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# Application of metagenomic next-generation sequencing (mNGS) to describe the microbial characteristics of diabetic foot ulcers at a tertiary medical center in South China

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## Abstract

**Background** Diabetic foot ulcers (DFUs) are characterized by dynamic wound microbiome, the timely and accurate identification of pathogens in the clinic is required to initiate precise and individualized treatment. Metagenomic next-generation sequencing (mNGS) has been a useful supplement to routine culture method for the etiological diagnosis of DFUs. In this study, we utilized a routine culture method and mNGS to analyze the same DFU wound samples and the results were compared.

**Methods** Forty samples from patients with DFUs at a tertiary medical center in South China were collected, the microorganisms were identified with mNGS and routine culture method simultaneously.

**Results** The results showed that the positive detection rate of microorganisms in DFUs with mNGS was much higher (95% vs. 60%). Thirteen strains of microorganisms were detected with routine culture method, and seventy-seven strains were detected with mNGS. Staphylococcus aureus was the most common microorganism detected with culture method, while Enterococcus faecalis was the most common microorganism detected with mNGS. The false negative rate of the culture method was 35%, that was, 14 samples with negative results with culture method were found to be positive with mNGS.

**Conclusion** The mNGS method had a higher positive detection rate and identified a broader spectrum of microorganisms in DFUs, thus, mNGS provided a more comprehensive understanding of the microbiome of DFUs to facilitate the development of timely and optimal treatment.

**Trial registration** The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethical Review Committee of the Fujian Medical University Union Hospital (approval number 2021KY054).

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**Keywords** Diabetic foot ulcer, Metagenomic next-generation sequencing, Microorganism culture, Wound microbiome

## Background

Diabetes mellitus affects 463 million individuals worldwide, and this number is projected to increase by 25% in 2030 and 51% in 2045 [1]. The high blood glucose levels of diabetes patients damages nerves and arteries and ultimately results in neuropathy and/or peripheral artery disease, leading to diabetic foot ulcers (DFUs). DFUs exhibit impairment in several precise stages of wound repair, including hemostasis, inflammation, growth, re-epithelialization, and remodeling [2]. These critical pathways that are dysregulated in DFUs maintain a chronic inflammatory state in the wound and impede vessel formation [3, 4], making them susceptible to the detrimental effects of microbes. When DFUs become infected, which is referred to as diabetic foot infection (DFI) [5], they are difficult to treat due to the presence of pathogenic microbes, especially biofilm-producing microbes, which exhibit firm adherence to the wound, immune evasion, and antibiotic resistance [6]. DFI was a major contributing factor to amputation, and the 5-year mortality rate of patients with diabetes who undergo amputation was reported to be more than 70% [7]. DFUs and the associated DFI increase the rate of emergency department visits and hospital admissions, resulting in a large burden for society [8, 9].

DFI is a common complication of DFUs, and the wound-associated microbiome are diverse and dynamic, making the diagnosis of infectious pathogens difficult [10]. Timely detection of DFI is critical for clinical treatment. The routine culture method is the gold standard for microbiological identification, but it is too time-consuming, and the sensitivity is often low [7]. Classical culture-based method that identify devastating pathogens in specimens that are obtained for aerobic and anaerobic culture usually take several days [10]. Additionally, the culture result might not be accurate because only the subset of microbes that grow well in the laboratory, which is not always the responsible pathogen, can be reported. The poor efficiency of microbiological detection delays treatment, increases patient suffering, and increases the economic burden. Therefore, a more accurate and timelier approach for microbe detection is urgently needed.

With the introduction and development of gene sequencing technology, metagenomic next-generation sequencing (mNGS) has become a useful approach that can rapidly detect multiple pathogen species, including bacteria, fungi, viruses and parasites, simultaneously with high sensitivity and accuracy. mNGS has been widely used in the diagnosis of pneumonia, sepsis, neurological infections, and other complicated infectious diseases, and the positive detection rate of mNGS is significantly higher than that of traditional culture method [7, 11–13]. The powerful detection ability of mNGS in unknown infectious diseases could identify the pathogen in a timely manner and avoid the unnecessary delay of diagnosis and then, effective anti-infective medication can be administered. mNGS enables the simultaneous identification of all potentially infectious agents in the wound, and furthermore, it provides additional genomic information about the wound microenvironment [14]. The large amounts of genetic information acquired by mNGS help clinicians quickly and reasonably make the best clinical decision. Currently, mNGS is a potential diagnostic tool to supplement routine method in the clinical management of patients.

In this study, we utilized a routine culture method and mNGS to analyze the same DFU wound samples. Comparing the results, we further evaluated the value of mNGS in the diagnosis of pathogenic microorganisms in DFUs and re-evaluated the clinical role of the routine culture method. The mNGS provided a large amount of genomic information about the microbiome in DFU wounds. We further elucidated the different compositions of microorganisms between the DFU subtypes, namely, peripheral neuropathy-related DFUs (N-DFUs), peripheral artery disease-related DFUs (A-DFUs), and peripheral neuropathy and artery disease mixed DFUs (M-DFUs), which were defined by their etiology [15].

## Methods

### Study patients

A total of 40 patients with DFUs who were admitted to Fujian Medical University Union Hospital from November 2020 to February 2022 were enrolled in this study. According to the etiology classification, DFUs were categorized into 3 subtypes: N-DFUs, A-DFUs, and M-DFUs. The research was approved by the Ethical Review Committee of the Fujian Medical University Union Hospital (approval number 2021KY054).

### Sample collection and routine culture detection

The wound surface underwent irrigation with sterile saline to facilitate debridement, during which the overlying surface covering and exudate were meticulously removed to the greatest extent possible. Subsequently, sections of the wound margin and underlying deep tissue were excised. The two tissue specimens at the base of wound were excised intraoperatively, and then simultaneously analyzed by routine culture methods and mNGS. For standard tissue culture, the samples were

immediately delivered to the clinical microbiology laboratory. Gram staining, acid-fast staining, and lactic acid phenol cotton blue staining were performed to identify potential pathogens.

### Metagenomic next-generation sequencing (mNGS) and bioinformatics analysis

The mNGS library preparation was performed with the NGS Automatic Library Preparation System (Cat. MAR002, MatriDx Biotech Corp. Hangzhou, China). Relevant reagents include the Nucleic Acid Extraction Kit (Cat. MD013, MatriDx Biotech Corp. Hangzhou, China), Cell-free DNA Library Preparation Kit (Cat. MD007, MatriDx Biotech Corp. Hangzhou, China) and Total DNA Library Preparation Kit (other sample types) (Cat. MD001T, MatriDx Biotech Corp, China). The libraries were pooled and then sequenced on an Illumina NextSeq500 system using a 75-cycle sequencing kit. A total of 10–20 million reads were obtained for each sample. Clean reads obtained after raw data demultiplexing and adapter trimming were subjected to microbial identification based on a reference database Clean reads obtained after raw data demultiplexing and adapter trimming were subjected to microbial identification based on a reference database (NCBI nt database (ftp://ftp.ncbinlm.nih.gov/blast/db/) and GenBank (ftp://ftp.ncbi.nlm.nih.gov/genomes/genbank/)) using Burrows Wheeler alignment (BWA, http://bio-bwa.sourceforge.net). All the species detected in the clinical samples using mNGS were first filtered with all the microorganisms detected in the parallel NTC (no template control) (background microorganisms) with a ratio of unique reads per million (RPM) above 10, and the RPM ratio = RPMsample/RPMNTC or RPM ratio = RPMsample if the organism was not detected in the parallel NTC. All species that were authentically present in clinical specimens were defined as microbiota. Substantially, all the species of microbiota were looked up in PubMed to determine whether the organisms caused DFI, and the positive pathogenic microorganisms were defined as pathogens. The raw data was archived in the Sequence Read Archive of The National Center for Biotechnology Information with a BioProject accession (PRJNA980579).

### Statistical analysis

The data were analyzed by SPSS 25.0 software. The difference in the results between routine culture method and mNGS was analyzed by the chi-square test. A $p$ value of $< 0.05$ was considered statistically significant.

## Results

### Clinical characteristics

The clinical characteristics of the patients are shown in Table 1. A total of 40 patients with DFUs were enrolled in this study, consisting of 28 males and 12 females, (65.2 ± 13.98) years old. According to the Wagner classification system, there were 0, 4, 4, 15, 16 and 1 patients with grades 0, 1, 2, 3, 4 and 5, respectively. According to the etiology, 13 patients were classified in the N-DFUs group, 12 patients were classified in the A-DFUs group, and 15 patients were classified in the M-DFUs group.

### The microbial characteristics of DFU obtained by routine culture method

In the results of the routine culture group, 13 strains of bacteria were detected in the DFUs of 40 patients. *Staphylococcus aureus* was the most common microorganism in all the culture-based groups (Table 2; Fig. 1). The microbial characteristic of the following DFU categories: N-DFUs, A-DFUs and M-DFUs subtypes were analyzed. Seven strains of microorganisms were detected in the N-DFUs group, and *Staphylococcus aureus* was the most common strain. Eight strains of microorganisms were detected in the A-DFUs group, and *Proteus species* were the most common strain. Six strains of microorganisms were detected in the M-DFUs group, and *Klebsiella pneumoniae* was the most common strain (Fig. 2).

### The microbial characteristics of DFU obtained by mNGS

In the sequencing results of the mNGS group, 77 strains of microorganisms were detected in all DFUs (Table 2). There were 70 bacteria, 5 fungi, 1 parasite and 1 virus (Fig. 3), and *Enterococcus faecalis* was the most common microorganism in the mNGS groups (Fig. 1). The microbial characteristic of three subtypes were further analyzed. Forty strains of microorganisms in the N-DFUs group were detected, and *Enterococcus faecalis* was the most common strain. Thirty-seven strains of microorganisms were detected in the A-DFUs group, and *Enterococcus faecalis* and *Finegoldia magna* were the most common strains. Thirty-eight strains of microorganisms were detected in the M-DFUs group, and *Enterococcus faecalis* and *Finegoldia magna* were the most common strains (Fig. 2).

### Correlation of mNGS with routine culture method

After a comprehensive evaluation, the mNGS positive detection rate was higher than that of routine culture method (95% vs. 60%), and the difference was statistically significant ($p < 0.05$, Table 2). The concordance between the two approaches was 55% (including 2 negative cultures), and the precision rate increased to 83.3% when the positive results of the two approaches were compared (Table 2).

The merged results showed that *Enterococcus faecalis* was the most common microorganism in DFUs. Furthermore, *Staphylococcus aureus* was the most common strain in the N-DFUs group, and *Enterococcus*

**Table 1** The clinical and microbial characteristics of 40 DFUs patients obtained by routine culture method and mNGS

| Patient ID | Sex | Age | Wagner's grade | DFU subtypes | Detection results | |
|---|---|---|---|---|---|---|
| | | | | | Culture method | mNGS |
| 1 | Male | 78 | 4 | A-DFUs | Pvu | Fma, Pan, Pha, Pvu |
| 2 | Male | 85 | 3 | A-DFUs | None | Apr, Efa, Fma, HHV-4, Kpn, Pco, Pvu, Slu |
| 3 | Male | 80 | 4 | A-DFUs | Efa | Bfr, Efa, Sma |
| 4 | Female | 53 | 4 | A-DFUs | Efa | Ala, Efa, Sau, Sep |
| 5 | Male | 75 | 4 | A-DFUs | Pae | Pae |
| 6 | Male | 74 | 3 | A-DFUs | Pvu | Ate, Efa, Fma, HHV-4, Pla, Pst, Pvu |
| 7 | Female | 71 | 4 | A-DFUs | Tri | Cca, Cgl, Cpa, Ean, Efa, Hha, Ppu, Tas, |
| 8 | Male | 63 | 4 | A-DFUs | Pmi | Bfr, Fps, Mmo, Pin, Pmi, Sau, Sor, Tva |
| 9 | Male | 63 | 3 | A-DFUs | None | Fma, San, Pde |
| 10 | Male | 75 | 4 | A-DFUs | None | Sau |
| 11 | Male | 77 | 4 | A-DFUs | Eco | Ala, Eho, Kpn, Pco, Ror, Sag |
| 12 | Female | 74 | 4 | A-DFUs | Sag, Sau | Ate, Fma, Pha, Sau, Sag, |
| 13 | Male | 51 | 4 | M-DFUs | Fma | Ala, Fma, Pas, Pin, Pco, Sin, Vpa |
| 14 | Male | 66 | 1 | M-DFUs | None | None |
| 15 | Female | 61 | 3 | M-DFUs | Efa | Efa, Eho |
| 16 | Male | 81 | 3 | M-DFUs | Eco | Cst, Eco, Efa, Fma, Pbi, Pgr, |
| 17 | Male | 65 | 3 | M-DFUs | Kpn | Efa, Fma, Kae, Pan, Pbi, Pha, Sag |
| 18 | Male | 80 | 3 | M-DFUs | None | Mma, Sha |
| 19 | Male | 59 | 4 | M-DFUs | Sau | Pme, Sau, Fma, Pha, Ssi, Sin, Fmu, Cst, Efa |
| 20 | Male | 71 | 3 | M-DFUs | None | None |
| 21 | Male | 66 | 5 | M-DFUs | Kpn | Apr, Efa, Pan, Pbi, Pvu, Vpa |
| 22 | Female | 76 | 4 | M-DFUs | None | Mmo, Kox, Pvu, Sal, Sdy |
| 23 | Male | 69 | 2 | M-DFUs | None | Spy |
| 24 | Male | 67 | 1 | M-DFUs | None | Cal, Sep |
| 25 | Female | 55 | 3 | M-DFUs | None | Bhe, Pin, Pmi, Sex |
| 26 | Female | 64 | 4 | M-DFUs | None | Ala, Bth, Fma, Hku, Pco, Pbu, Spy |
| 27 | Male | 79 | 4 | M-DFUs | Sag | Apr, Mmo, Sag, Sau |
| 28 | Male | 75 | 3 | N-DFUs | None | Cpe, Ecl, HHV-4, Kpn, Kra, Kor, Lfe, Lpa, Sag |
| 29 | Male | 38 | 2 | N-DFUs | Sau | Cst, Sau, Sdy |
| 30 | Female | 66 | 2 | N-DFUs | Sha | Sau |
| 31 | Male | 22 | 3 | N-DFUs | Eav | Dpn, Pen, Pin, Pmi, Pst, Sco |
| 32 | Female | 70 | 3 | N-DFUs | None | Efa, Kpn, Mmo, Pae, Pmi |
| 33 | Male | 78 | 1 | N-DFUs | Sau | Sau |
| 34 | Male | 29 | 1 | N-DFUs | None | Efa |
| 35 | Female | 56 | 3 | N-DFUs | Pae | Bfr, Efa, Fma, Mmo, Pae, Pin, Spy |
| 36 | Male | 51 | 2 | N-DFUs | Sau | Cre, Dho, Efa, Fma, Hku, Pha, Sau, Sag |
| 37 | Male | 59 | 3 | N-DFUs | None | Aha, Fmu, Pas, Pco, Pti, Sdy |
| 38 | Male | 47 | 3 | N-DFUs | None | Era, Lri, Pan, Pde, Pmi, San, |
| 39 | Female | 63 | 4 | N-DFUs | Mmo, Efa | Efa, Mmo, Sma |
| 40 | Female | 76 | 4 | N-DFUs | Sag | Sag |

* N-DFUs: Peripheral Neuropathy-related DFUs; A-DFUs: Peripheral Artery disease-related DFUs; M-DFUs: Peripheral Neuropathy and Artery disease Mixed DFUs. **Ala,** *Anaerococcus lactolyticus*; **Apr,** *Anaerococcus prevotii*; **Ate,** *Anaerococcus tetradius*; **Aha,** *Arcanobacterium haemolyticum*; **Bfr,** *Bacteroides fragilis*; **Bhe,** *Bacteroides heparinolyticus*; **Bth,** *Bacteroides thetaiotaomicron*; **Cal,** *Candida albicans*; **Cca,** *Candida carpophila*; **Cgl,** *Candida glabrata*; **Cpa,** *Candida parapsilosis*; **Cpe,** *Clostridium perfringens*; **Cre,** *Corynebacterium resistens*; **Cst,** *Corynebacterium striatum*; **Dho,** *Dermabacter hominis*; **Dpn,** *Dialister pneumosintes*; **Ean,** *Elizabethkingia anopheles*; **Ecl,** *Enterobacter cloacae*; **Eho,** *Enterobacter hormaechei*; **Eav,** *Enterococcus avium*; **Efa,** *Enterococcus faecalis*; **Era,** *Enterococcus raffinosus*; **Eco,** *Escherichia coli*; **Fma,** *Finegoldia magna*; **Fmu,** *Fusobacterium mucleatum*; **Fps,** *Fusobacterium pseudonecrophorum*; **Hha,** *Haematonectria haematococca*; **Hku,** *Helcococcus kunzii*; **HHV-4,** *Human herpesvirus 4*; **Kae,** *Klebsiella aerogenes*; **Kox,** *Klebsiella oxytoca*; **Kpn,** *Klebsiella pneumoniae*; **Kor,** *Kosakonia oryzae*; **Kra,** *Kosakonia radicincitans*; **Lfe,** *Lactobacillus fermentum*; **Lpa,** *Lactobacillus paracasei*; **Lri,** *Lancefieldella rimae*; **Mmo,** *Morganella morganii*; **Mma,** *Mycolicibacterium mageritense*; **Pmi,** *Parvimonas micra*; **Pco,** *Peptoniphilus coxii*; **Pgr,** *Peptoniphilus grossensis*; **Pha,** *Peptoniphilus harei*; **Pla,** *Peptoniphilus lacydonensis*; **Pan,** *Peptostreptococcus anaerobius*; **Pst,** *Peptostreptococcus stomatis*; **Pas,** *Porphyromonas asaccharolytica*; **Pen,** *Porphyromonas endodontalis*; **Pbi,** *Prevotella bivia*; **Pbu,** *Prevotella buccalis*; **Pde,** *Prevotella denticola*; **Pin,** *Prevotella intermedia*; **Pme,** *Prevotella melaninogenica*; **Pti,** *Prevotella timonensis*; **Pmi,** *Proteus mirabilis*; **Pvu,** *Proteus vulgaris*; **Pst,** *Providencia stuartii*; **Pae,** *Pseudomonas aeruginosa*; **Ppu,** *Pseudomonas putida*; **Ror,** *Raoultella ornithinolytica*; **Sma,** *Serratia marcescens*; **Sal,** *Shewanella algae*; **Sex,** *Slackia exigua*; **Sau,** *Staphylococcus aureus*; **Sep,** *Staphylococcus epidermidis*; **Sha,** *Staphylococcus haemolyticus*; **Slu,** *Staphylococcus lugdunensis*; **Ssi,** *Staphylococcus simulans*; **Sag,** *Streptococcus agalactiae*; **San,** *Streptococcus anginosus*; **Sco,** *Streptococcus constellatus*; **Sdy,** *Streptococcus dysgalactiae*; **Sin,** *Streptococcus intermedius*; **Sor,** *Streptococcus oralis*; **Spy,** *Streptococcus pyogenes*; **Tva,** *Trichomonas vaginalis*; **Tas,** *Trichosporon asahii*; **Tri,** *Trichosporon sp.* **Vpa,** *Veillonella parvula*

**Table 2** Comparation of positive detection rate between routine culture method and mNGS

| | Culture method | mNGS | Total | *p* value |
|---|---|---|---|---|
| Positive | 24 | 38 | 62 | |
| Negative | 16 | 2 | 18 | 0.002 |
| Total | 40 | 40 | 80 | |

*faecalis* was the most common strain in the A-DFUs group. *Enterococcus faecalis* and *Finegoldia magna* were the most common strains in the M-DFUs group (Table 2; Fig. 2). Then the differences were analyzed, 14 samples that were negative by routine culture method were found to be positive by mNGS. The false negative incidence of routine culture method was 35%. In the 14 samples that were only identified as positive by mNGS, 44 strains of microorganisms were detected (Table 2). *Klebsiella species* was the most common microorganism in the only mNGS-positive group.

## Discussion

DFUs are the most frequently recognized complication of diabetes mellitus, and the lifetime incidence of a person with diabetes developing DFUs is estimated to be 15 to 25% [16]. More than half of DFUs become infected, which is known as DFI, and 20% of patients with moderate or severe DFI have to undergo amputation [5, 17, 18].

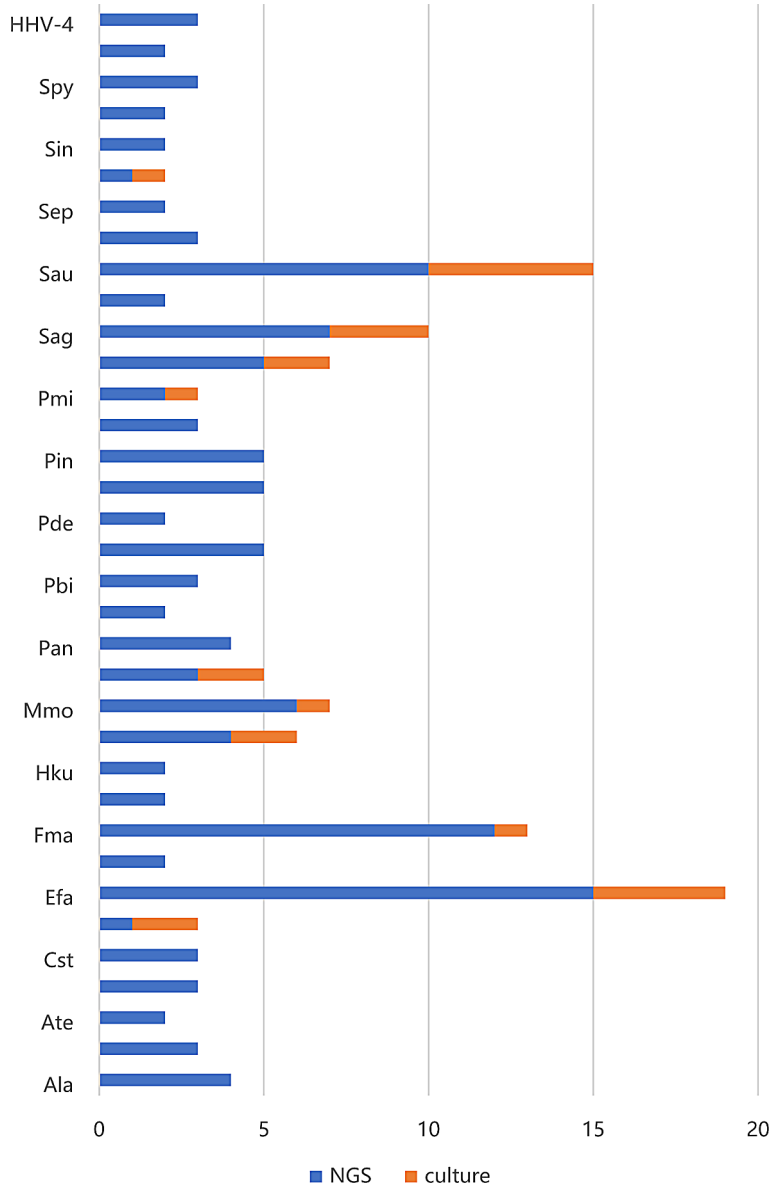


**Fig. 1** The microbial characteristics of DFUs obtained by routine culture method and mNGS (frequency ≥ 2). The results showed that *Enterococcus faecalis* was the most common microorganism in DFUs, for details, *Staphylococcus aureus* was the most common microorganism in the culture method group and *Enterococcus faecalis* was the most common microorganism in the mNGS group

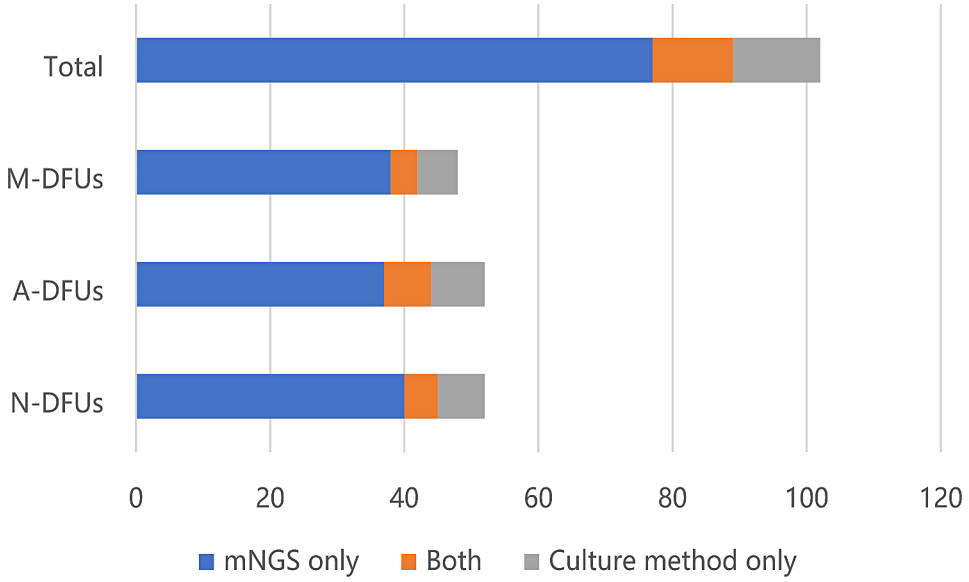


**Fig. 2** The distribution of microorganisms detected by culture method and mNGS in DFUs subtypes. The results showed that mNGS identified a much broader spectrum of microorganisms in DFUs subtypes

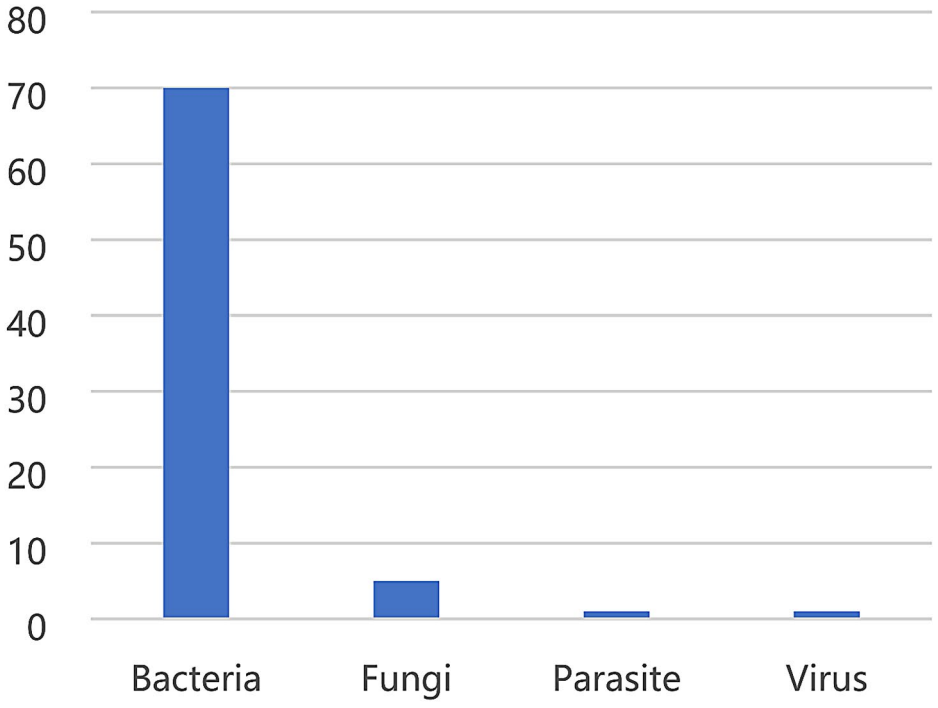


**Fig. 3** The microbial characteristics of DFUs detected by mNGS method. There were 70 bacteria, 5 fungi, 1 parasite and 1 virus detected by mNGS method, the results indicated that bacteria was the most common microbial species in DFUs

The disability resulting from amputation directly affects the quality of life of patients and markedly increases the risk of death [19, 20]. Curing diabetes mellitus is an ongoing issue, and dealing with DFUs and associated DFI might be a higher priority in daily clinical work. Due to the lack of local or systemic symptoms of DFI, accurately and quickly determining the microbial composition responsible for wound infection is critical for effective therapeutic interventions. The microbiome of the skin is a complex and dynamic microbial community, especially when infection occurs [21]. The immune responses are impaired in persons with diabetes, and the colonization of pathogenic bacteria in wounds can no longer be prevented [10]. Pathogenic bacteria often form biofilms that

irreversibly attach to wounds and produce extracellular polymers, which ultimately delay wound healing [22]. This characteristic was verified again by the finding that an increasing number of genes related to biofilm formation were identified by sequencing DFU samples [23]. The diverse and complex communities of microorganisms in DFUs make it difficult for the standard culture method to detect responsible pathogens. The routine culture method often takes too long, and worse, the likelihood of false negative or positive cultures is still unavoidable. The imprecise results result in the use of excessive or ineffective treatments [24, 25]. mNGS is able to generate large datasets to provide more insight into community-wide microbiome in wounds depending on its indiscriminate sequencing of all genes for all organisms.

Here, we simultaneously analyzed the DFUs wound samples by the mNGS and culture-based method. The concordance rate of positive detection between the two approaches was 83.3%, meaning that the microorganisms identified by the routine culture method were mostly identified in the mNGS sequencing results. Moreover, mNGS identified more 64 strains of microorganisms in this study. *Enterococcus faecalis* was the most common microorganism in all the mNGS results. *Enterococcus faecalis* is a gram-positive bacterium and is one of the most prevalent microorganisms in several chronic infections [26]. Li et al. reported that *Enterococcus faecalis* is the second most common in DFUs [27]. *Enterococcus faecalis* is an opportunistic multidrug-resistant pathogen because of its biofilm development, which causes persistent DFUs infection [28]. In all culture-based analyses, *Staphylococcus aureus* was the most common microorganism in DFUs. *Staphylococcus aureus* is also a gram-positive bacterium, and it often causes severe wound infection. The virulence factors of *Staphylococcus aureus*, such as toxic shock syndrome toxin-1, leukocidins, enterotoxins, and exfoliatins, seriously damage the tissue, and the appearance of multidrug-resistant strains, such as methicillin-resistant *S. aureus*, negatively impacts treatment and ultimately hinders wound healing [29].

We further summarized the characteristic distributions of microorganisms of the 3 subtypes of DFUs. The results of the two approaches showed several distinct constitutions. Given the more abundant results of mNGS, mNGS results might be more admissible than those of routine culture method. In mNGS analyses, *Enterococcus faecalis* was the most common microorganism in each DFU subtype, while *Finegoldia magna* was also the most common microorganism in A-DFUs and M-DFUs. *Finegoldia magna* is an anaerobic gram-positive bacterium that commensally colonizes the skin. Due to the difficulties of obtaining good-quality specimens and laboratory incubation of *Finegoldia magna*, its incidence in clinical analyses of infections was mostly underestimated [30]. Ariane Neumann et al. found that *Finegoldia magna* was able to activate neutrophils to release reactive oxygen species and initiate host defense mechanisms [31]. When the skin barriers are destroyed, *Finegoldia magna* might become an opportunistic pathogen that could cause opportunistic invasive infections. The distinct descriptions of microorganism distributions in DFUs subtypes can provide individualized diagnosis, which helps clinicians quickly understand the potential pathogens in several subtypes. We also plan to collect more samples to for further analysis according to Wagner classification to provide additional descriptions of every DFUs subtype.

Sequencing technologies have been confirmed to detect more anaerobes and gram-positive bacilli than routine culture method [32]. mNGS showed a stronger detection capability than the culture-based method and could provide more genomic information related to microbiome analyses and antibiotic resistance [33, 34]. The more comprehensive and accurate description of the microbiome in DFUs assists clinicians in better selecting the most appropriate clinical management. There were also 2 cases whose detection results were negative according to both the routine culture method and mNGS. This did not exclude the possible error of the sampling operation, so it is worth mentioning again that the regular clinical identification of the causative agent in DFUs is necessary to avoid misjudgments.

Moreover, mNGS identified 14 false-negative samples that were reported by the culture-based method in this study; thus, mNGS successfully overcomes the weakness of the routine culture method. We had to note that various factors of each culture procedure might decrease the ability of the culture method to identify the causative microorganisms. First, obtaining a high-quality sample fundamentally affects the accuracy of the results, and the bias lies in the standard operating procedure that is performed by a specialized surgery. After the samples are collected, they need to be carefully and quickly delivered to the laboratory and appropriately inoculated on the media; then, quite some time is required to allow the growth of microorganisms. During the in vitro culture stage, only organisms that grow easily in the laboratory culture environment are ultimately reported. Therefore, the routine culture method might limit the identification some microorganisms of DFUs that are difficult to culture, which might be the responsible causative organisms [10]. This underestimation of the biodiversity in DFUs results in incorrect results delivered to clinicians. mNGS might be a good choice to supplement the shortage of culture method because it can identify any microorganism regardless of the required growth environment. In our study, 44 strains of microorganisms were detected by mNGS in the negative culture group, and *Klebsiella species* was the most abundant microorganism in this group.

*Klebsiella pneumoniae* has become notorious worldwide due to its substantial pathogenicity, especially since the appearance of carbapenem-resistant *Klebsiella pneumoniae*. A higher risk for hypervirulent *Klebsiella pneumoniae* infection in patients with diabetes was observed in multiple studies [35]. Jin et al. suggested that defective neutrophil extracellular trap (NET)-mediated killing in diabetes contributed to the development of relative infections, including DFUs, although enhanced NET formation was observed in patients with diabetes compared to healthy controls [36]. The mechanism underlying NET impairment might be the dysregulation of protein components of NETs, so the timely detection of *Klebsiella pneumoniae* in DFUs makes sense for appropriate treatment. We had to reaffirm that the strong ability of pathogen detection by mNGS played an important role in supplementing the routine culture method.

Discordance of positive results between mNGS and routine culture method occurred in 4 cases. The culture-based method detected 4 strains of microorganisms in these 4 samples, while mNGS identified 19 strains of microorganisms. This difference might result from the unique principles of the two approaches. In terms of culture method, only live microorganisms were able to proliferate and be passaged in the laboratory, and the in vitro culture environment allowed only dominant microorganisms to easily reproduce. Therefore, it mostly relies on the growth characteristics and the existing proportion of the main pathogen in DFUs. These objective limitation inevitably affect the possible error of unexpected microorganism being listed in culture-based results. mNGS can indiscriminately identify both viable and dead microorganisms due to the direct sequencing of DNA or RNA from samples. However, the discordance described above indicated that the supposedly powerful mNGS might still miss pathogens. A similar phenomenon was also described in another study [11]. Any contamination in the culture procedures, such as sample processing, could bias the results and dramatically skew the results. A higher sequencing depth of mNGS was also recommended to avoid omission [37]. We must be rational about the role of mNGS in pathogen diagnosis. The accuracy of mNGS detection depends on comprehensive sequencing and specialized bioinformatics analyses. There were several parameters in the dataset construction and data interpretation, including the sequencing reads, genomic coverage, and relative abundance of each organism. However, how to utilize these indicators to interpret mNGS results still lacks a unified standard [38]. It is difficult to distinguish the pathogens and colonizing microorganisms or contaminant microorganisms in the specimens. The final mNGS reports must be repeatedly analyzed and interpreted by bioinformatics specialists and clinicians, and each distinctive condition always requires a greater combination of computational knowledge and the clinical situation, which is more expensive.

In summary, the microbiome of DFUs is a highly dynamic microbial community, and it is very difficult to determine the responsible pathogen when DFUs become infectious wounds. The routine culture method can identify viable detrimental microorganisms, but false negatives are difficult to avoid due to the features of culture-based systems. mNGS is a new approach for pathogen diagnosis that is more accurate and timelier, which allow it to compensate for the deficiencies of routine culture method. We found that mNGS can indiscriminately identify specimens and thus identify potential causative agents in samples that were considered negative according to culture-based method, and mNGS can further provide additional bioinformation about the DFUs microenvironment. The complementary role of mNGS to routine culture method for the etiological diagnosis of DFI can rapidly assist clinicians in developing tailored treatments and largely reduce costs. mNGS might be widely used in the regular surveillance of DFI to implement precision medicine.

## Conclusions

The microorganism spectrum of DFUs is complex and dynamic, but the detection efficiency of routine culture method is limited due to its process and principle. The mNGS method had a higher positive detection rate and identified a broader spectrum of microorganisms in DFUs. Staphylococcus aureus and Enterococcus faecalis are the most common microorganism detected in DFUs. The mNGS provided a more comprehensive understanding of the microbiome of DFUs, the detail description of the microbiome helped clinicians quickly pick the responsible pathogen of infection and develop a more effective therapeutic regime.

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#### Author contributions

Conceptualization, Shun Chen and Zhaorong Xu; Formal analysis, Hongteng Xie, Guohua Wu, Pei Wei and Teng Gong; Investigation, Guohua Wu, Pei Wei, Teng Gong and Shun Chen; Methodology, Hongteng Xie and Guohua Wu; Supervision, Zhaorong Xu; Validation, Zhaohong Chen; Writing– original draft, Hongteng Xie; Writing– review & editing, Zhaohong Chen, Shun Chen and Zhaorong Xu. All authors have read and agreed to the published version of the manuscript.

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**Data availability**
The data presented in this study are openly available in the NCBI database, the web link: https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA980579, the accession number: PRJNA980579.

## Declarations

**Ethics approval and consent to participate**
The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethical Review Committee of the Fujian Medical University Union Hospital (approval number 2021KY054). Informed consent was obtained from all subjects involved in the study.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare no competing interests.

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