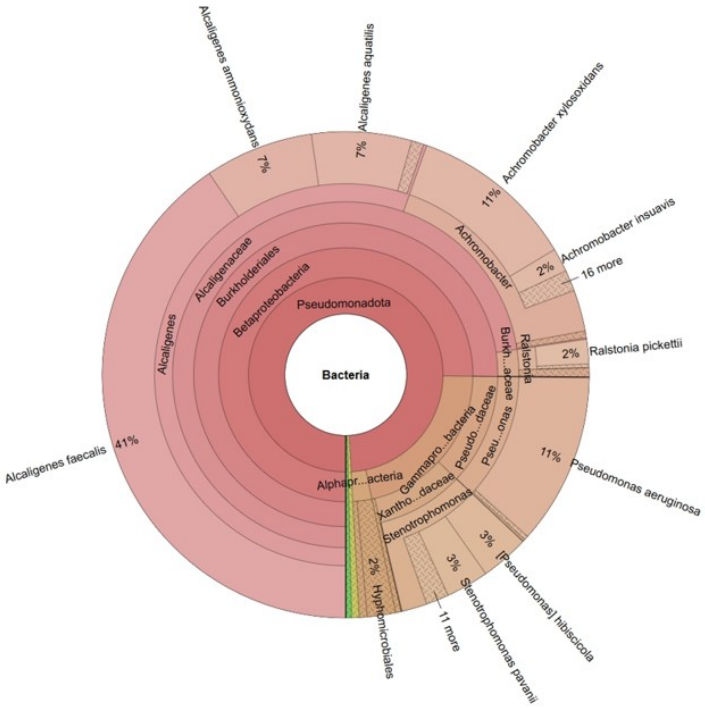

Figure 2. Metagenomic profile of the microbiome in sample 7AB (antibiotic-treated).

Software, PBC, USA) version 4.2.0 were used for visualization and downstream analysis. Alpha diversity was assessed using the Chao1 and ACE richness estimators, as well as Shannon, Simpson, inverse Simpson (InvSimpson), and Fisher's alpha diversity indices, to characterize bacterial richness and diversity within each sample. Beta diversity was evaluated using Bray–Curtis dissimilarity and visualized through principal coordinates analysis (PCoA) to compare bacterial community structures among samples. Microbial community profiling was conducted using Oxford Nanopore Technology (ONT)-based full-length 16S rRNA gene sequencing, covering the complete V1–V9 regions (~1,500 bp) of the 16S rRNA gene sequence. This approach provides enhanced taxonomic resolution and facilitates more accurate bacterial identification at the species level compared with partial 16S rRNA amplicon sequencing [24][25].

3. RESULTS AND DISCUSSIONS

3.1. Characteristics of Patients with Diabetic Wounds

Four wound swab specimens were obtained from four patients with diabetic ulcers. The microbial diversity profiles of the four samples were analyzed using metagenomic techniques (Table 1). One wound swab sample (code 11) was collected as a control, which did not receive antibiotic therapy. Three wound samples (4, 7AB, and 8) were samples that had received one or more courses of antibiotic therapy. Three samples were obtained from three different patients. Antibiotic administration in three patients with diabetic wounds (patients 4, 7AB, and 8) did not improve the condition of the wounds. The predominant diabetic ulcer grading system is the Wagner FW (1981) wound classification, which categorizes wounds according to depth, tissue involvement, and the occurrence of gangrene and osteomyelitis, using the following scale: grade 0, pre- or post-traumatic lesions; grade 1, superficial wounds limited to the skin and subcutaneous tissue; grade 2, ulcers have spread to the joint capsule, tendons, and bone; grade 3, the wound is deeper with bone involvement and osteitis; grade 4, gangrene in part of the leg; grade 5, gangrene covering an entire leg [26].

Wounds create exposed areas that allow bacteria to enter and infect surrounding tissues. These bacteria can cause infections that may hinder the wound-healing process [27]. Bacteria identified as the causes of diabetic wound infections include *Proteus sp, E. coli, Citrobacter sp, Klebsiella sp, Enterococcus, Enterobacter sp, Streptococcus sp, S.*

aureus, Staphylococcus sp koagulase negatif, *Morganella sp, Acinetobacter sp, Serratia sp, Achromobacter xylosoxidans, Ochrobacterium anthropi, Enterococcus faecalis, Staphylococcus aureus* resisten methicillin, *Corynebacterium, Citrate spp. Enterobacter cloacae, Enterobacter aerogenes, Enterobacteriaceae, Stenotrophomonas maltophilia, Alcaligenes, Finegoldia magna, Staphylococcus epidermidis, Morganella morganii, Citrobacter spp, Providencia rettgeri, Dermabacter hominis, Anaerococcus spp., Finegoldia spp, Firmicutes, Actinobacteria , Proteobacteria, Bacteroidetes, Fusobacteria* [7]-[9][28][29] *Alcaligenes faecalis* NRBC 13111, *Shigella flexneri* ATCC 29903, *Enterococcus faecalis* ATCC 19433, *Proteus mirabilis* ATCC 29906, *Proteus mirabilis* JCM 1669, *Acinetobacter soehaensis* SW-100, *Klebsiella sp., S. aureus, Proteus mirabilis, P. aeruginosa, Proteus vulgaris*, *Enterobacter sp, Proteus mirabilis, Basillus. sp* [10][30][31] *Finegoldia, Corynebacterium, Streptococcus, Pseudomonas, Staphylococcus, Enterococcus, Stenotrophomonas, Prevotella, Acinetobacter, Anaerococcus, Serratia, Bacteroides, Enterobacter, Delftia, Propionibacterium, Proteus, Peptoniphilus, Flavobacterium, Fusobacterium* [32], *Burchlorderia* [33]. The microorganisms responsible for diabetic wound infections are inadequately documented. Consequently, it is essential to examine the variety of microbiomes using metagenomic methods. The examination of microbiome diversity in diabetic wounds is crucial for identifying the predominant bacterial types, as well as their quantity and variety, which can influence the management of bacterial infections and the development of effective therapies for diabetic wounds [34].

3.2. Bacterial Populations based on Metagenomic Analysis (Unculturable) in Patients with Diabetic Ulcers

The results of the metagenome analysis of four diabetic wound swab samples, including 11 as a control without antibiotic therapy and three samples (4, 7AB, and 8) with antibiotic therapy, showed microbiome diversity. Molecular detection of bacterial diversity in diabetic ulcers has revealed the presence of opportunistic pathogenic bacterial communities. Figure 1 shows the bacterial diversity in patients with diabetic ulcers who received antibiotics versus the control group (who did not receive antibiotics).

Sample 11, which met the criteria of no antibiotic therapy, wound duration of 2 weeks, and wound severity based on wound grade I. Sample 11 showed higher diversity than the other three samples, which had received antibiotic therapy and had more severe wounds (grades II and IV). Sample

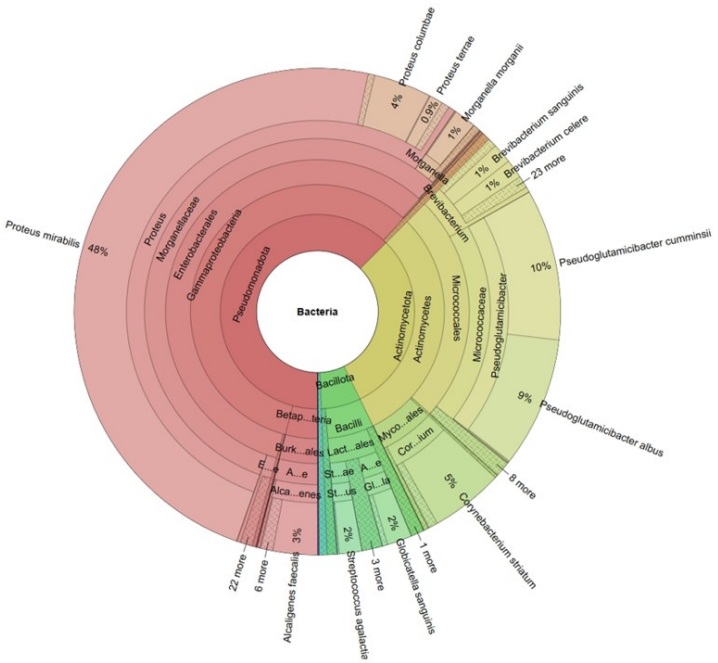

Figure 3. Metagenomic profile of the microbiome in sample 4 (antibiotic-treated).

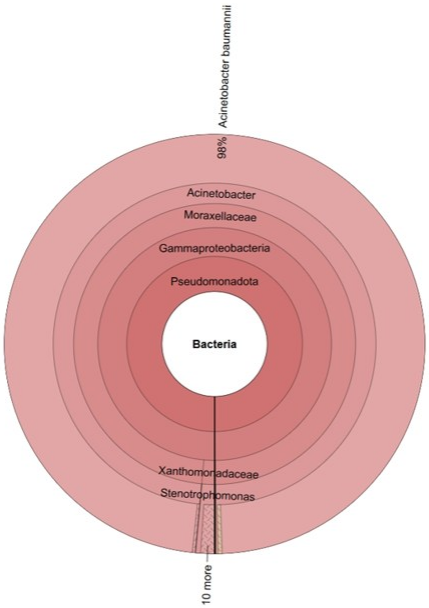

Figure 4. Metagenomic profile of the microbiome in sample 8 (antibiotic-treated).

7AB met the criteria for receiving cefixim 200 mg antibiotic therapy, a wound duration of 4 weeks, and a wound severity grade of II. Sample 4 met the following criteria: receiving azithromycin 500 mg antibiotic therapy, a wound duration of 24 months, and a wound severity grade of II. Sample 8, which met the sample criteria, received antibiotic therapy with cefixime 200 mg, had a wound duration of 2 weeks, and had a wound severity based on a wound grade of IV. Sample 7AB exhibited a higher diversity level than sample 4, which, in turn, had a higher diversity level than that of sample 8. In contrast, sample 8 showed lower diversity than the other three samples. Sample 8, exhibiting grade IV wound severity, demonstrated a comparatively more homogeneous bacterial composition than samples 7AB and 4, both of which presented grade II wound severity. Sample 8 was predominantly characterized by *Acinetobacter baumannii* and *Acinetobacter calcoaceticus.* The two samples (7AB and 4) that underwent antibiotic treatment exhibited considerable bacterial diversity, suggesting that the bacterial communities in the patients' wounds were resistant to the administered antibiotics, notably cefixime in patient 7AB and azithromycin in patient 4. Concurrently, patient 8 had a comparatively diminished bacterial community; however, the bacteria retained their susceptibility to cefixime upon exposure.

Figure 1 depicts the ten species derived from four origins in this study. The species with the highest abundance in sample 4 were *Acinetobacter junii, Delftia lacustris,* and *Stutzeromonas stutzeri.* The *Stutzerimonas stutzeri* group, *Stutzerimonas stutzeri subgroup, Aeromonas veronii, Brevundimonas diminuta, Pseudomonas aeruginosa group, Klebsiella pneumoniae*, sample 4 contained the most abundant species of *Proteus mirabilis, Pseudoglutamicibacter cumminsii, Pseudoglutamicibacter albus,* and *Alcaligenes faecalis*. Sample 7AB exhibited the highest concentrations of *Alcaligenes faecalis, Pseudomonas aeruginosa, Pseudomonas aeruginosa* group, *Achromobacter xylosoxidans* and *Alcaligenes ammonioxydans.* The species exhibiting the highest abundance in sample 8 were *Acinetobacter baumannii* and the *Acinetobacter calcoaceticus*/baumannii complex. The control sample (11) and the other three samples (4, 7AB, and 8) showed changes in diversity in terms of relative abundance. Both untreated samples (11) and patients who received antibiotic therapy (4, 7AB, and 8) were identified as having *P. aeruginosa*. It is possible that *P. aeruginosa* had become resistant to the antibiotics administered.

P. aeruginosa and *S. aureus* are often isolated from non-healing foot ulcers. Co-infection with both bacteria can lead to worse outcomes than infection with either species alone. Both pathogenic bacteria can form biofilms, enabling them to cause

persistent infections and survive antibiotic treatments [35]. The capability of *P. aeruginosa* and *S. aureus* to form strong biofilms allows them to survive harsh environmental challenges, such as high levels of antibacterial agents. Interactions between *P. aeruginosa* and *S. aureus* that can influence drug tolerance in various ways have been previously reported [35]. *P. aeruginosa* is known as an opportunistic gram-negative bacterium that causes infections in the community, in hospitals, and can even be life-threatening [36]. This pathogen is associated with a broad spectrum of infections, including skin and soft tissue infections, keratitis, diabetic foot infections, bacteremia, pneumonia, and urinary tract infections (UTIs), particularly in hospitalized and immunocompromised patients [36] [37]. Of all nosocomial infections, *P. aeruginosa* accounts for 11–13.8% of cases, and its prevalence is significant owing to antibiotic resistance and exotoxin production. Various methods have been attempted to develop an effective vaccine against *P. aeruginosa*; however, none have been successful [37].

Ulcer classification according to the grading system: Patient 11 had ulcer grade I, patients 4 and 7AB had ulcer grade II, and patient 8 had ulcer grade IV ulcers. When analyzed according to the duration of the diabetic ulcers, each of the four samples exhibited peculiarities. Diabetic patients (11) with a diabetic ulcer duration of 2 weeks, diabetic patients (4) with a diabetic ulcer duration of 24 months, and diabetic patients (7AB and 8) each with a diabetic ulcer duration of four weeks. *S. aureus* was detected in sample 11 but not in the other three samples. Sample 11 represents a two-week course of diabetic ulcer. *E. coli* was exclusively identified in sample 4 and was absent in the other samples. Sample 4 was characterized by a length of 24 months of diabetic ulceration.

Successful management of diabetic wound infections necessitates an understanding of the microbial variety present in these wounds. Increasing wound depth and severity are generally accompanied by greater microbial complexity and polymicrobial colonization. Early stage wounds are commonly dominated by *Staphylococcus aureus*, *Enterococcus* spp., *Corynebacterium* spp., and coagulase-negative *Staphylococcus* spp. As the infection progresses, gram-negative bacteria such as *Pseudomonas aeruginosa* and members of the *Enterobacteriaceae* family, along with diverse anaerobic microorganisms, become increasingly prevalent and are frequently associated with moderate-to-severe diabetic foot infections [38].

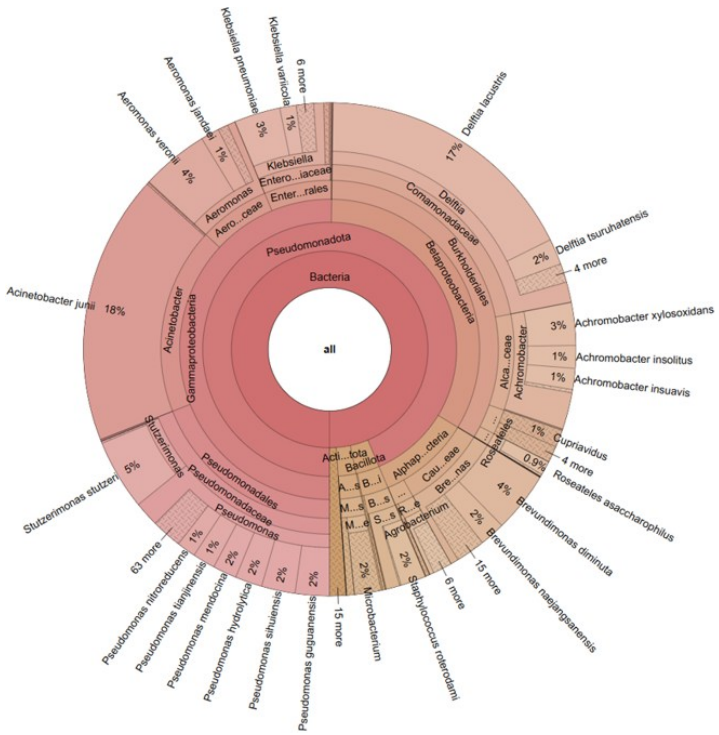

Figure 5. Metagenomic profile of the microbiome in Sample 11 (non-antibiotic-treated).

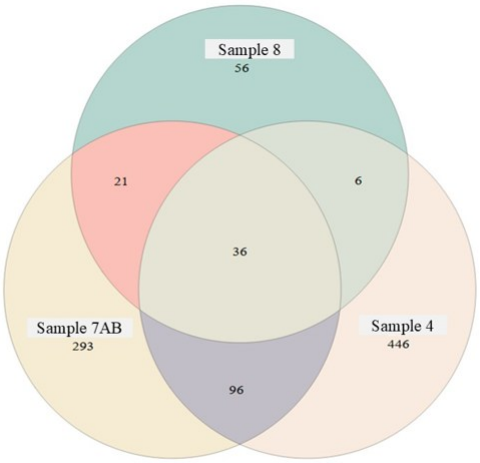

Figure 6. A Venn diagram for each of the three diabetic ulcer specimens (samples 7AB, 4, and 8 with antibiotic therapy).

Diabetic wound infections are associated with *S. aureus, P. aeruginosa*, and species *Escherichia coli* [7]-[10]. *P. mirabilis* was identified in grade 2 and 4 diabetic wounds [39]. In this study, *P. mirabilis* was also identified in samples code 4 and 7AB with ulcer grade II and sample 8 with ulcer grade IV.

S. aureus is an opportunistic pathogen with significant virulence and resistance to most antibiotic treatments [40]. *S. aureus* has several virulence factors, including hemolysin, leukocidin, lipase, invasive protease, nuclease, hyaluronidase, staphylokinase. Hemolysin plays a vital role in colonization and pathogenesis [41] by penetrating the host cell membrane, allowing the entry of water and other harmful substances, resulting in cell swelling, rupture, and necrosis [40]. *S. aureus* easily grows on the skin surface and mucosa of diabetic patients and is capable of producing lipase, protease, hyaluronidase, nuclease, hemolysin alpha (α), hemolysin beta (β), hemolysin gamma (γ), hemolysin delta, collagenase, which important in bacterial proliferation and tissue invasion [42][43].

Biofilms are groups of bacteria that reside in an extracellular polysaccharide matrix and exhibit altered phenotypes and behaviors. Biofilms contribute to the persistence of diabetic foot ulcers. Biofilms can inhibit wound healing and complicate infection treatment by preventing access to antibiotics and the host immune system. Most biofilms consist of *S. aureus,* and bacteria involved in chronic diabetic foot ulcers are generally resistant to multiple drugs [44].

K. pneumoniae was identified in sample 11 at 3% and in samples 4 and 7AB, whereas it was not present in sample 8 (Figure 1). *K. pneumoniae* is a major pathogen in diabetic foot ulcers, posing significant challenges due to its high treatment resistance and aggressiveness. Individuals with diabetes are particularly vulnerable to these infections, which can lead to severe complications, such as amputation and systemic disease. *K. pneumoniae* is often isolated from diabetic foot ulcers, typically ranking as the predominant or one of the principal pathogens in these wounds [45]. Infections are often polymicrobial; however, *K. Pneumoniae* is notable for its correlation with more severe outcomes and an elevated likelihood of multidrug resistance [45] [46].

Pneumoniae isolates from diabetic ulcers demonstrate multidrug resistance (MDR) and extensive drug resistance (XDR) or produce extended-spectrum beta-lactamases (ESBLs) and MBL (also called metallo-beta-lactamases), complicating treatment [46]-[48]. Resistance is particularly pronounced against frequently used antibiotics, including ampicillin, cephalosporins, and carbapenems, whereas some susceptibility persists to tigecycline, colistin, and amikacin [47], The bacteria *K. Pneumoniae* in diabetic ulcers frequently possess virulence genes (e.g., magA, rmpA, and K1/K54 serotypes) that augment its capacity to circumvent the immune system and induce severe infections [48]-[50]. Individuals with

diabetes have a higher risk of infection with hypervirulent strains, causing multisystem infections [50].

Figure 1 shows the ten most common species in each sample (4, 7AB, and 8). Sample code 11 consisted mainly of *Acinetobacter junii* (18%), *Delftia lacustris* (17%), *Stutzerimonas stutzeri* (5%), *Aeromonas veronii* (4%), *Brevundimonas diminuta* (4%), *Pseudomonas aeruginosa* group (3%), *Klebsiella pneumonia* (3%), *Achromobacter xylosoxidans* (3%), *Stutzerimonas stutzeri* group (2%), and *Stutzerimonas stutzeri* subgroup (1.08%). Sample code 4 consisted mainly of *Proteus mirabilis* (48%), *Pseudoglutamicibacter cumminsii* (10%), *Pseudoglutamicibacter albus* (9%), and *Alcaligenes faecalis* (3%) (sample code 7AB consisted mainly of *Alcaligenes faecalis* (41%), *Pseudomonas aeruginosa* (11%), *Achromobacter xylosoxidans* (11%), *Alcaligenes ammonioxydans* (7%), and *Pseudomonas aeruginosa* group (3.42%). Sample 8 consisted mainly of *Acinetobacter baumannii* (98%) and *Acinetobacter calcoaceticus*/ baumannii complex (0.58%) on Figures 1 – 4. Among the bacteria identified in the metagenomic analysis were opportunistic pathogenic species, including *Pseudomonas aeruginosa, Proteus mirabilis, Acinetobacter baumannii, Alcaligenes faecalis, Acinetobacter junii, Klebsiella pneumoniae, Achromobacter, Aeromonas veronii, Brevundimonas diminuta*, and *Stutzerimonas stutzeri. Pseudoglutamicibacter cumminsii*, which has low pathogenic potential, has rarely been reported (Figures 1 – 5).

Acinetobacter is a gram-negative, coccobacillus, aerobic bacterium that does not ferment glucose, is catalase-positive, oxidase-negative, and nonmotile [51] and inhabits aquatic environments, soil, domestic animals, arthropods, and livestock. This bacterium can inhabit the skin, wounds, respiratory tract, digestive tract, and oral mucosa of humans, potentially leading to pneumonia if aspirated into the lower respiratory tract. Several Acinetobacter species are involved in nosocomial infections, including catheter-related bloodstream infections, ventilator-associated pneumonia, catheter-associated urinary tract infections, and surgical site infections (SSIs). *A. junii* was recovered from urine cultures of patients who underwent left nephrostomy tube insertion and had a history of antibiotic usage. The colonies on blood agar media exhibited non-hemolytic characteristics. The isolate was found to be catalase-positive [52].

Delftia is an aerobic, gram-negative, motile, oxidase-positive bacterium that does not ferment glucose. Delftia species are extensively found in aquatic and terrestrial environments. *Delftia lacustris* was initially isolated in 2009 in Denmark from freshwater sources. *D. lacustris* has been extracted from patients exhibiting keratitis and potential endophthalmitis. *D. lacustris* has been documented to induce bloodstream infections. *D. lacustris* is resistant to Amikacin and Gentamicin [53]. *Delftia lacustris* strain LzhVag01 was isolated from vaginal fluid. possesses 11 virulence genes (motA, flip, flhA, bauD, pvdF, sugC, pilU tapT, HsiC1/vipB/tssC, gspE, and narH), as well as eight virulence factors, which include Pyoverdine, the HSI-1 Type four pili transporter, flagella, recycling Trehalose ABC, the Gsp, Nitrat reductase. Virulence factors are primarily associated with bacterial motility, adhesiveness, iron uptake, metabolism, and secretion. *Delftia lacustris* (strain LzhVag01) exhibits resistance to aminoglycosides (gentamicin), nitroimidazoles (metronidazole), fluoroquinolones (levofloxacin), and lincosamides (clindamycin). The *Delftia lacustris* (strain LzhVag01) harbours 16 multidrug-resistant genes, including fusA, rpsJ, OXA-3, ceoB, rpsL, OqxB, rpoB, thyA, and fabI [54]. *Aeromonas veronii* is a Gram-negative bacterium of the *Aeromonadaceae* family, commonly found in soil, food sources, and aquatic habitats. The virulence genes of *Aeromonas veronii* comprise serine protease cytotoxic enterotoxin, aerolysin, type III secretion system, and DNase [55]. *Achromobacter* is associated with otitis media, bacteremia, catheter infections, meningitis, pneumonia, endocarditis, and contamination of hospital water [56].

Diabetes mellitus can increase the number of infections caused by *Pseudomonas spp. Enterococci, Klebsiella spp.*, fungi and *P. mirabilis* [44]. *Proteus* and *Providencia* groups of Gram-negative Bacilli, opportunistic pathogens from the order *Enterobacterales,* family *Morganellaceae*, and group of *Proteae*, are commonly implicated in healthcare-associated illnesses in immunocompromised patients. Their therapeutic significance, which is considered a last-line

treatment, has increased because of their intrinsic resistance to polymyxins [57][58].

Sample code 4, treated with azithromycin, resulted in the presence of *P. mirabilis* at a rate of 48% (Figure 3). Sample code 7AB, when treated with the antibiotic cefixime, produced *Alcaligenes faecalis* (41%) and *P. aeruginosa* (11%) colonies. Sample code 8, treated with the antibiotic cefixime, showed a prevalence of *A. baumannii* of 98% (Figure 2). The bacteria with the highest prevalence in the three samples likely exhibited resistance to azithromycin and cefixime. *P. mirabilis* is a predominant bacterial agent associated with urinary tract infections and other opportunistic diseases. *P. mirabilis* demonstrates resistance to several medicines, such as macrolide antibiotics like azithromycin [59]. Occurrence resistance is commonly correlated with macrolide resistance determinants, notably mphA, in conjunction with other multidrug resistance determinants, including blaTEM, sul1, and sul2 [60][61]. Azithromycin is not recommended for treating *P. mirabilis* infections because of its resistance to this agent is commonly observed. Instead, therapeutic options like carbapenems, ceftazidime, or amikacin should be considered, guided by antimicrobial susceptibility testing, as these drugs frequently remain active against multidrug-resistant strains [61][62]. *P. mirabilis* possesses several virulence factors, including pmfA, mrpA, ucaA (fimbriae), atfA, zapA, hpmA, ptA (proteases), ireA (siderophore receptors), and hlyA (hemolysins) [63].

Pseudoglutamicibacter has a low pathogenic potential. No serious infections attributed to *Pseudoglutamicibacter* have been reported. *Pseudoglutamicibacter cumminsii* is a gram-positive, catalase-positive, aerobic coccobacillus commonly isolated from the soil. Recent taxonomic revisions have led to the reclassification of this species, which was formerly known as *Arthrobacter cumminsii* [64][65]. Information on *Pseudobacter* infection is limited [66]. *A. faecalis*, an opportunistic pathogen, is resistant to many drugs, for example cephalosporins such as cefixime (CFM). This resistance hampers the efficacy of the therapy. *A. faecalis* frequently exhibits resistance to various cephalosporins, whereas select isolates demonstrate susceptibility to specific third-generation cephalosporins, including ceftazidime and cefepime; however, they remain resistant to cefixime and cefuroxime [67]. Mechanisms of resistance include production of ESBLs (extended-spectrum beta-lactamase), beta-lactamases including PER-1, TEM-type, and OXA-type enzymes, which hydrolyze broad-spectrum cephalosporins [68]. *A. faecalis* demonstrates significant resistance to amoxicillin, cefuroxime, ceftriaxone, and cefixime [67]. The use of cefixime for *A. faecalis* infections is not recommended because of increasing resistance [67].

Acinetobacter baumannii is an opportunistic pathogen with multiple virulence factors that contribute to its persistence in diabetic foot infections (DFIs). Among these factors, biofilm formation is considered to be one of the most

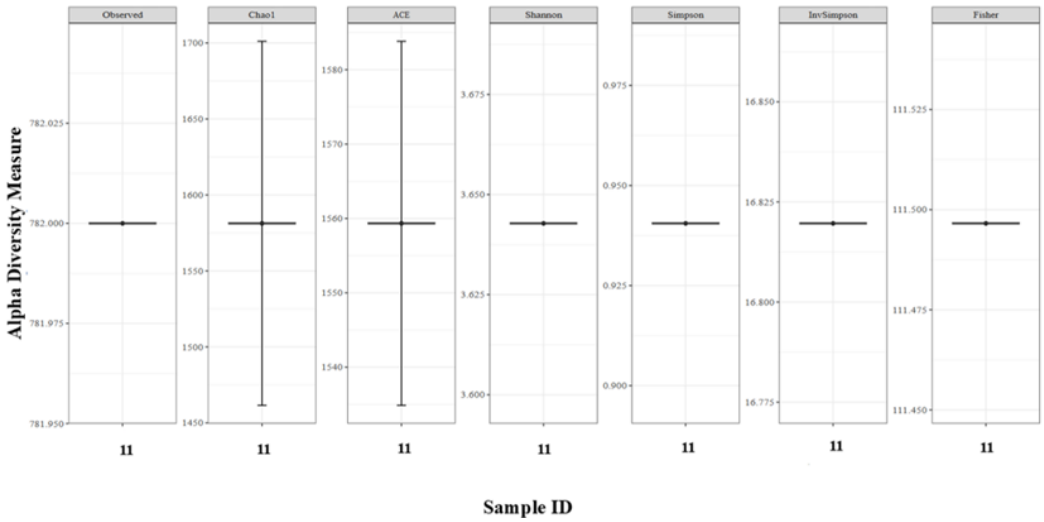

Figure 7. Alpha diversity of sample 11 obtained from an individual not treated with antibiotics.

important mechanisms that facilitate long-term colonization and chronic infection. Biofilm-associated growth enhances bacterial survival by reducing antibiotic penetration, promoting horizontal gene transfer, protecting bacterial cells from host immune defenses, and facilitating adaptive changes in gene expression, thereby increasing bacterial persistence in the wound environment [44][69]. Furthermore, biofilms are widely recognized as major contributors to delayed wound healing, chronic inflammation, and treatment failure in diabetic foot ulcers. Although *A. baumannii* is not commonly regarded as a dominant member of the normal skin microbiota, it has been increasingly reported as a clinically significant pathogen in DFIs and other chronic wound infections [44]. The clinical importance of this organism is further amplified by the emergence of carbapenem-resistant *A. baumannii* strains, which represent a growing challenge in diabetic patients owing to their association with multidrug resistance, prolonged hospitalization, and poor clinical outcomes [70].

A. baumannii, an opportunistic bacterium can rapidly acquire multidrug resistance, particularly to cephalosporins, such as cefixime. *A. baumannii* demonstrates a very high level of non-susceptibility to 3rd generation cephalosporin antibiotics, such as cefixime, cefotaxime, and ceftriaxone, with resistance observed in 80 persent of tested isolates [71][72]. Several mechanisms, including the synthesis of AmpC beta-lactamases, carbapenemases, and extended-spectrum β-lactamases, mediate this antibiotic resistance. ESBLs, including OXA and NDM-1, hydrolyze cephalosporins, rendering antibiotics ineffective [7][72]. Genomic plasticity and horizontal gene transfer can expedite the dissemination of gene resistance [73]. *Acinetobacter baumannii* is an opportunistic pathogen equipped with a broad repertoire of virulence factors that facilitate host colonization, persistence, and infection of diabetic foot ulcers (DFUs). These virulence determinants include capsular polysaccharides; outer membrane proteins (such as OmpA, Omp33-36, CarO, and OprD-like proteins); lipopolysaccharides; outer membrane vesicles; phospholipases; pili; secretion systems; metal acquisition systems for iron, zinc, and manganese; efflux pumps; and multiple regulatory proteins involved in stress adaptation and antimicrobial resistance. Collectively, these factors promote biofilm formation, immune evasion, nutrient acquisition, tissue damage, and survival under adverse environmental conditions, thereby enhancing the ability of *A. baumannii* to persist within the diabetic wound environment and contributing to chronic infection, delayed wound healing, and treatment failure in DFUs [74][75]. Due to the high frequency of antimicrobial resistance, cefixime and other cephalosporin agents are considered ineffective for the management of *A. baumannii* infections [71][72][76]. *A. baumannii* is a particularly concerning organism, known for its treatment resistance and associated fatality rates of 60% for pneumonia and 43.4% for bloodstream infections [73]. *A. baumannii* infections include infections of the soft tissues and skin, urinary tract, pneumonia associated with ventilators, tracheitis, and bacteremia [76].

In the Venn diagram (Figure 6), 36 species were common to samples, 4, 7AB, and 8. The identified bacteria in the three samples included *Pseudomonas aeruginosa, Ralstonia pickettii, Acinetobacter, Acinetobacter baumannii, Alcaligenes faecalis, Proteus, Proteus mirabilis, Streptococcus agalactiae, Weissella confusa, Moraxella osloensis, Rothia kristinae, Acinetobacter junii, Stenotrophomonas, Stenotrophomonas maltophilia, Corynebacterium striatum, Achromobacter xylosoxidans, Pseudomonas hibiscicola, Pseudoglutamicibacter albus, Rummeliibacillus pycnus, Ralstonia mannitolilytica, Pseudoglutamicibacter cumminsii, Brevibacterium sanguinis, Alcaligenes aquatilis, Megasphaera sueciensis, Megasphaera paucivorans, Paludibacterium yongneupense, Stenotrophomonas pavanii, Segatella cerevisiae, Clostridium fermenticellae, Alcaligenes ammonioxidans, Stenotrophomonas cyclobalanopsidis.* These bacteria can cause diabetic ulcer infections or exacerbate wounds.

Wound healing may be markedly compromised by polymicrobial consortia and microbial resistance in the wound environment [33]. Microbiome data indicate that diabetic foot infections persisting beyond six weeks are frequently polymicrobial. Our investigation revealed a comparable occurrence of

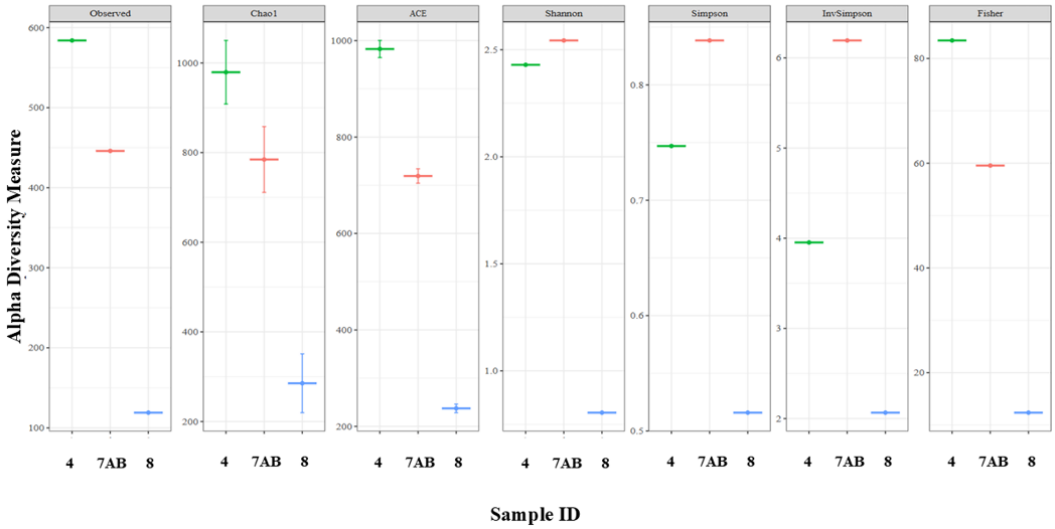

Figure 8. Alpha diversity of samples 4, 7AB, and 8 obtained from individuals treated with antibiotics.

antibiotic resistance; specifically, there was an increased number of antibiotic-resistant gene subtype categories in DFIs persisting beyond six weeks. This may indicate that the categories of antibiotic-resistant gene subtypes proliferate with the growth of microbial taxa. Extended exposure of wounds to the environment results in contamination by environmental bacteria and increases the number of antibiotic-resistant genes inside the ulcer tissue. Microbial resistance to antibiotics develops with prolonged antibiotic use. Antibiotic resistance may emerge from DNA mutations or the uptake of exogenous genetic material. Genes responsible for antimicrobial resistance are frequently embedded within mobile DNA structures, including plasmids and transposable elements, which facilitate the development of multidrug resistance and promote horizontal dissemination among bacterial populations [77].

Functional profiling of metagenomic data demonstrated increased levels of antibiotic resistance, biofilm-associated functions, and virulence-related genes in infected diabetic foot ulcers, leading to amputation ($P < 0.001$). Additionally, genes involved with bacterial virulence factors beyond mechanisms of resistance were linked to IDFU, resulting in amputation; many were related to biofilm formation, toxin production, and quorum sensing. The use of molecular sequencing approaches has provided insights into the intricate bacterial communities of IDFU, revealing a substantial occurrence of anaerobic gram-negative microorganisms. Sequence data revealed potential associations between particular genera and medical outcomes and species, notably the link between the genus Bacteroides and amputated but *Staphylococcus aureus* with less severe wounds. Metagenomic investigations have revealed associations between gene functions linked to bacterial virulence and amputation [18].

Metagenomic studies have transformed our understanding of bacterial communities in DFUs, revealing complex, polymicrobial, and often anaerobe-rich ecosystems. Through 16S rRNA gene sequencing and shotgun metagenomics, diabetic wounds have been shown to harbor highly diverse microbial communities, with members of the phyla *Firmicutes, Proteobacteria, Actinobacteria,* and *Bacteroidetes* being frequently reported among the dominant bacterial groups. These culture-independent approaches have greatly enhanced the detection and characterization of wound-associated microorganisms, thereby providing valuable insights into the microbial ecology of chronic diabetic wounds [17][78][79]. Common genera/species include *P. aeruginosa, S. aureus, C. striatum, Enterococcus, Streptococcus, Porphyromonas, Bacteroides, Prevotella*, and varied anaerobic microorganisms [16][18][78]. More severe or long-standing ulcers and higher Wagner grades are enriched with obligate anaerobes and gram-negative bacteria, such as *Bacteroides, Prevotella, Morganella,* and *Gammaproteobacteria* [18]. Metagenomic analysis has been developed as an effective technique for the swift detection of various pathogen species, including bacteria, with

high sensitivity and accuracy, circumventing diagnostic delays and facilitating the timely administration of appropriate anti-infective therapies. Metagenomic sequencing facilitates the concurrent detection of all potentially pathogenic agents in wounds [16].

3.3. Alpha and Beta Diversity of Microbial Communities in Diabetic Ulcer Samples

The alpha diversity of sample 11, collected from an individual not receiving antibiotic treatment, is shown in Figure 7. Conversely, the alpha diversity of samples 4, 7AB, and 8, obtained from antibiotic-treated individuals, is presented in Figure 8. Alpha diversity analysis revealed a high level of bacterial richness and diversity in sample 11 (Figure 7). A total of 782 observed OTUs were detected, while the estimated richness based on the Chao1 and ACE indices reached 1,581.30 and 1,559.34 OTUs, respectively. The substantially higher Chao1 and ACE values compared with the observed OTUs suggest the presence of numerous low-abundance taxa that were not fully captured during sequencing, indicating that the microbial community is likely richer than directly observed. The relatively low standard errors for Chao1 (119.75) and ACE (24.47) indicate a reliable estimation of species richness. Furthermore, the Shannon diversity index (3.64) demonstrated moderate-to-high microbial diversity with a relatively even distribution of taxa within the community. This finding was supported by the high Simpson index value (0.94) and inverse Simpson index value (16.82), indicating low dominance by individual taxa and a highly diverse bacterial community. In addition, the Fisher's alpha value (111.50) further confirmed the substantial richness and heterogeneity of bacterial taxa present in the sample. Overall, the alpha diversity metrics indicate that sample 11 harbors a highly complex, species-rich, and well-distributed bacterial community.

Alpha diversity analysis revealed distinct differences in bacterial community richness and diversity among samples 4, 7AB, and 8 (Figure 8). Sample 4 exhibited the highest observed richness (584 OTUs), followed by sample 7AB (446 OTUs) and sample 8 (119 OTUs). Richness estimators showed a similar trend, with sample 4 displaying the highest Chao1 (979.28) and ACE (982.64) values, followed by sample 7AB (Chao1 = 784.61; ACE = 719.24) and sample 8 (Chao1 = 285.36; ACE = 237.29). The higher estimated richness compared with observed OTUs in all samples suggests the presence of numerous low-abundance taxa that remained undetected.

In terms of community diversity, sample 7AB exhibited the highest Shannon diversity index (2.54), slightly exceeding that of sample 4 (2.43), while sample 8 showed substantially lower diversity (0.80). Likewise, Simpson (0.84) and inverse Simpson (6.19) indices were highest in sample 7AB, indicating a more even distribution of bacterial taxa and lower dominance by individual species. In contrast, sample 4 showed intermediate diversity (Simpson = 0.75; inverse Simpson = 3.95), whereas sample 8 exhibited the lowest diversity (Simpson = 0.52; inverse Simpson = 2.07), suggesting a community dominated by a limited number of taxa.

Fisher's alpha further demonstrated that sample 4 possessed the greatest taxonomic richness (83.45), followed by sample 7AB (59.55) and sample 8 (12.37). Overall, sample 4 was characterized by the highest species richness, whereas sample 7AB displayed the highest community diversity and evenness. Sample 8 exhibited the lowest richness, diversity, and taxonomic complexity among all analyzed samples.

Interestingly, sample 11, which originated from a grade I diabetic ulcer, exhibited the highest bacterial richness and diversity, whereas Sample 8, derived from a grade IV diabetic ulcer representing the most severe wound condition, showed the lowest diversity across all alpha diversity indices. Samples 4 and 7AB, both obtained from grade II diabetic ulcers, displayed intermediate levels of richness and diversity, with sample 4 characterized by higher species richness and sample 7AB exhibiting greater community evenness. The observed pattern, characterized by high bacterial diversity in the grade I ulcer (sample 11), intermediate diversity in the grade II ulcers (samples 4 and 7AB), and low diversity in the grade IV ulcer (sample 8), suggests a potential decline in microbial diversity with increasing ulcer severity. Advanced diabetic wounds are often characterized by prolonged inflammation, extensive tissue damage, impaired vascularization, and biofilm

formation, conditions that may favor the dominance of a limited number of highly adapted bacterial taxa while reducing overall community diversity [80].

Principal Coordinate Analysis (PCoA) based on Bray–Curtis dissimilarity revealed a clear separation among samples 4, 7AB, and 8 (Figure 9), indicating distinct bacterial community compositions across diabetic wound samples. The first and second principal coordinates (PCoA1 and PCoA2) explained 57.27% and 42.73% of the total variation, respectively, collectively accounting for 100% of the observed variation. Sample 8, derived from a grade IV diabetic ulcer, was clearly separated from samples 4 and 7AB along PCoA1, suggesting substantial differences in microbial community structure. In contrast, samples 4 and 7AB, although both originating from grade II diabetic ulcers, occupied distinct positions within the ordination space, indicating considerable inter-sample variability in bacterial composition.

The distinct clustering pattern observed in the PCoA plot suggests that diabetic wound microbiota differ markedly among samples. Notably, the grade IV ulcer (sample 8) exhibited a bacterial community structure that was clearly separated from those of the grade II ulcers (samples 4 and 7AB), supporting the alpha diversity findings that indicated reduced diversity in the most severe wound. This observation is consistent with the notion that progressive wound deterioration may be accompanied by shifts in microbial community structure and a reduction in overall microbial diversity. Furthermore, the observed pattern of high bacterial diversity in the grade I ulcer (sample 11), intermediate diversity in the grade II ulcers (samples 4 and 7AB), and low diversity in the grade IV ulcer (sample 8) suggests a potential decline in microbial diversity with increasing ulcer severity.

Furthermore, the separation between samples 4 and 7AB, despite sharing the same ulcer grade, highlights the highly heterogeneous nature of diabetic wound microbiota. This finding suggests that ulcer severity alone is insufficient to explain the observed variation in bacterial community composition. Previous studies have demonstrated that multiple factors, including host physiological condition, wound chronicity, antibiotic exposure, tissue oxygenation, immune responses, and biofilm development, can influence microbial colonization and succession within diabetic wounds [81][82]. Biofilm formation, in particular, may create highly selective microenvironments that favor the persistence and dominance of specific bacterial taxa while limiting the establishment of less-adapted microorganisms. In the present study, sample 11, obtained from a grade I diabetic ulcer, had not received antibiotic treatment and exhibited the highest alpha diversity. In contrast, samples 4 and

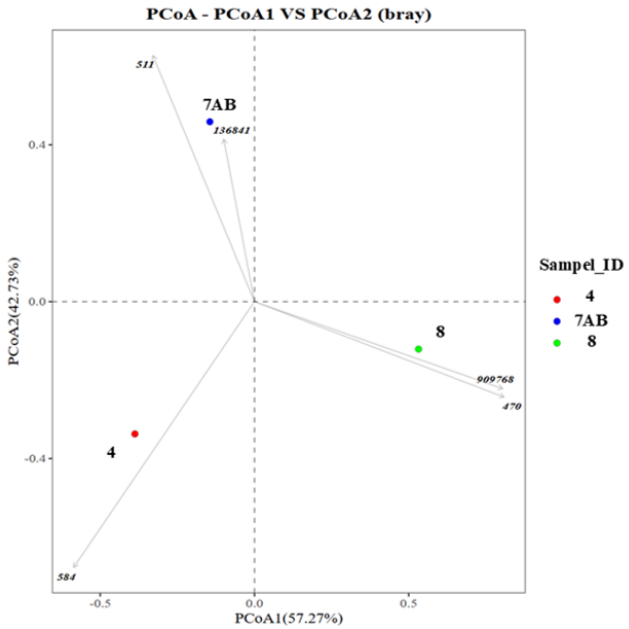

Figure 9. Beta diversity of samples 4, 7AB, and 8 obtained from individuals treated with antibiotics.

7AB (grade II ulcers) and sample 8 (grade IV ulcer) had undergone antibiotic therapy prior to sample collection. These observations suggest that, in addition to ulcer severity, prior antibiotic exposure may have contributed to the variation in bacterial community composition observed among the samples. Consequently, diabetic wounds with similar clinical grades may still harbor substantially different microbial communities, reflecting the influence of both clinical and treatment-related factors.

In summary, these findings indicate that the microbiota of diabetic wounds are shaped by a complex interplay between wound severity and local ecological factors. The marked separation of sample 8 from the grade II ulcers, coupled with its lower alpha diversity, suggests that advanced ulceration may be associated with a less diverse but more specialized bacterial community structure. Understanding these microbial dynamics is clinically important because future treatment regimens should be tailored according to the bacterial composition of diabetic wounds. Antimicrobial therapies should therefore be selected based on the predominant pathogenic species present in the wound environment, particularly targeting clinically relevant bacteria such as *P. aeruginosa*, *S. aureus*, *P. mirabilis*, *A. faecalis*, and *A. baumannii*, which were identified as dominant bacterial taxa in the metagenomic analysis conducted in the present study. These microorganisms are frequently associated with persistent infections, antimicrobial resistance, biofilm formation, and delayed wound healing, highlighting their clinical relevance in diabetic wound management. However, given the limited sample size, further studies involving a larger number of diabetic wound samples and integrating clinical, microbiological, and host-related parameters are required to elucidate the mechanisms underlying microbial community shifts during diabetic wound progression.

In addition to targeted antimicrobial therapy, future diabetic wound management strategies should also emphasize the maintenance of optimal glycemic control, as persistent hyperglycemia is known to impair tissue repair, promote oxidative stress, and increase susceptibility to chronic wound infections. Improving glucose homeostasis may help create a more favorable wound microenvironment, thereby supporting tissue regeneration and reducing the persistence of pathogenic bacterial communities. In this context, Arman et al. [83] reported that gallic acid exhibited significant anti-diabetic and anti-lipid peroxidative activities in diabetic rats, suggesting its potential as an adjunctive therapeutic agent for improving metabolic status and supporting wound healing. Nevertheless, further clinical studies are required to determine whether these beneficial effects can be translated into diabetic wound care in human patients.

4. CONCLUSIONS

Metagenomic profiling of diabetic ulcer samples demonstrated distinct microbial community structures associated with ulcer severity and prior antibiotic exposure. Despite differences in treatment history, clinically important bacterial taxa, including opportunistic pathogens such as *P. aeruginosa, S. aureus, P. mirabilis, A. faecalis,* and *A. baumannii*, remained prevalent across the analyzed samples. These findings highlight the potential value of metagenomic approaches for identifying dominant wound-associated pathogens and supporting more targeted antimicrobial selection in diabetic wound care. Furthermore, the results suggest that future treatment strategies should consider both microbial composition and host-related factors, including glycemic control and prior antibiotic exposure, to improve wound healing outcomes. The present study expands current knowledge of bacterial diversity in diabetic ulcers and provides a foundation for future investigations integrating microbiological, clinical, and host-related parameters to optimize diabetic wound management. However, the relatively small sample size limits the generalizability of the findings; therefore, studies involving larger patient cohorts are required to validate the observed microbial patterns and further elucidate the dynamics of diabetic wound microbiomes.

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Conflicts of Interest

The authors declare no conflict of interest.

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DECLARATION OF GENERATIVE AI

Not applicable.

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