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Microbiological characterization of neuropathic diabetic foot infection: a retrospective study at a Portuguese tertiary hospital

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Abstract

Diabetic foot infection imposes a significant burden and is the major cause of nontraumatic limb amputation. Adequate patient management with effective antibiotic therapy is crucial.

This retrospective cohort study aimed to characterize the microbiology and resistance patterns of moderate to severe neuropathic diabetic foot infection in patients hospitalized at a tertiary referral hospital between January 2020 and June 2023. Deep tissue specimens from ulcers were collected for culture.

Sixty inpatients were included (62% male, mean age 59.1 ± 11.5 years). Osteomyelitis was present in 90% of the patients. Among 102 microorganisms (average of 1.91 ± 1.25 pathogens per patient), 60.8% were gram-positive bacteria, 31.4% were gram-negative, 3.92% were anaerobic bacteria, and 3.92% were fungi. *Staphylococcus aureus* (19%) and *Enterococcus faecium* (17%) were the most common. *Pseudomonas aeruginosa* (8%) and bacteria of the *Enterobacterales* family (24%) accounted for all the isolated gram-negative bacteria. Sixteen percent of *Staphylococcus aureus* and 67% of coagulase-negative *Staphylococci* were resistant to methicillin. Resistance to ampicillin was found in 11% of *Enterococci*. All *Pseudomonas aeruginosa* isolates were sensitive to piperacillin-tazobactam, ceftazidime, or cefepime. Among the *Enterobacterales*, resistance rates were 35% for piperacillin-tazobactam, 38% for ceftazidime, 21% for cefepime, and 13% for carbapenems.

Although the prevalence of methicillin-resistant staphylococci was lower than that in other studies, carbapenem resistance among gram-negative bacteria warrants attention. This study highlights the importance of understanding local epidemiology for effective diabetic foot infection management and resistance mitigation.

Keywords Diabetic foot infection, Diabetic foot ulcer, Microbiology, Epidemiology, Antibiotics

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Introduction

Diabetes mellitus (DM) is a significant global health issue that affects approximately 10.5% of the adult population worldwide [1]. Diabetic foot disease is recognized as one of the most serious complications of DM. The estimated lifetime incidence of diabetic foot ulcers (DFUs) ranges from 15 to 34% [2–4]. DFUs arise from a complex interplay of risk factors, including diabetic neuropathy and peripheral artery disease. The combination of loss of protective sensitivity, foot deformities, and limited joint mobility leads to abnormal foot loading, thereby increasing the likelihood of ulceration [4]. Bacterial colonization occurs due to skin breakdown and can lead to infection, facilitated by immunosuppression related to DM [3, 5].

DFUs are the most frequent cause of disability among individuals with DM and are the main cause of nontraumatic lower limb amputations. In Portugal, in 2021, 2,445 lower limb amputations were performed in patients with DM [6], resulting in substantial burdens on healthcare systems in terms of hospitalizations and costs. Additionally, DFUs are associated with a 5-year mortality rate of approximately 50% [2–4].

The management of neuropathic diabetic foot infection (NDFI) requires crucial interventions, including offloading, appropriate antibiotic therapy, debridement surgery, and optimal glycemic control [4]. Current guidelines recommend collecting specimens for microbiological culture before initiating empiric antibiotic therapy [4]. The selected antibiotics should effectively target all potential pathogens while minimizing the risk of antibiotic resistance [2, 4]. Therefore, knowledge of local microbiological epidemiology is crucial for adjusting empirical antibiotic therapy and improving patient outcomes.

The aim of our study was to characterize the microbiological profile and susceptibility pattern of NDFI in patients requiring hospitalization at our centre.

Methods

Study design and participants

We performed a retrospective observational study evaluating all patients with moderate to severe NDFI who were hospitalized in the Endocrinology Department of a tertiary hospital in Portugal between January 2020 and June 2023. Admission criteria for NDFI were i) severe NDFI (PEDIS 4); ii) PEDIS 3 cases where surgical debridement was likely due to the extent of infection wound; iii) clinical deterioration despite oral antimicrobial therapy; and iv) need for intravenous antibiotic-directed therapy [7]. All patients were treated by the same multidisciplinary team, including Endocrinology, Infectious Diseases and Orthopedics, all of whom had expertise in the treatment of NDFI, following national and international guidelines and recommendations. Peripheral arterial disease was excluded by the Vascular Surgery team.

Data collection and definitions

For clinical characterization, demographic and clinical data were collected from medical records. The analysed parameters included age, sex, type and duration of DM, last hemoglobin A1c (HbA1c) (accepted if collected within the last 3 months), current antidiabetic drugs, presence of microvascular (diabetic peripheral neuropathy, diabetic kidney disease, and diabetic retinopathy) and macrovascular disease (ischemic heart disease, cerebrovascular disease), heart failure, tobacco abuse, and risk factors for multidrug-resistant microorganisms (MDROs). The definitions of microvascular and macrovascular complications were based on the guidelines outlined in the *Standards of Care in Diabetes-2024* [8–10]. The risk factors for MDROs included a history of previous antibiotic therapy or hospitalization within the last three months and undergoing hemodialysis [11].

DFU was defined as a full-thickness lesion that was present distal to the malleolus [4]. The severity of the NDFI was assessed using the International Working Group of the Diabetic Foot PEDIS system, which categorizes DFUs into four grades based on perfusion, extension/size, depth/tissue loss, infection, and sensation [12]. Additionally, for NDFI characterization, we recorded the wound location, analytic parameters (C-reactive protein (CPR) and erythrocyte sedimentation rate (ESR) at admission), and magnetic resonance imaging (MRI) scan findings (including osteomyelitis, abscess, and other relevant observations).

Specimens for aerobic culture were collected using four methods: blood culture, biopsy from the base of the ulcer, aspiration of purulent exudate, and intraoperative biopsy (proximal and distal bone samples). These procedures were performed under strict aseptic techniques. Blood cultures, biopsies from the base of the ulcer, and aspiration of the purulent exudate were collected before starting empirical antibiotic therapy. If clinical instability occurred, empirical antimicrobial therapy was started, however, it was stopped 48 h to one week before surgery. For biopsy from the base of the ulcer and for aspiration of the purulent exudate, the nursing team performed necrotic tissue debridement, followed by cleaning of the area with a simple saline solution before sampling [13]. During intraoperative biopsies, tissues were washed with diluted hydrogen peroxide between sample collection, and surgical equipment and materials were replaced [4, 14]. In cases where aseptic collection was not possible or when the patient was at risk (due to insupportable pain induction or the risk of ulcer enlargement), we did not collect samples.

Minor amputation was defined as being distal to the tarsometatarsal joint (i.e., transphalangeal or transmetatarsal amputation of single or multiple digits), whereas major amputation was defined as being proximal to this joint [15]. The described surgical procedure represents the most radical intervention carried out (i.e., if a patient underwent a toe amputation and an above-knee amputation, the surgery type considered was major amputation).

All microbiological isolates were considered, and their susceptibility test results were analysed. Pathogens isolated from more than one sample were counted only once. If a bacterium was isolated multiple times with different susceptibility profiles, the less favourable profile was considered due to the likelihood of antibiotic resistance development.

Considering the frequent use of β-lactams for the treatment of diabetic foot infections, we also attempted to identify which microorganisms exhibit resistance mechanisms to β-lactams, particularly the expression of carbapenemases and AmpC-type and ESBL (extended spectrum β-lactamases) β-lactamases.

Succinctly, carbapenemases are members of the molecular class A, B, and D β-lactamases of the Ambler classification and are capable of inactivating most of the β-lactams, including carbapenems. AmpC β-lactamases and ESBL belong to the class C and A of the Ambler classification, respectively, and can inactivate a broad spectrum of β-lactam antibiotics, including penicillins, cephalosporins, and monobactams; however, ESBLs are especially effective against extended-spectrum cephalosporins, such as cefepime [16].

The clinical microbiology laboratory at our hospital center employs the Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) method for bacterial susceptibility profiling. Based on this profile, agents displaying phenotypic behavior characteristic of carbapenemase or ESBL production are identified. The identification of potentially AmpC-producing strains was performed through a subsequent analysis of susceptibility profiles by the authors.

Statistical analysis

The data analysis was performed from the perspective of admissions and using SPSS Statistics 27® software. Continuous variables with a normal distribution were presented as the mean ± standard deviation (SD), while continuous variables with a nonnormal distribution were expressed as the median and interquartile range (IQR). The normality of the data distribution was examined using skewness and kurtosis, except for the variable 'pack-year', for which we performed the Kolmogorov–Smirnov test of normality. Categorical variables were displayed as frequencies and percentages. To determine the prevalence of antibiotic resistance for each pathogen, only those pathogens with available susceptibility test results were considered. All procedures performed in this study were in accordance with the ethical standards of the institution.

Results

Participants

This study enrolled a total of 60 admissions for the NDFI. The mean age of the participants was 59.1 ± 11.5 years, and 37 (61.7%) were males. Most inpatients were diagnosed with type 2 DM ($n=46$, 76.7%). The average time since DM diagnosis was 22.5 ± 11.9 years, and the mean HbA1c was 77.0 ± 1.00 mmol/mol. Among them, 40 (66.7%) were receiving insulin treatment, with a median total daily insulin dosage of 0.47 (0.00 – 0.92) UI/Kg. Diabetic retinopathy ($n=38$, 64.4%) and diabetic kidney disease ($n=36$, 60.0%) were the most common complications. In 41 (68.3%) patients, at least one risk factor for MDROs was identified. The remaining clinical characteristics are provided in Table 1.

Diabetic foot ulcers

Of the diabetic foot ulcers (DFUs), 37 (61.7%) were categorized as PEDIS 3, while 23 (38.3%) were classified as PEDIS 4. Upon admission, inpatients displayed elevated inflammatory parameters, with a mean CPR of 122.7 ± 88.4 mg/L and a mean ESR of 79.0 ± 29.4 mm/hr. Osteomyelitis was diagnosed in 54 (90.0%) of the inpatients. Abscesses were present in 27 (45.0%) patients, and septic arthritis was diagnosed in 6 (10.0%) patients based on MRI scans. Surgical debridement was performed in 49 (81.7%) inpatients, and the overall amputation rate was 50.0%, with 43.3% of patients having minor amputations and 6.67% having major amputations. Further details can be found in Table 2.

Microbiology

Tissue specimens for microbial analysis were collected from 90.0% of the individuals. An absence of microbial growth was observed in 7 patients (11.7%). Among patients who underwent intraoperative biopsies, 38 (77.5%) had started antibiotic therapy before surgery, with a median antimicrobial exposure time of 6.50 (1.50 – 11.5) days. The antimicrobial regimen was described in the Table Supplementary. Table 3 provides further details on the etiological characteristics of the positive cultures. Biopsies from the base of the ulcer were the most successful at isolating at least one pathogen, achieving a success rate of 96%. However, most pathogens ($n=64$, 62.7%) were isolated from intraoperative biopsies, followed by biopsies from the base of the ulcer ($n=40$,

Table 1 Demographics and clinical characteristics of NDFIs hospitalization ($n=60$) in total of 48 patients

Age (years)	59.1	±11.5
Gender—n (%)		
Male	37	(61.7)
Female	23	(38.3)
Diabetes mellitus – n (%)		
Type 1	9	(15.0)
Type 2	46	(76.7)
Others (Post-transplant diabetes mellitus, MODY, steroid-induced diabetes)	5	(8.30)
Time since diabetes diagnosis (years)[b]	22.5	±11.9
HbA1c (mmol/mol)[c]	77.0	±1.00
On insulin treatment – n (%)	40	(66.7)
TDI (UI/Kg)[c]	0.47	(0.00 – 0.92)
On oral or injectable antidiabetics – n (%)		
Metformin	28	(46.7)
SGLT2i	21	(35.0)
DDP4 inhibitors	15	(25.0)
GLP-1 agonist	12	(20.0)
Sulphonylureas	6	(10.0)
Thiazolidinediones	1	(1.70)
Diabetic kidney disease – n (%)	36	(60.0)
End-stage kidney disease – n (%)	7	(19.4)
Diabetic retinopathy – n (%)[d]	38	(64.4)
Diabetic peripheral neuropathy – n (%)	60	(100)
Ischemic heart disease – n (%)	6	(10.0)
Cerebrovascular disease – n (%)	10	(16.7)
Heart failure – n (%)	9	(15.0)
Active or past tobacco abuse – n (%)[e]	33	(68.9)
Pack-year[f]	25.0	(-18—68)
Risk factors for MDROs – n (%)	41	(68.3)
Previous antibiotic therapy and/or hospitalization in last three months[a]	38	(63.3)
Haemodialysis	3	(5.00)

MODY Maturity-Onset Diabetes of the Young, *HbA1c* Hemoglobin A1C, *TDI* Total daily insulin, *SGLT-2i* Sodium- glucose transport protein 2 inhibitors, *GLP-1* Glucagon-like peptide-1 receptor, *DPP-4* Dipeptidyl Peptidase, *MDROs* Multidrug-resistant microorganisms

[a] Thirty-three (55.0%) patients were on antimicrobials prior to admission for diabetic foot infection

[b] missing: 3 (5.00%)

[c] missing: 1 (2.50%)

[d] missing: 1 (1.67%)

[e] missing: 12 (20.0%)

[f] missing: 6 (18.2%)

Table 2 Clinical characterization of ulcers in NDFIs and surgical management

PEDIS classification – n (%)		
PEDIS 3	37	(61.7)
PEDIS 4	23	(38.3)
Location of ulcer – n (%)[a]		
Toe or interdigital space	33	(55.0)
Plantar surface	17	(25.8)
Dorsal surface	3	(4.50)
Borders of foot	4	(6.10)
Hindfoot	7	(10.6)
Ankle	1	(1.50)
Extension to the leg or thigh	1	(1.50)
C-protein reactive (mg/L)	122.7	±88.4
Erythrocyte sedimentation rate (mm/hr)[b]	79.0	±29.4
MRI scan findings – n (%)		
Osteomyelitis	54	(90.0)
Abscess	27	(45.0)
Brodie abscess	1	(1.70)
Septic arthritis	6	(10.0)
Chronic Charcot foot	4	(6.67)
Surgery – n (%)		
Non-surgical debridement	11	(18.3)
Surgical debridement	14	(23.3)
Surgical debridement and ostectomy	5	(8.30)
Minor amputation	26	(43.3)
Major amputation	4	(6.67)
Time until surgery (days)	10.0	(1.00 – 19.00)
Surgical reintervention – n (%)[c]	17	(34.7)
Postoperative complication or repeat surgical site infection control– n (%)	8	(47.1)

According to the PEDIS system, in Grade 3, ulcers have an area of more than 1 to 3 cm2, involving deeper tissues such as muscle, fascia, or tendon, without the presence of systemic inflammatory response syndrome (SIRS). Patients with grade 4 disease exhibit SIRS [12]

MRI Magnetic resonance imaging

[a] In six (10.0%) patients, there was more than one location of ulcer

[b] missing: 7 (11.7%)

[c] Other reasons for surgical reintervention included two-stage procedures and vascular or plastic surgery. Four (8.89%) patients underwent more than 2 procedures

39.2%) and aspiration of purulent exudate ($n=15$, 14.7%). Bacteremia was found in one patient (1.92%).

A total of 102 pathogens were isolated, resulting in an average of 1.91 ± 1.25 organisms per admission when tissue specimens were collected for microbiology culture. Polymicrobial infection was detected in 32 (53.3%) individuals. The prevalence of the isolated pathogens and their susceptibility profiles are described in Tables 4 and 5, respectively. Among the total isolates, four (3.92%) were fungi, specifically *Candida* spp. Gram-positive bacteria accounted for 62 (60.8%) of the isolates, gram-negative bacilli were identified in 32 (31.4%) of the isolates, and anaerobic bacteria were identified in four (3.92%). *Staphylococcus aureus* ($n=19$, 18.6%) was the most frequently identified pathogen, followed by *Enterococcus faecalis* ($n=17$, 16.7%) and coagulase-negative *Staphylococci* ($n=14$, 13.7%).

Table 3 Characteristics of positive cultures ($n=80$)

Percentage of patients who had a positive culture out of the total number of patients from whom sample could be collected– n (%)		
Blood	1	(1.92)
Biopsy from the ulcer base	24	(96.0)
Aspiration of purulent exudate	10	(90.9)
Intraoperative biopsy – distal bone samples[a]	39	(79.6)
Intraoperative biopsy – proximal bone samples	6	(60.0)
Number of pathogens isolated in each sample – n (%)[b]		
Biopsy from the ulcer base	40	(39.2)
Aspiration of purulent exudate	15	(14.7)
Intraoperative biopsy – distal samples to amputation[1]	64	(62.7)
Intraoperative biopsy – proximal samples to amputation	7	(6.86)
Polymicrobial infection – n (%)[c]	32	(53.3)

A total of 95 tissue specimens for aerobic culture were collected from 54 patients (90%). This included 25 biopsies from the ulcer base, 11 aspirations of purulent exudate, and 60 intraoperative biopsies were performed (comprising 49 distal and 10 proximal samples). More than one sample was collected from 32 patients (53.3%). Blood cultures were not collected in 8 patients (13.3%). At least one microorganism was isolated in 80 specimens (85.1%). No pathogens were isolated in 7 individuals (11.7%)

[a] Includes samples collected intraoperatively when no amputation was performed

[b] One hundred and five distinct pathogens were isolated, among which 21 were found in more than one sample, and three bacteria appeared to have developed resistance in response to antibiotic pressure

[c] missing: 13 (21.7%)

Table 4 Pathogens isolated from inpatients with NDFIs ($n=102$)

Pathogen	Frequency – n (%)	
Gram-positive aerobic bacteria	62	(60.8)
Staphylococcus aureus[a]	19	(18.6)
Enterococcus faecalis	17	(16.7)
Coagulase-Negative *Staphylococcus*[b]	14	(13.7)
β hemolytic *Streptococcus*[c]	8	(7.84)
Corynebacterium spp.[d]	2	(1.96)
Enterococcus faecium	2	(1.96)
Gram-negative aerobic bacteria	32	(31.4)
Pseudomonas aeruginosa	8	(7.84)
Enterobacterales	**24**	**(23.5)**
Enterobacter cloacae	7	(6.86)
Klebsiella pneumoniae	6	(5.88)
Proteus mirabilis	5	(4.90)
Morganella morganii	2	(1.96)
Escherichia coli	2	(1.96)
Providencia rettgeri	1	(0.98)
Serratia marcescens	1	(0.98)
Anaerobic bacteria[e]	4	(3.92)
Fungi	4	(3.92)
Candida parapsilosis	3	(2.94)
Candida albicans	1	(0.98)

[a] In the case of bacteremia, a single strain of Staphylococcus aureus was isolated

[b] Included *Staphylococcus capitis*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus lentus*, and *Staphylococcus hominis*

[c] Including *Streptococcus agalactiae*, *Streptococcus anginosus*¸ and *Streptococcus constellatus*

[d] Including *Corynebacterium tuberculostearicum* and *Corynebacterium jeikeium*

[e] Included *Fusobacterium varium*, *Bacteroides fragilis*, *Gardanella vaginallis*, and *Actinobaculum schaalii*

Among the gram-negative bacteria, *Pseudomonas aeruginosa* was the most prevalent, with eight (7.84%) isolates. Twenty-four (23.5%) bacteria were isolated from the *Enterobacterales* family, with *Enterobacter cloacae* ($n=7$, 6.86%) being the most frequently isolated agent.

Susceptibility pattern

Three (15.8%) *Staphylococcus aureus* and eight (66.7%) coagulase-negative *Staphylococci* isolates were methicillin resistant (MRSA). The susceptibility of these agents to methicillin was inferred by their susceptibility to oxacillin. Among the patients from whom methicillin-resistant *Staphylococcus* spp. were isolated, two (18.2%) had no risk factors for MDROs (such as a history of previous antibiotic therapy or hospitalization within the last three months and who were undergoing hemodialysis). Resistance to clindamycin was identified in seven (36.8%) *Staphylococcus aureus* isolates and 2 (16.7%) coagulase-negative *Staphylococci* isolates. Furthermore, two (10.5%) *Enterococcus* spp. were ampicillin resistant, both of which were sensitive to vancomycin. Additionally, both patients exhibited risk factors for MDROs. None of the isolated strains of *Pseudomonas aeruginosa* were resistant to piperacillin-tazobactam, ceftazidime or cefepime. Among the *Pseudomonas aeruginosa* isolates, one (25.0%) was resistant to carbapenems. Among the *Enterobacterales* isolates, 17 (85.0%) were resistant to amoxicillin/clavulanic acid, nine (37.5%) were resistant to ceftazidime, eight (34.8%) were resistant to piperacillin-tazobactam, five (20.8%) were resistant to cefepime, and three (13.0%) were resistant to carbapenems. Extended-spectrum β-lactamases (ESBL), *Klebsiella pneumoniae* carbapenemases (KPC), and AmpC β-lactamases were found in one (4.17%), four (16.7%), and four (17.4%) of the isolated *Enterobacterales*, respectively. These bacteria were isolated from patients who displayed risk factors for MDROs.

Discussion

This study investigated the microbiology of NDFI and detailed and essential data pertaining to the etiological pathogens responsible for NDFI and their corresponding susceptibility profiles in our population. This pivotal prevalence study contributes to the understanding of local epidemiology and provides guidance for empirical antibiotic therapy in NDFI patients.

Table 5 Pathogen resistance profile

A. Gram-positive aerobic bacteria						
Resistance – n (%)	*Staphylococcus spp.*				*Enterococcus spp.*	
	Staphylococus aureus		Coagulase-Negative Staphylococus[a]			
Ampicillin	-		-		2	(10.5)
Oxacillin	3	(15.8)	8	(66.7)	-	
Clindamycin	7	(36.8)	2	(16.7)	-	
Tetracycline	3	(15.8)	4	(33.3)	-	
Co-trimoxazole	1	(5.26)	4	(33.3)	-	
Vancomycin	-			-	0	(0.00)
B. Gram-negative aerobic bacteria						
Resistance – n (%)	*Pseudomonas aeruginosa*[b]		*Enterobacterales*[c]			
Amoxicillin clavulanic acid	-		17	(85.0)		
Ceftazidime	0	(0.00)	9	(37.5)		
Cefepime	0	(0.00)	5	(20.8)		
Aztreonam	0	(0.00)	-			
Imipenem / meropenem	1	(25.0)	3	(13.0)		
Piperacillin tazobactam	0	(0.00)	8	(34.8)		
Quinolones	1	(14.3)	5	(20.8)		
Co-trimoxazole	-		7	(30.4)		
ESBL	0	(0.00)	1	(4.17)		
KPC	0	(0.00)	4	(16.7)		
AmpC β-lactamases	-		4	(17.4)		

ESBL Extended-spectrum β-lactamases, *KPC* Klebsiella pneumoniae carbapenemases

[a] missing: oxacillin, clindamycin, tetracycline, co-trimoxazole – 2 (14.3%)

[b] missing: cefepime, imipenem/meropenem – 4 (50.0%), quinolones – 1 (12.5%)

[c] missing: amoxicillin clavulanic acid—4 (16.7%), imipenem/meropenem, piperacillin tazobactam, co-trimoxazole, ESBL, AmpC β-lactamases – 1 (4.20%)

The sociodemographic and clinical data related to DM in our population overlapped with previous national and international studies [5, 17–20], showing that NDFI is more prevalent in men and tends to affect professionally active individuals. In our cohort, the prevalence of ischemic heart disease [5, 17, 20] was lower, as expected given our focus on the NDFI. The high prevalence of both microvascular and macrovascular diseases reflects poor diabetes control and prolonged disease duration [21, 22], contributing to increased immunosuppression and compromised wound healing [3, 4].

Our findings underscore the severity of NDFI within our patient population, with 38.3% of cases displaying systemic repercussions according to the PEDIS classification. Furthermore, elevated inflammatory parameters, namely, osteomyelitis, abscess, and septic arthritis, were present in 90.0%, 46.7%, and 10.0% of the patients, respectively, underscoring the strength of the situation. These findings highlight a greater prevalence of bone involvement in this study than in other studies (50–65%) [3, 5, 18, 23, 24]. MRI, the most sensitive modality (95.6%) and more specific than plain radiography used in other reports (80.7% vs. 68.0%) [3], was employed for osteomyelitis diagnosis in our study.

In contrast to previous studies [5, 17, 18, 25–27], this research exclusively relied on deep tissue and bone sampling techniques for microbiological diagnosis. Tissue specimens are considered the gold standard [4], offering greater sensitivity and specificity in causative pathogen identification while excluding colonizers or contaminants [4, 28]. Although most pathogens were isolated from intraoperative biopsies, the proportion of positive cultures was lower than that of preoperative samples. This difference may be attributed to the initiation of empirical antibiotic therapy prior to surgery in some patients, owing to the severity of the NDFI and the extended period until surgery. This approach could hinder pathogen identification. To minimize the influence on microbiological findings, we discontinue antibiotics for a period before obtaining intraoperative biopsies. The duration of antibiotic therapy might be crucial; one study reported that administering antibiotics for fewer than seven days did not affect culture yield [29].

Importantly, among the collected proximal bone samples, 60% yielded positive cultures, indicating persistent bone infection after amputation. Although findings on this matter remain controversial, this persistence might correlate with poorer outcomes, including postoperative

complications and readmissions [4, 30]. Conversely, two studies showed a significantly lower rate of positive histology results in the distal specimen compared to microbiological results, supporting the possibility of false positive residual bone cultures [31, 32].

It is crucial to acknowledge that not every identified agent may have an active role as a potential pathogen, especially for coagulase-negative *Staphylococcus*, *β-hemolytic Streptococcus*, and *Corynebacterium spp*., which are known skin commensals [14]. Furthermore, *Gardenerella vaginalis* was identified as a contamination case. Nevertheless, the collection of all specimens from deep tissues under aseptic conditions reinforced our confidence that the isolates were true pathogens. Aerobic gram-positive cocci usually lead to monomicrobial infections in acute, untreated cases, while polymicrobial infections commonly involve gram-positive *cocci*, *Enterobacterales*, *Pseudomonas*, and anaerobes, especially in chronic or deep wounds [16, 17, 27, 28]. *Enterococcus* spp. are considered commensal but assume pathogenic roles in patients with diabetes, especially chronic ulcers [5]. Although a correlation between prior antibiotic exposure and isolated microorganisms could not be established due to the small sample size, our clinical practice suggests the predominant isolation of gram-negative bacilli in MDRO-risk patients. Similarly, with other studies, the prevalence of anaerobes might be underestimated due to challenges in isolation by routine clinical microbiology laboratories [26].

Our findings are in line with previously reported results [5, 17–20, 25–27], where gram-positive bacteria prevailed. A recent review analysed the global epidemiology of diabetic foot infection, and *Staphylococcus aureus* remained the most common pathogen (11–46%), particularly in Western countries [23]. The incidence of gram-negative bacteria has surged, especially in Asia, and gram-negative bacteria are becoming the most frequently isolated pathogen. The worldwide prevalence of *Pseudomonas aeruginosa* ranges from 10 to 26.6% [23].

Two additional bacterial surveys were conducted in Portugal, where *Staphylococcus aureus* prevailed (19.7 – 21.8%) [5, 17]. In the most recent study, a significant proportion of the isolates were gram-negative bacteria (48.9%), particularly from the *Enterobacterales* family (30.6%) [17]. Our study contrasts with these reports, which revealed a greater prevalence of coagulase-negative *Staphylococcus* (4.37%), whereas certain gram-negative bacteria, such as *Pseudomonas aeruginosa* (13.7%), were more common [17]. The incidences of *Enterococcus spp.* (8.84%), β-hemolytic *Streptococcus* (4.08%), and *Enterobacterales* (10.9%) were lower, while the incidence of *Corynebacterium spp.* (8.16%) was higher in another survey [5].

Compared with both international and national data, our observed MRSA prevalence was lower (15.8%). National surveys reported MRSA proportions ranging from 41.7 to 53.1% [5, 17], while recent European surveys reported MRSA proportions ranging from 24.7–27.1% [18, 32]. Globally, the incidence of MRSA has been steadily increasing and ranges from 16 to 44% [19]. Limited information exists on coagulase-negative *Staphylococci* methicillin resistance, but our survey indicated a significant rate (66.7%). This rate remained below that of a middle-income country (91.8%) [19], exceeding that of another high-income country (55.4%) [32], and that of a national survey (27.2%) [5]. *Enterococcus spp.* displayed notable susceptibility to ampicillin (10.5%), which is consistent with other international reports (2.00 – 17.4%) [19, 33, 34] and is attributed to the minority of *Enterococcus faecium* isolates [19]. A Portuguese study reported increased ampicillin resistance (33.3%) [34]. Notably, no vancomycin-resistant *Enterococcus* (VRE) strains were detected. The prevalence of VRE among *Enterococcus spp*. ranges from 0.00% to 7.69% [5, 17, 18, 32–36] in previous reports.

These disparities can be attributed to several factors: in several studies, peripheral arterial disease was identified as a risk factor for MDROs [23]. This study analysed the microbiology of NDFI. Additionally, other national studies occurred in reference centres where most complex cases are transferred, likely presenting identified risk factors for MDROs, such as previous hospitalization, amputation, and antibiotic exposure [23, 37]. National data predate 2018; in recent years, the incidence of MRSA has been declining due to the implementation of hospital infection control measures [37]. In our centre, we only used empirical broad-spectrum antibiotics in patients with risk factors for MDROs. This approach to antibiotic use may contribute to a lower prevalence of resistance [5, 17].

Regarding gram-negative bacterial susceptibility, our study revealed a favourable profile, particularly concerning fluoroquinolone resistance. This rate was lower than that reported in most international and national studies (*Pseudomonas aeruginosa*: 14.3% vs. 18.2–57.3% [17–19, 23, 32, 36]; *Enterobacterales*: 20.8% vs. 40.0–50.0% [19, 23, 32]). This divergence might stem from global fluoroquinolone overuse in recent decades, despite declining prescriptions in last years [38]. Our centre avoids fluoroquinolones for empirical therapy in both outpatient and inpatient settings. The significant prevalence of resistance to amoxicillin-clavulanic acid among *Enterobacterales* was likely due to these patients exhibiting risk factors for MDROs. The imipenem or meropenem resistance rates (*Pseudomonas aeruginosa*: 25.0%; *Enterobacterales*: 20.8%) were concerning. The prevalence of *Pseudomonas*

was similar to that in other southern European countries (9.1–23.5%) [30, 34] but greater than that in northern European countries (5.4%) [15]. In Asia, resistance rates are as high as 55% [20, 36]. *Enterobacterales* rates approach those reported in Asia (13.4–16.5%) [20, 27, 37, 38]. In other Western countries, the resistance rate was substantially lower (1.73–7.5%) [20, 31, 34]. Our ESBL prevalence matches that of national studies (4.17% vs. 2.40–6,25% [5, 17]); however, it was lower than that of other European reports (20.0–29.8% [32, 39]). Conversely, our lower KPC incidence aligns with the findings of national and European studies (16.7% vs. 1.79–5.00% [17, 32]). Additionally, the prevalence of ESBL, KPC and AmpC β-lactamases in our study was considerably lower than that in Asiatic series, reaching 50.0% [23, 29].

Our study has limitations that must be acknowledged. First, due to its retrospective nature, some crucial data, including the duration and regimen of antibiotic therapy before admission, were missing from medical records. Information about previous instances of NDFI, hospitalizations for NDFI, amputations, the healthcare setting before admission (community or hospital), adverse events during hospitalization, histology of distal bone samples, and outcomes after discharge was not available. MRI is valuable for detecting osteomyelitis, yet histological and microbiological methods are the gold standard. Even though our samples were collected from deep tissues, it is important to note that the assumption that all isolated pathogens were causative agents of NDFI might not be entirely accurate, as previously mentioned. Specimen collection before empirical antibiotic therapy is ideal.

Our study's strength lies in the comprehensive description of resistance profiles among NDFI pathogens. To our knowledge, this is the most detailed study conducted in Portugal, offering important insights. All cultures were obtained through purulent aspiration and deep tissue or bone biopsies under aseptic techniques, reducing contamination risk and clarifying the causative pathogens of NDFI. Given the variations in epidemiology among different centers, understanding the local epidemiology and resistance pattern is crucial for tailored empirical antibiotic therapy protocols. Our findings suggest considering a higher dose of amoxicillin-clavulanic acid for empirical antibiotic therapy in patients without risk factors for MDROs. This selection, which offers effective bone penetration [40] and coverage against gram-positive aerobes and anaerobes, could be crucial. However, it may fail to cover gram-negative bacteria. For patients with risk factors for MDROs, we suggest vancomycin along with piperacillin tazobactam or ceftazidime. This combination would provide coverage against MRSA, coagulase-negative methicillin-resistant *Staphylococcus*, and gram-negative bacteria. Nonetheless, it is important to note that approximately 34.8% of the *Enterobacterales* were not covered, underscoring the necessity of obtaining specimens as early as possible for guiding antibiotic therapy decisions.

In conclusion, local microbiology analysis proves invaluable in establishing antimicrobial stewardship practices and selecting empiric antibiotic therapy critical for managing severe infections with the potential for amputation and disability, particularly among working individuals. Timely and appropriate interventions have the potential to enhance quality of life and reduce morbidity and mortality in affected individuals. Hence, continued vigilance in this domain is imperative for improving outcomes in managing NDFI.

Abbreviations

DM	Diabetes mellitus
DFU	Diabetic foot ulcer
NDFI	Neuropathic diabetic foot infection
HbA1c	Hemoglobin A1c
MDROs	Multidrug-resistant microorganisms
SIRS	Systemic inflammatory response syndrome
CPR	C-reactive protein
ESR	Erythrocyte sedimentation rate
MRI	Magnetic resonance imaging
ESBL	Extended-spectrum β-lactamases
MALDI-TOF MS	Matrix-assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry
SD	Standard deviation
IQR	Interquartile range
MRSA	Methicillin-resistant *Staphylococcus aureus*
KPC	*Klebsiella pneumoniae* Carbapenemases
VRE	Vancomycin-resistant *Enterococcus*

Supplementary Information

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Supplementary Material 1: Table Supplementary. Antibiotic therapy regimen started before surgery in 38 (77.5%) patients who underwent intraoperative biopsies.

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Authors' contributions
Conceptualization, J.G. and A.G.; methodology, J.G. and A.G.; software, J.G. and A.G.; validation, J.P. N.N., R.S.S. L.S. and J.Q.; formal analysis, J.G. and A.G.; investigation, J.G., A.G., H.U.F., S.R., T.M., M.B.C., I.M., P.F., N.N., R.S.S.; data curation, J.G. and A.G.; writing original draft preparation, J.G. and A.G.; writing, review and editing, M.B.C., F.S., J.P., N.N., R.S.S., L.S. and J.Q.; visualization, all authors; supervision, J.P., N.N., L.S. and J.Q. All authors have read and agreed to the published version of the manuscript. J.G. and A.G. are considered co-first authors and contributed equally to this work.

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Availability of data and materials
The dataset for this study is available from the corresponding author upon reasonable request and ethical approval.

Declarations

Ethics approval and consent to participate

This study was performed in accordance with the principles of the Declaration of Helsinki. Ethical approval was obtained from the ethics committee of Unidade Local de Saúde de São João (CES 330/23). For this type of study (retrospective and observational), formal consent was not needed.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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