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Care Delivery

Enhancing diabetic foot osteomyelitis diagnosis with metagenomics next-generation sequencing, proof of concept

Madjid Morsli[1] | **Chloé Magnan**[1] | **Florian Salipante**[2] | **Adeline Dubois**[1] | **Sophie Schuldiner**[3] | **Nicolas Cellier**[4] | **Albert Sotto**[5] | **Jean-Philippe Lavigne**[1] | **Catherine Dunyach-Remy**[1]

[1]VBIC, INSERM U1047, Université Montpellier, Service de Microbiologie et Hygiène Hospitalière, Clinique du Pied Diabétique Gard Occitanie, CHU Nîmes, Nîmes, France

[2]Service de Biostatistique, Épidémiologie, Santé Publique, et Information Médicale, Univ Montpellier, CHU Nîmes, Nîmes, France

[3]VBIC, INSERM U1047, Université Montpellier, Service des Maladies Métaboliques et Endocriniennes, Clinique du Pied Diabétique Gard Occitanie, CHU Nîmes, Nîmes, France

[4]Service d'Orthopédie, Université Montpellier, Clinique du Pied Diabétique Gard Occitanie, CHU Nîmes, Nîmes, France

[5]VBIC, INSERM U1047, Université Montpellier, Service des Maladies Infectieuses et Topicales, Clinique du Pied Diabétique Gard Occitanie, CHU Nîmes, Nîmes, France

Correspondence
Madjid Morsli, Service de Microbiologie et Hygiène Hospitalière, CHU Nîmes, Place du Professeur Robert Debré, 30029 Nîmes cedex 09, France.
Email: mor_madjid@hotmail.com

Abstract

Diabetic foot osteomyelitis (DFOM) is a serious medical condition that necessitates robust diagnostic tools for effective clinical management. Conventional diagnostic methods for DFOM rely heavily on bacterial culture, which is time-consuming and may fail to capture the full microbial diversity present in infections. This pilot study explored the utility of metagenomics next-generation sequencing (mNGS) as a complementary diagnostic tool for DFOM. We retrospectively analysed ten bone biopsies from nine diabetic persons using both routine microbiological culture and mNGS. Routine culture identified 11 bacterial species across seven biopsies, while mNGS detected 84 species, including all those found by culture. High microbial diversity (Shannon index = 1.10) was associated with severe osteomyelitis, leading to amputation in three of seven DFOM cases. Interestingly, one culture-negative biopsy revealed high bacterial diversity by mNGS and progressed to a severe infection within 7 days. mNGS also identified resistance genes, providing additional insights for targeted therapy. Integrating mNGS into routine clinical microbiology may serve as a complementary method to conventional diagnostics, particularly for distinguishing infection from colonization and predicting clinical outcomes. However, challenges such as human DNA contamination and limited sequencing depth must be addressed to optimize its clinical application. These findings support the integration of mNGS into diagnostic workflows for bone biopsies for improved management of DFOM.

KEYWORDS

bone biopsy, diabetic foot osteomyelitis, metagenomics next-generation sequencing, microbial diversity, osteomyelitis management

1 | BACKGROUND

Diabetic foot infections (DFIs) represent a severe and advanced complication of non-healing wounds in people with diabetes mellitus, accounting for 40%–80% of all diabetic foot ulcers.[1] The clinical outcomes of DFIs are largely influenced by the bacterial pathogens infecting the soft tissues of the wound.[2] In 20% of cases, these infections progress from soft tissue to bone infection, leading to diabetic foot osteomyelitis (DFOM).[1] When antibiotic therapy fails, surgical intervention remains the only available treatment option for DFOM, increasing healthcare management costs and leading to a high risk of amputation and a 50% mortality rate in people with culture-positive biopsies.[1,3,4]

DFI and DFOM are consistently reported as polymicrobial infections. The clinical complications and wound outcomes are usually determined by the microbial communities colonizing the wound. The ability of the infecting bacteria to persist in bone through mechanisms such as intracellular life, small colony variant formation and the production of virulence factors, toxins and biofilm contributes to therapeutic failure.[4–7]

Current routine DFOM microbiology investigation relies primarily on conventional culture techniques, which are optimized for detecting bacteria commonly associated with diabetes infections.[8] The most frequently isolated species include *Staphylococcus aureus*, particularly methicillin-resistant *S. aureus* (MRSA), *Staphylococcus epidermidis*, *Proteus mirabilis* and *Pseudomonas aeruginosa*, while anaerobes remain underrepresented.[9–11] Bone biopsies remain the gold standard for confirming DFOM, as clinical signs alone are often insufficient for early detection.[12–15] However, this routine method is hindered by significant delays, requiring a 2-week antibiotic washout period and up to 14 days for culture results, thereby postponing critical treatment decisions.

Recent advances in metagenomics next-generation sequencing (mNGS) have revealed a broader microbial diversity in DFOM samples.[2,13] The use of 16S rDNA gene sequencing has enhanced our understanding of the microbial communities involved in DFOMs. This provides additional information about the diversity and abundance of bacteria, as well as the prevalence of anaerobic Gram-negative bacilli.[10] Nevertheless, 16S rDNA gene sequencing is limited by short read lengths and often only allows taxonomic resolution at the genus level.[14] This poses a challenge for emergency diagnosis and biopsy management. However, real-time metagenomic sequencing offers a promising alternative, enabling rapid and comprehensive microbial identification directly from clinical samples. This approach not only shortens diagnostic timelines but also provides insights into the role of detected microorganisms in biopsy outcomes.[15,16]

In this study, we retrospectively applied real-time metagenomics directly to bone biopsies collected from people with suspected DFOM at the Diabetic Foot reference Center (Clinique du Pied diabétique Gard-Occitanie) of Nîmes University Hospital (France). Our objective was to estimate the microbial diversity in both culture-positive DFOM cases and bone biopsies routinely considered as negative cultures.

What's new

What is already known?

Diabetic foot osteomyelitis (DFOM) is a potentially life-threatening condition that requires accurate diagnostic tools. Conventional methods, such as bacterial culture, are time-consuming and may not detect all microbes involved in the infection.

What this study has found?

This pilot study compared routine culture and metagenomics next-generation sequencing (mNGS) on bone biopsies from diabetic patients with DFOM. Although culture identified 11 bacterial species in 7 biopsies, mNGS detected 84 species, including all those identified by culture. High microbial diversity detected by mNGS correlated with severe osteomyelitis and a higher risk of amputation. mNGS also identified antibiotic resistance genes, providing additional clinical insights. One culture-negative case showed high bacterial diversity by mNGS and quickly developed severe infection.

What are the implications of the study?

The mNGS could complement conventional diagnostics by improving detection of microbial diversity, differentiating infection from colonization, and aiding prognosis in DFOM. Challenges like human DNA contamination and sequencing depth need addressing before routine clinical implementation. Integrating mNGS into diagnostic workflows may improve management and outcomes in DFOM patients.

2 | METHODOLOGY

2.1 | Sample collection and ethics

A total of 10 leftover bone biopsy samples from nine adult participants (with biopsies 4 and 9 originating

from the same individual) who were clinically suspected of having DFOM were retrospectively submitted for microbial analysis in this study using both conventional culture and mNGS. These samples were obtained from people admitted to the Diabetic Foot reference center at Nîmes University Hospital (Clinique du Pied diabetique Gard Occitanie) between March and May 2017. The inclusion was limited to adult participants (age >18 years) presenting clinical signs of infection, with no systemic antibiotic treatment in the 2 weeks prior to the consultation. The wounds were evaluated by a trained diabetologist using the International Working Group on the Diabetic Foot (IWGDF) classification system to evaluate the severity of infection.[17] Foot osteomyelitis was suspected when at least two of the following criteria were met: a wound persisting for ≥2 weeks over a bony prominence with a surface area >2 cm^2 or depth >3 mm; a positive probe-to-bone test; and abnormalities consistent with the diagnosis of osteomyelitis on plain radiographs, radionuclide imaging (triphasic bone scintigraphy and/or labelled leukocyte imaging) or MRI. Following surgical debridement, transcutaneous bone biopsies were performed by a qualified orthopaedic surgeon in accordance with established guidelines.[13] A systematic clinical follow-up was conducted at 1 month to evaluate DFOM progression. Epidemiological and clinical data were collected for all participants. This study was approved by the Ethics Committee of the Nîmes University Hospital (Institutional Review Board, CHU Nîmes), under reference number: 24.07.05. No additional sampling was performed specifically for this study.

2.2 | Microbiological analysis

Tissue and bone samples were processed according to European guidelines.[18] Each biopsy was cultured for 14 days using an in-house protocol. Briefly, 5 mL of sterile water was added to the sample, which was then homogenized using an IKA Ultra-Turrax grinder (power 9, 5 min). The homogenate was inoculated onto Chocolate PolyViteX agar (bioMérieux, Marcy l'Etoile, France) incubated in a 5% CO_2 atmosphere, Columbia 5% sheep blood agar (bioMérieux) under aerobic conditions and *Brucella* agar under anaerobic conditions, at 35 ± 2°C for 7 days.[18] To enhance culture sensitivity, Schaedler broth was inoculated and incubated aerobically at 35 ± 2°C with daily visual reading for 14 days. At the end of incubation, the broth was subcultured onto the same culture media as previously and incubated for 48 h. The cultured bacteria were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Vitek MS®, bioMérieux). Antibiotic susceptibility testing was conducted using the disk diffusion method (Bio-Rad, Marnes La Coquette, France) on Mueller-Hinton agar, with or without horse blood, following European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines version 2024 available on: (https://www.eucast.org/bacteria/clinical-breakpoints-and-interpretation/clinical-breakpoint-tables/). Fungal cultures were performed on BBL™ CHROMagar™ Candida (Becton Dickinson, Le Pont de Claix, France). In cases of high clinical suspicion, an in-house PCR targeting the *rpoB* gene of *S. aureus* was performed, as *S. aureus* is a key pathogen in diabetic foot infections (DFI).[19]

2.3 | Metagenomics investigation

Total DNA was extracted from 200 µL of bone suspension using a previously validated in-house protocol.[20] The suspension was incubated overnight at 56°C with 20 µL of proteinase K, followed by a 20-min incubation at 100°C with glass powder. Samples were then vortexed for 90 s at 6.5 m/s using a Fast-Prep apparatus (MP Biomedicals, Santa Ana California, USA). After centrifugation at 13,000*g* for 5 min, DNA was extracted from 200 µL of the supernatant and eluted in 50 µL using the EZ1 DNA Tissue Kit (Qiagen, Courtaboeuf, France).

mNGS was performed using both paired-end and single long-read platforms. Oxford Nanopore libraries were prepared using a native barcoding protocol as previously developed,[21] with 48 µL of extracted DNA and sequenced for a 24-h run on an Oxford Nanopore MinION (Oxford Nanopore Technologies, Oxford, UK). Real-time analysis was conducted by EPI2ME online software (Oxford Nanopore Technologies). Additionally, Nanopore reads were analysed using Kraken 2 software (version 2.1.1), and results were visualized with the Pavian metagenomics data explorer online software (https://fbreitwieser.shinyapps.io/pavian/). Pathogen virulence and antibiotic resistance genes were predicted using ResFinder and Virulence Finder tools on the CGE platform (http://www.genomicepidemiology.org/services/).

2.4 | Statistical analysis

All statistical analyses were conducted using R software (version 4.2.0). Microbiome data were processed using the phyloseq package. Data were rarefied prior to analysis. Alpha diversity was assessed using the observed number of operational taxonomic units (OTUs) and the Shannon diversity index. Beta diversity was

evaluated using Bray-Curtis dissimilarity, and visualized via Principal Coordinate Analysis. Relative abundances were displayed using bar plots. Differential abundance was tested using the Mann-Whitney, with a significant threshold set at 5%. False discovery rate (FDR) corrections were applied to account for multiple comparisons.

3 | RESULTS

3.1 | Patient characteristics

This retrospective pilot study analysed ten bone biopsy samples collected from nine diabetic people (with biopsies 4 and 9 obtained from the same patient during a single visit). The cohort included seven men and two women, with ages ranging from 31 to 71 years (mean age: 59.5 years). Both conventional microbiological culture and mNGS were performed on all samples. Seven biopsies were culture-positive and classified as confirmed DFOM cases (DFOM group), while three biopsies were culture-negative despite clinical suspicion of infection and were used as controls (control group). The anatomical origins of the biopsies were diverse, including two cases of hallux osteomyelitis, one metatarsophalangeal joint arthrodesis, one left foot radius osteomyelitis, two toe amputations from areas presumed to be non-infected and one left foot amputation from a clinically non-infected site. Clinical records were unavailable for two participants (Table 1). In the 3 months preceding admission, four participants had received antibiotics, including β-lactams (3/4), fluoroquinolones (2/4) and rifampicin (1/4). Based on clinical follow-up, complete wound healing was observed in three participants, while four experienced stagnation or worsening of their condition. Two participants ultimately required amputation due to severe wound deterioration (Table 1).

3.2 | Conventional microbiological findings

In the DFOM group, routine culture over a 14-day incubation period identified *S. aureus* in five of the seven positive biopsies, including two mono-microbial infections. *P. mirabilis* was isolated in two biopsies. Additional mono-microbial isolates included *Streptococcus agalactiae*, unidentified coagulase-negative staphylococci (CoNS), *Pseudomonas aeruginosa*, *Morganella morganii*, *Escherichia coli*, *Citrobacter koseri*, *Corynebacterium jeikeium*, *Corynebacterium striatum* and the anaerobe *Finegoldia magna*. In the control group, Biopsy-10 revealed a polymicrobial aerobic profile interpreted as colonization, while no bacterial growth was detected in Biopsy-08 and Biopsy-09 (Table 1).

PCR-based detection of *S. aureus* was positive in three of the seven DFOM biopsies. No PCR positivity was noted in the remaining samples. Notably, four discrepancies were identified between culture and PCR results. *S. aureus* was cultured from Biopsy-1, -6 and -7, yet PCR failed to detect its DNA. Conversely, Biopsy-8 tested positive for *S. aureus* by PCR despite a negative culture result. These findings highlight the limitations of PCR-based detection in bone biopsy specimens (Table 1).

3.3 | Metagenomics findings

The ten bone biopsies yielded between 5742 reads (Biopsy-5) and 2,060,455 reads (Biopsy-2), with an average of 85.86% of reads successfully classified. The proportion of classified reads corresponding to microbial genomes averaged 1.28% in the DFOM group and 0.23% in the control group (Table S1). No microbial-specific sequences were detected in the negative control, except for trace reads of *Shigella* and Phage T4, which likely originated from library preparation reagents. At the phylum level, Pseudomonadota dominated (56.5%), followed by Actinomycetota (29.7%), Cyanobacteriota (11.3%) and Bacillota. Other phyla each accounted for less than 0.6% of classified reads. Biopsies from the DFOM group exhibited higher microbial diversity (Shannon index: 1.10) compared to biopsies from the control group (Shannon index: 0.14). The most abundant genera in DFOM samples included *Escherichia*, *Enterobacter*, *Nostoc*, *Mycobacterium*, and *Klebsiella* (>10%) (Figure 2a). *Proteus*, *Aeromonas*, *Citrobacter* and *Streptococcus* were also present at >2% (Table S2). In contrast, *Mycobacterium*, *Nostoc* and *Escherichia* were predominant in biopsies from the control group. Across all samples, 74 bacterial species from 38 genera were identified. Of these, 15 species were shared between the DFOM and control groups, while 59 species were exclusive to DFOM samples. No species were uniquely associated with the control group (Figure 1). The most commonly shared species included *E. coli*, *Enterobacter cloacae, C. koseri*, *P. mirabilis, Enterobacter hormaechei* and the environmental species *Mycobacterium branderi*.

Among the top ten most abundant species in the DFOM group, *E. coli* and *E. hormachei* were predominant, followed by *P. mirabilis*, *C. koseri* and *S. aureus*. Interestingly, Enterobacterales (*E. coli, C. koseri, E. hormaechei, P. mirabilis*) were also frequently detected in the control group.

TABLE 1 Microbiological results of the 10 bone biopsies obtained by routine culture, PCR (targeting *S. aureus*) and mNGS, in nine participants with DFOM.

Samples	Bone origin	Culture results	PCR	Main results obtained by mNGS	Patient data		
					Age (y)	Sex	Outcome at 1 month
DFOM group							
Biopsy-1	Hallux osteitis	*S. aureus*	Negative	38 species including: *S. aureus, Escherichia coli, Citrobacter koseri, Proteus mirabilis, Klebsiella pneumoniae, Enterobacter hormaechei, Enterobacter cloacae, Salmonella enterica*, anaerobes (*Clostridium botulinum, Clostridium perfringens*), *Bacillus thuringiensis, Priestia megaterium, Mycobacterium branderi*	37	Male	Stagnation
Biopsy-2	NP	*S. aureus*	Positive	10 species, including ***S. aureus***, *E. coli, C. koseri, P. mirabilis, E. hormaechei, Klebsiella michiganensis, Mycobacterium branderi*	55	Male	Complete healing
Biopsy-3	2nd radius of the left foot	*S. aureus*, *P. aeruginosa, Corynebacterium* sp., *Candida* sp.	Positive	7 species, including ***S. aureus***, *E. coli, C. koseri, P. mirabilis, E. hormaechei, Enterobacter kobei, Mycobacterium branderi*	70	Female	Complete healing
Biopsy-4	NP	CoNS, *Corynebacterium jeikeium, Finegoldia magna*	Negative	22 species, including *E. coli, E. hormaechei, Enterobacter cloacae complex, Klebsiella aerogenes, K. pneumoniae, Leclercia adecarboxylata, Raoultella ornithinolytica,* ***Corynebacterium jeikeium, Finegoldia magna,*** *Mycobacterium branderi*	69	Male	Amputation
Biopsy-6	NP	*S. aureus, P. mirabilis, M. morganii*, *C. striatum*	Negative	14 species, including ***S. aureus***, *E. coli, C. koseri,* ***P. mirabilis***, *E. hormaechei, K. pneumoniae, Serratia marcescens, Porphyromonas somerae, Bacillus thuringiensis,* ***Corynebacterium***, *Anaerococcus obesiensis, Mycobacterium branderi*	31	Male	Worsening evolution (surgery)

(Continues)

TABLE 1 (Continued)

Samples	Bone origin	Culture results	PCR	Main results obtained by mNGS	Patient data		
					Age (y)	Sex	Outcome at 1 month
Biopsy-7	Left foot necrosis+ amputation	*S. aureus, S. agalactiae, E. coli, Actinomyces odontolyticus*, Anaerobes	Negative	28 species, including *S. aureus, Streptococcus agalactiae, E. coli, C. koseri, P. mirabilis, E. hormaechei, E. cloacae, K. pneumoniae, P. somerae, Porphyromonas asaccharolytica, Bacillus cereus, Listeria monocytogenes*, **anaerobes** (*Prevotella melaninogenica, Peptoniphilus harei, A. obesiensis, Anaerococcus vaginalis, Finegoldia magna, Prevotella buccalis, Clostridium pasteurianum, Fusobacterium necrophorum, Clostridium perfringens, Clostridium botulinum*), *Mycobacterium branderi*	69	Female	Transtibial amputation
Biopsy-8	Left hallux last phalanx osteitis	*P. mirabilis, C. koseri*	Positive	61 species, including *S. aureus, E. coli, C. koseri, P. mirabilis, E. hormaechei, K. pneumoniae*, other *Citrobacter* sp., *Salmonella enterica, S. marcescens, M. morganii, P. vulgaris, Aeromonas veronii*, anaerobes (*Fusobacterium gonidiaformans, Parvimonas micra*), *Mycobacterium branderi*	61	Male	Worsening evolution
Control group							
Biopsy-5	Metatarsophalangeal joint arthrodesis	Negative	Negative	6 species, including *E. coli, C. koseri, E. hormaechei, S. enterica, S. aureus, M. branderi*	71	Male	Complete healing
Biopsy-9[a]	Fourth toe amputation osteitis	Negative	Negative	10 species, including *C. koseri, E. cloacae, E. hormaechei, E. roggenkampii, E. coli, M. branderi, P. toebii, P. somerae, P. mirabilis* and *S. aureus*	69	Male	Amputation

TABLE 1 (Continued)

Samples	Bone origin	Culture results	PCR	Main results obtained by mNGS	Patient data		
					Age (y)	Sex	Outcome at 1 month
Biopsy-10	Amputation due to severe lesions	Negative	Negative	19 species, including *A. obesiensis, B. cereus, B. cuenoti, C. freundii, C. koseri, E. asburiae, E. coli, E. hormaechei,* ***F. magna, F. gonidiaformans****, K. pneumoniae, M. branderi, Nostoc, P. asaccharolytica, P. harei, P. micra, P. mirabilis, P. melaninogenica, P. somerae and P. toebii. Among them, B. cuenoti, M. branderi, Nostoc and P. toebii are strict aerobes; A. obesiensis, F. magna, F. gonidiaformans, P. micra, P. harei, P. asaccharolytica, P. somerae and P. melaninogenica are strict anaerobes; while B. cereus, C. freundii, C. koseri, E. asburiae, E. coli, E. hormaechei, K. pneumoniae and P. mirabilis are facultative anaerobes*	63	Male	Super-infected necrotic evolution (surgery)

Note: In bold, species identified in culture and in mNGS.

Abbreviation: CoNS, coagulase-negative staphylococci; NP, no precision.

[a]Same patient with Biopsy-4.

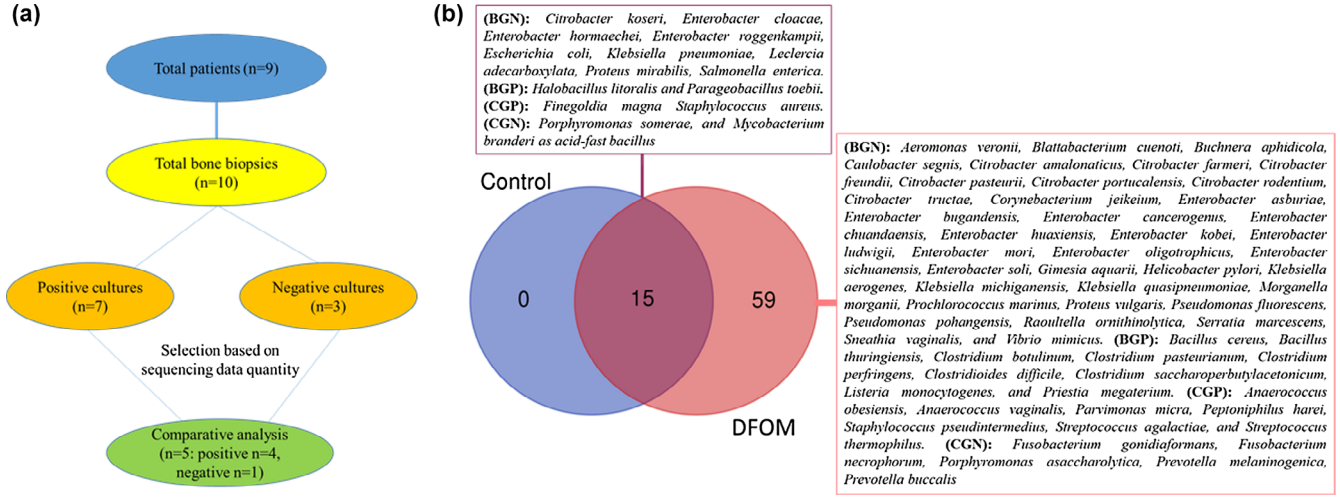

FIGURE 1 Biopsy inclusion and microbial composition of DFOM and negative bone biopsies. (a) Overview of biopsy inclusion and collection of bone biopsies. (b) Microbial composition of DFOM and negative bone biopsies. GNB, Gram-negative bacilli; GNC, Gram-negative cocci; GPB, Gram-positive bacilli; GPC, Gram-positive cocci.

Less abundant but clinically relevant bacteria such as *Porphyromonas somerae*, *Salmonella enterica* and various anaerobes were identified in both groups, along with *M. branderi* (Table 1).

Antibiotic resistance gene analysis revealed the presence of the beta-lactamase gene *blaACT-14* exclusively in Biopsy-4 (Table S3). Additionally, a total of 81 pathogenicity-associated genes from Gammaproteobacteria were detected in this sample, including 79 genes linked to *Citrobacter* pathogenicity, a fimbrial usher protein from *P. mirabilis* and a ribosomal-binding factor A from *S. enterica* involved in bacterial stress response and virulence.[22] No resistance or virulence encoding genes were detected in the remaining samples.

3.4 | Microbial comparison based on sequencing depth

Five biopsies with sufficient sequencing depth were selected for comparative analysis: four from the DFOM group (Biopsies 2, 3, 4 and 6) and one from the control group (Biopsy-10) (Figure 1a). Notably, the patient corresponding to Biopsy-10 experienced rapid clinical deterioration within 7 days, progressing to a super-infected necrotic wound. All bacterial species identified in Biopsy-10 were also found in confirmed DFOM samples (Figure 1b). Among the 15 shared species, *E. hormaechei*, *E. cloacae*, *P. mirabilis, Leclercia adecarboxylata*, *F. magna* and *S. aureus* were the most frequently associated with DFI. The microbial diversity of Biopsy-10 (Shannon index = 1.1) closely resembled that of confirmed DFOM cases (Figures 1 and 2).

3.5 | Comparison between routine culture and mNGS

In the DFOM group, mNGS was fully concordant with routine culture in three biopsies. For example, *S. aureus* was the sole isolate in Biopsies 1 and 2 by culture, while mNGS additionally identified 37 and nine other species, respectively. In Biopsy-8, culture identified *P. mirabilis* and *C. koseri*, whereas mNGS detected 59 additional species (Table 1). Biopsies 4, 6 and 7 showed general agreement between methods, with minor discrepancies (e.g. CoNS, *M. morganii*, *A. odontolyticus* detected only by culture). In Biopsy-3, both methods identified *S. aureus*, but mNGS failed to detect *P. aeruginosa*, *Corynebacterium* sp. and *Candida* sp. found by culture.

In the control group, mNGS identified 15 bacterial species, including *P. mirabilis* associated with *S. enterica, P. somerae, E. cloacae* complex and *L. adecarboxylata* (Figures 1b and 2a, Table 1).

Anaerobes were detected by mNGS in 6 out of 10 biopsies, but were largely missed by culture, except in Biopsy-7, where both methods identified anaerobic microorganisms.

3.6 | Limitations

The primary limitation of this study was the small sample size and the limited number of culture-negative biopsies. The storage conditions of the samples significantly affected DNA quality and yield, with 6 out of 10 samples producing less than 10 ng/μL of total extracted DNA, thereby limiting the depth of sequencing. Additionally, contamination from library preparation

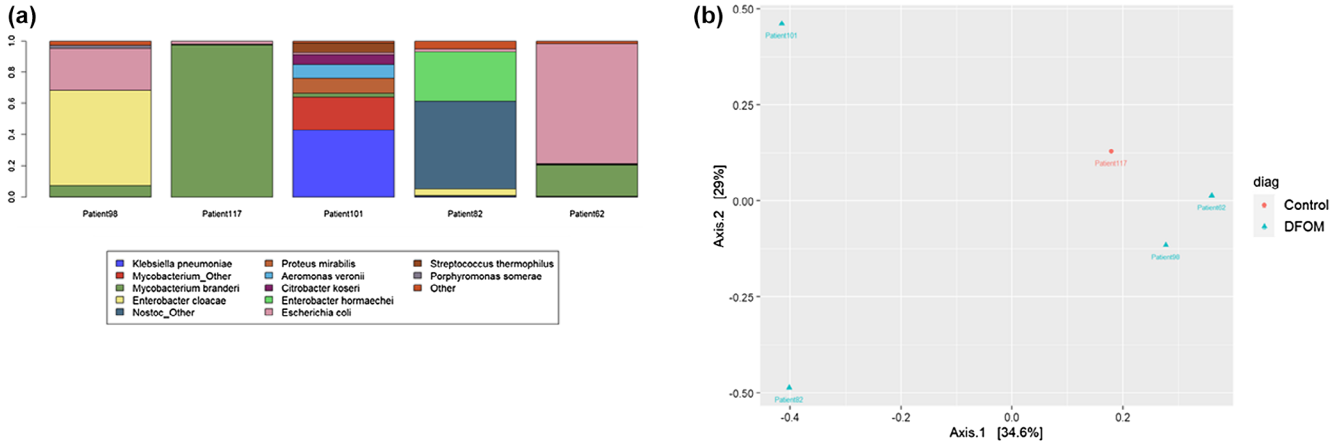

FIGURE 2 Microbial analysis of DFOM and negative bone biopsy (Patient 117), according to microbial data quantity. (a) Bar plot showing the most identified species in DFOM and negative bone biopsies. (b) Beta-diversity analysis of four confirmed DFOM and one negative bone biopsy. Comparing all samples and highlighting those with the highest abundance (≥3000 reads).

enzymes and reagents introduced background reads, particularly from *E. coli* and *Shigella*. Despite clear differences between DFOM and control groups, the sample size was insufficient for robust statistical validation. Larger-scale studies are needed to confirm these findings and validate the clinical utility of real-time mNGS in DFOM diagnosis.

4 | DISCUSSION

To date, no reliable molecular diagnostic tools have been specifically validated for the routine diagnosis of DFOM. Current microbiological investigations rely primarily on conventional culture methods, with 16S rDNA gene sequencing recommended only in selected cases.[23] Although mNGS offers a promising, time- and cost-effective alternative that bypasses the limitations of targeted PCR, international guidelines currently advise against its routine use on tissue or bone biopsies from people with DFI or DFOM.[12,17,24] Among the few molecular tools developed, the Unyvero ITI multiplex PCR assay (Curetis, Holzgerlingen, Germany) targeted a restricted panel of 14 bacterial genera and five fungi commonly associated with DFI and DFOM.[25] Despite promising preliminary results, its limited detection spectrum and inability to capture the full microbial diversity led to its commercial withdrawal. As a result, conventional culture remains the current diagnostic gold standard.

In this pilot study, we applied direct mNGS to ten bone biopsies from diabetic people with suspected DFOM (Table 1). While conventional culture recovered 11 bacterial species across seven biopsies, mNGS detected a total of 84 species, including all but one of those isolated by culture (Figure 1). These findings underscore the potential of mNGS to uncover underdiagnosed infections that are often missed by routine methods. A recent study has suggested that microbial diversity itself may serve as a biomarker of infection severity.[26] Our results support this hypothesis: high microbial diversity was associated with severe DFOM cases, including three participants who subsequently required amputation. The traditional focus on *S. aureus* as the primary pathogen in DFOM.[27] may overlook other clinically relevant bacteria. For example, *P. somerae*, frequently isolated from bone and soft-tissue infections in diabetic people,[28] members of the *E. cloacae* complex, recognized healthcare-associated pathogens,[29] and *L. adecarboxylata*, implicated in necrotizing soft-tissue infections,[30,31] were all detected in our study. Six of the seven DFOM biopsies showed strong concordance between culture and mNGS, while all three culture-negative biopsies in the control group were positive by mNGS. Enterobacterales were particularly prevalent (3/3), notably *E. coli*, *C. koseri*, *E. hormachei* and *P. mirabilis*, detected in 8 to 10 biopsies, regardless of clinical outcome. However, *K. pneumoniae* was found in six biopsies, all associated with poor clinical outcomes, suggesting a potential role in accelerating wound deterioration. Notably, this species was consistently co-detected with anaerobes, reinforcing recent evidence that anaerobic-aerobic synergy may contribute to DFOM progression.[11] Interestingly, in one control biopsy initially reported as negative, mNGS revealed high microbial diversity, including *K. pneumoniae* and anaerobes. This patient developed a severe infection requiring surgical intervention within 7 days, suggesting that mNGS may help predict early progression of DFI. These findings highlight the potential of microbial diversity and specific bacterial signatures as

indicators of the severity of infection in DFOM. Future research should focus on understanding and describing the interactions between all bacteria present during these infections, their crosstalk and their propensity to organize in biofilm and pathogroups, influencing wound outcomes.[32–34] The exclusive detection of anaerobic bacteria signatures identified in bone biopsies by mNGS further supports the growing recognition of anaerobes as key contributors to DFOM severity and poor outcomes and their impact in the management of DFOM.[11] This is consistent with the poor outcomes observed in the patient (Biopsy-10) whose considered as clinically non-infected. The frequency co-occurrence of anaerobes with major pathogens such as *K. pneumoniae* argues against simple contamination and instead supports the existence of synergistic microbial consortia.[35] These findings emphasize the need to improve anaerobic sampling strategies, specimen transport and routine anaerobic culture protocols to better capture the true microbiological landscape of DFOM.

The direct application of mNGS on bone biopsies provided clinicians with more information to assess wound status and propose appropriate wound management and adequate treatment. Beyond taxonomic profiling, mNGS also provided functional insights. In one biopsy, detection of the beta-lactam resistance gene *bla*ACT-14 together with 81 virulence-associated genes illustrates the added value of mNGS in guiding antimicrobial therapy.[36] Nevertheless, several limitations must be acknowledged. A major technical constraint for shotgun mNGS remains the high proportion of human DNA in the sequenced samples, which reduces analytical sensitivity and limits sequencing depth for microbial genomes. This necessitates further optimization of nucleic acids extraction protocols combined with efficient human DNA depletion to enhance microbial signal and improve bioinformatics resolution. Microbial diversity has been shown as an additional factor associated with infection, which can assist clinicians in the early identification of DFI, thereby improving biopsy management and wound treatment. Importantly, the detection of bacterial DNA by mNGS does not confirm bacterial viability, especially in biopsies from clinically healthy areas. This distinction is critical for interpreting results and guiding clinical decisions.

Despite these limitations, our study provides proof of concept that direct mNGS can characterize the DFOM microbiome with high taxonomic resolution. Microbial diversity emerged as a potential marker of infection severity and adverse outcome. Given its simplicity, reliability, speed (<24 h) and affordability (<200€),[21] mNGS represents a promising adjunct to conventional microbiology for the management of complex DFIs/DFOMs. Its future integration into routine diagnostic workflows could significantly enhance the management of infected diabetic foot wounds, particularly DFOM, by providing clinicians with comprehensive information.

AUTHOR CONTRIBUTIONS

MM: literature search, study design, data collection and cleaning, interpretation, validation and writing of the original manuscript. CD-R, CM and AD: sample collection, storage and data collection. FS: data cleaning, interpretation and formal analysis. AS and J-PL: validation, critical review of the manuscript, conceptualization and direction of the study. MM, AS and J-PL: final validation and approval of the manuscript. All authors have read and approved the final manuscript.

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The authors have nothing to report.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All metagenomics data had been submitted to NCBI GenBank under the submission number: SUB14505094.

ETHICAL APPROVAL

The retrospective study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of CHU Nîmes (protocol code MINION DFOM-2024 and under N°: 24.07.05). The bone biopsies were conducted within the context of medical care and were classified as non-interventional research. This type of research only requires the non-opposition of the patient during sampling, in accordance with articles L1221-1.1, L1211-2 and N°: DC-2020-4155 of the French Public Health Code.

INFORMED CONSENT

All participants provided written informed consent and approved the use of data for publication.

ORCID

Madjid Morsli https://orcid.org/0000-0002-8074-1794

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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