ORIGINAL ARTICLE

IWJ WILEY

The contribution of intestinal *Streptococcus* to the pathogenesis of diabetic foot ulcers: An analysis based on 16S rRNA sequencing

Yunyang Wang[1] | Hong Zhang[2] | Guixin Ma[3] | Zibin Tian[4] | Bin Wang[3]

[1]Department of Endocrinology and Metabolism, the Affiliated Hospital of Qingdao University, Qingdao, Shandong, China

[2]School of Public Health, Qingdao University, Qingdao, Shandong, China

[3]School of Basic Medicine, Qingdao University, Qingdao, Shandong, China

[4]Department of Gastroenterology, the Affiliated Hospital of Qingdao University, Qingdao, Shandong, China

Correspondence
Zibin Tian, Department of Gastroenterology, the Affiliated Hospital of Qingdao University, No, 16, Jiangsu Road, Qingdao 266000, Shandong Province, China.
Email: tianzbsun@163.com

Bin Wang, School of Basic Medicine, Qingdao University, No. 308, Ningxia Road, Qingdao 266071, Shandong Province, China.
Email: wangbin532@126.com

Abstract

In this study, we intend to determine the microbial communities that are differentially expressed in diabetic foot ulcers (DFUs) from the view of species abundance difference and compositions. The EMBL-EBI database and QIIME2 platform were used to obtain and process 16S rRNA sequencing data of normal healthy and DFU samples. The LEfSe software was utilised to retrieve key intestinal bacteria differentially expressed in DFUs. Additionally, PICRUSt2, FAPROTAX and BugBase functional analyses were performed to analyse the potential microbial functions and related metabolic pathways. The correlations between intestinal microbiota and clinical indexes were evaluated using the Spearman correlation analysis. Significant differences existed in intestinal microbiota between DFU and normal healthy samples regarding species abundance difference and compositions at Kingdom, Phylum, Class, Order, Family, Genus and Species levels. Seven microbiota were demonstrated differentially expressed in DFUs that contained *Bacteroidaceae*, *Prevotellaceae*, *Streptococcaceae*, *Lactobacillales*, *Bacilli*, *Veillonellaceae* and *Selenomonadales*. Insulin signalling pathway may be the key pathway related to the functional significance of *Streptococcus* and *Bacillus* in the DFUs. The intestinal microbiota in DFUs exhibited susceptibility to sulphur cycling while displaying pathogenic potential. Last but not least, a close relationship between *Streptococcus* and the occurrence of DFUs was revealed. Taken together, this study mainly demonstrated the high abundance of *Streptococcus* in DFUs and its correlation with the disease occurrence.

KEYWORDS

16S rRNA sequencing, *Bacillus*, diabetic foot ulcers, gut microbiota, insulin pathway, *Streptococcus*

Key Points
- High abundance of gut microbiota is determined in diabetic foot ulcer samples
- 7 kinds of key bacteria are differentially expressed in diabetic foot ulcers
- *Streptococci* and *Bacillus* involve in the diabetic foot ulcers *via* insulin pathway
- *Streptococcus* is closely related to the occurrence of diabetic foot ulcers
- This study suggests the possible bacteria contributing to diabetic foot ulcers

1 | INTRODUCTION

Diabetic foot ulcers (DFUs) are identified as one of many complications induced by poorly controlled diabetes.[1] It occurs in approximately 30% of patients with diabetes.[2] Neuropathy and trauma, as well as concurrent peripheral artery occlusive disease, are thought to be implicated in the pathophysiology of DFUs.[3] Recommended wound care protocols for DFUs include adequate blood supply, control of infection, debridement and wound management.[4] Unfortunately, DFUs may lead to considerable suffering and multiple recurrences and correlate with a high death rate and high treatment expenditure, which impose a substantial burden to health care.[5,6] It is estimated that mortality rates related to the progression of a DFU is 5% in the first 12 months and 42% within 5 years.[7] In this context, it is of significance to further discover and understand the possible pathogenesis of DFUs in order for better management.

The gut microbiota, a complex ecological group, exert a variety of crucial roles within a host.[8] It has been unfolded that intestinal microbiota is related to insulin resistance a through controlling chronic subclinical inflammation.[9] Strikingly, a diversity of bacterial communities have been identified in DFUs.[10] Notably, microbiota have been highlighted to produce an impact on therapeutic interventions and clinical prognosis of chronic DFUs.[11] Sequencing of bacterial 16S rRNA has identified bacteria including *Streptococcus*, *Staphylococcus* and *anaerobes* to be a main part of the microbiome in many chronic wounds such as DFUs.[12] It has also been found that the abundance of faecal microbiota including *Streptococcus* shares a positive correlation with body mass index and can regulate the insulin sensitivity and its secretion in overweight/obese adults.[13] A previous study has shown that the main differences in bacterial species between DFU samples and normal healthy samples were Proteobacteria, Streptococcus, Anaerobes and Staphylococcus, but only Staphylococcus and *Streptococcus* were closely associated with clinical symptoms of patients with DFU.[14] A 16S rRNA gene-based metagenomic analysis including 122 wound (100 diabetic and 22 non-diabetic) samples has indicated that the Gram-negative microbes were more abundant in the wound microbiome.[15] Staphylococcus, Streptococcus, Anaerobes and Proteobacteria have been referred to as functionally equivalent pathogroups, which may coaggregate symbiotically in a pathogenic biofilm and act synergistically to cause a chronic wound infection.[16] In addition, Price et al. have also revealed that *Streptococcus* was more prevalent in patients with chronic wounds.[17] However, the understanding about the structure and composition of gut microbiota in DFUs is insufficient yet.

Insulin pathway is a conserved one that plays an important role in controlling biological processes in organisms.[18] These biological processes may involve growth and development, resistance to stress and metabolic homoeostasis, etc.[19] Interestingly, the activation of insulin signalling in keratinocytes underlies the suppressive role of ganglioside GM3 depletion in diabetic wound healing.[20] Taking the above-mentioned findings into consideration, we thus aimed to explore the structure and distribution of gut microbiota in DFUs and to identify the possible mechanisms underlying their role in the pathogenesis of this disease.

2 | MATERIALS AND METHODS

2.1 | Public data retrieval

The items related to the 'diabetic foot ulcer' phenotype were retrieved through the EMBL-EBI database (https://www.ebi.ac.uk/ena/browser/search), and the phenotypic information of samples for all items were downloaded (biological item No. PRJNA287759). Through the NCBI Sequence Read Archive (SRA) database (https://www.ncbi.nlm.nih.gov/sra/), 16S rRNA sequencing data of item samples were obtained. In total, 115 DFU samples and 221 normal healthy samples were included. The project samples were from the University of Pennsylvania, Microbiota colonising plantar DFUs were collected every 2 weeks. Sample details were shown in Table S1. All analyses in this study were carried out using R version 3.6.3.

2.2 | Sequencing data analysis

The FASTQC software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was utilised to assess the quality of the raw reads. After the reads containing adapters and low-quality reads were removed from raw sequences, the BBDuk program in the BBTools software suite (BBMap – Bushnell B.– sourceforge.net/projects/bbmap/) was applied for cleaning (ktrim = r; k = 23; mink = 11; hdist =1; tpe tbo qtrim = rl' trimq = 10). In order to obtain the optimum parameters of non-chimeric data, an optimisation table was generated for paired- and single-end reads for each primer set.

The QIIME2 pipeline (version 2020.2, http://qiime2.org) was used to merge and process the 337 raw sequence datasets at a uniform standard, which could achieve quality control and sequence read analysis.[21] DADA2 was utilised to denoise demultiplexed, single-end sequence data. Finally, taxonomic classification against the Greengenes database v13_8 (http://greengenes.secongenome.com) was performed using Naïve Bayesian classifier.

2.3 | α and β diversity analyses

α and β diversity analyses were performed at a sampling depth of 12 480. β diversity was analysed utilising the UniFrac distance. An unsupervised grouping pattern of the microbiome was visualised using principle coordinate analysis (PCoA), a dimension-reducing approach that can illustrate the correlation between samples based upon a distance matrix. Selected microbiome-associated information can be depicted as a clear separation or a tendency by colouring samples in PCoA.

2.4 | Differential abundance analysis

Differential taxa between groups were identified via the linear discriminant analysis effect size (LEfSe), an algorithm to find high-dimensional biological marker which can determine metagenomic characteristics featuring differences between at least two biological conditions.[22] Coupling standard tests were performed to determine statistical significance and additional tests conducted to encode biological consistency and effect size, after which LEfSe analysis was utilised to identify the characteristics most probably to decipher the differences between the classes. With the linear discriminant analysis (LDA) threshold of 4 in the training cohort, each of the differential characteristics determined by LEfSe was scored using LDA, where a higher LDA score indicated a greater difference in terms of characteristics between the groups.

2.5 | Kyoto encyclopedia of genes and genomes pathway analysis

R package was utilised for transforming QIIME data, and the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) software was applied to perform KEGG for predicting metagenome pathways for each primer set.[23,24] Comparisons of metabolic pathways were visualised through Statistical Analysis of Metagenomic Profiles (STAMP).[25]

2.6 | Functional annotation of prokaryotic taxa and BugBase

On the basis of the generated sequencing data, gene functions were annotated against the Functional annotation of prokaryotic taxa (FAPROTAX) dataset (http://www.loucalab.com/archive/FAPROTAX/; accessed on August 17, 2020).[26] FAPROTAX dataset, a manually established database mapping prokaryotic taxa (e.g., genera or species), was used to predict the major ecological functions of the microbiota. Phenotypes of the gut microbiota were predicted utilising the BugBase tool. Additionally, BugBase was applied for characterising the relative representation of microbiome features based on six categories: Gram staining, oxygen tolerance, capability to form biofilms, content of mobile element, pathogenicity, as well as oxidative stress tolerance.

2.7 | Spearman correlation analysis

Spearman correlation coefficient was utilised to produce a correlation network, followed by filtering of edges based on the false discovery rate adjusted P-value. Finally, Corrplot package in the R software was utilised for heatmap analysis.

3 | RESULTS

3.1 | Significant differences in gut microbiota between DFU and normal healthy samples

Differences in gut microbiota between DFU and normal healthy samples were compared in the current study. Firstly, we analysed the α diversity in gut microbiota of 115 DFU samples and 220 normal healthy samples. No significant difference was noted in gut microbiota between DFU and normal healthy samples on the basis of the α diversity index (Kruskal-Wallis H = 0.7794,
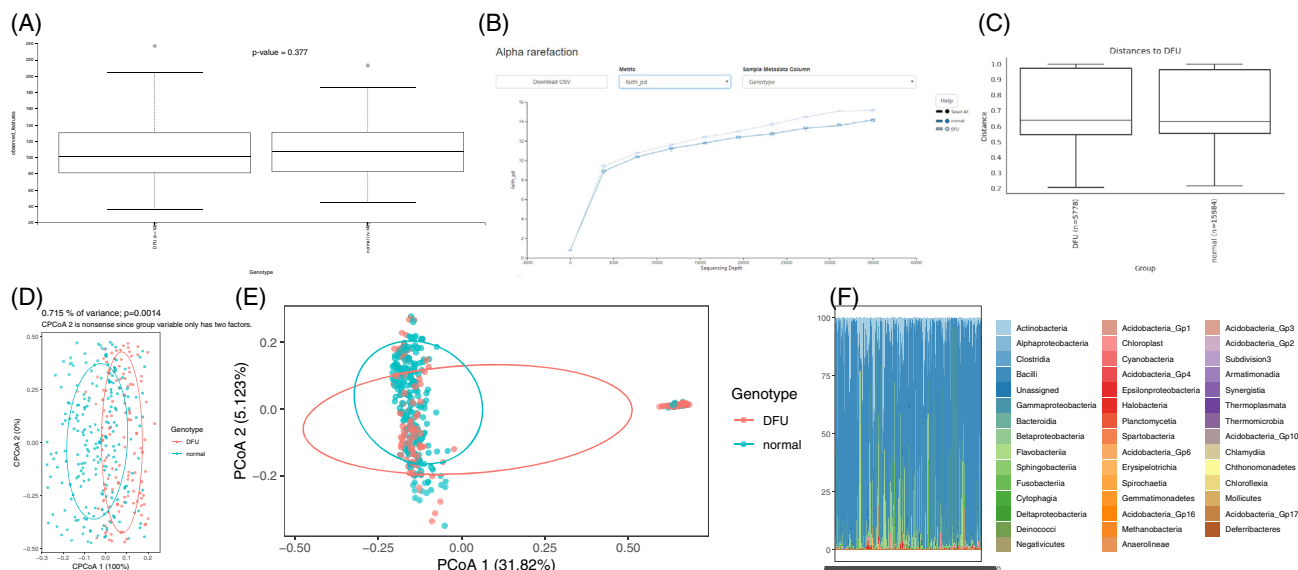
FIGURE 1 Significant differences in gut microbiota between diabetic foot ulcer (DFU) and normal healthy samples. A, Kruskal-Wallis H test results of gut microbiota in DFU and normal healthy samples. B, The α rarefaction curve for gut microbiota in DFU and normal healthy samples. C, The β diversity (unweighted unifrac β index) in DFU and normal healthy samples. D, The CPCoA plot for DFU and normal healthy samples. E, The PCoA plot for DFU and normal healthy samples. F, Species composition analysis of DFU and normal healthy samples

$P = 0.3773$) (Figure 1A). In addition, the results of α rarefaction curve revealed markedly higher abundance of gut microbiota in DFU samples than in normal healthy samples (Figure 1B).

We further compared the β diversity between DFU and normal healthy samples. According to unweighted unifrac β index, the abundance of gut microbiota in DFU samples was shown to be appreciably higher than that in normal healthy samples ($P = 0.001$) (Figure 1C). Based on the results of CPCoA plot and PCoA plot, a significant difference was detected in gut microbiota between DFU samples and normal healthy samples (Figure 1D, E). As depicted in Figure 1F, a notable difference was determined in terms of species composition between DFU and normal healthy samples.

3.2 | Significant differences in specie abundance and composition between DFU and normal healthy samples

Meanwhile, differential analysis of bacterial abundance in DFU and normal healthy samples was conducted at the kingdom level, and it was shown that the abundance of bacteria and archaea in normal healthy samples was higher than that in DFU samples (Figure 2A, Figure S1A).

Additionally, the difference in terms of bacterial abundance between DFU and normal healthy samples was analysed at the phylum level. *Firmicutes*, *Fusobacteria*, *Cyanobacteria*, *Bacteroidetes* and *Acidobacteria* in DFU

samples exhibited markedly higher abundance versus normal healthy samples (Figure 2B, Figure S1B).

The differential analysis of bacterial abundance at the class level showed the considerably higher abundance of *Clostridia*, *Fusobacteria*, *Bacteroidia*, *Chloroplast*, *Bacilli*, *Negativicutes* and *Acidobacteria Gp2* in DFU samples as compared to that of normal healthy samples (Figure 2C; Figure S1C).

According to differential analysis of bacterial abundance at order level, *Lactobacillales*, *Enterobacteriales*, *Selenomonadales*, *Clostridiales*, *Fusobacteriales*, *Bacteroidales*, *Bacillales* and *Xanthomonadales* showed remarkably higher abundance in DFU samples than in normal healthy samples (Figure 2D; Figure S1D).

The differential analysis in regard to bacterial abundance at family level displayed markedly higher abundance of *Clostridiales Incertae Sedis XI*, *Streptococcaceae*, *Enterobacteriaceae*, *Peptoniphilaceae*, *Prevotellaceae*, *Porphyromonadaceae* and *Fusobacteriaceae* in DFU samples than in normal healthy samples (Figure 2E; Figure S1E).

As displayed in Figure 2F and Figure S1F, the abundance of *Finegoldia*, *Pantoea*, *Lactococcus*, *Anaerococcus*, *Fusobacterium*, *Porphyromonas*, *Helcococcus* and *Streptococcus* was observed to be significantly higher in DFU samples than in normal healthy samples at genus level.

Furthermore, at species level, *Finegoldia magna*, *Pantoea rwandensis*, *Staphylococcus capitis subsp. capitis*, *Anaerococcus murdochii*, *Lactococcus taiwanensis*, *Anaerococcus vaginalis*, *Staphylococcus schleiferi subsp.*
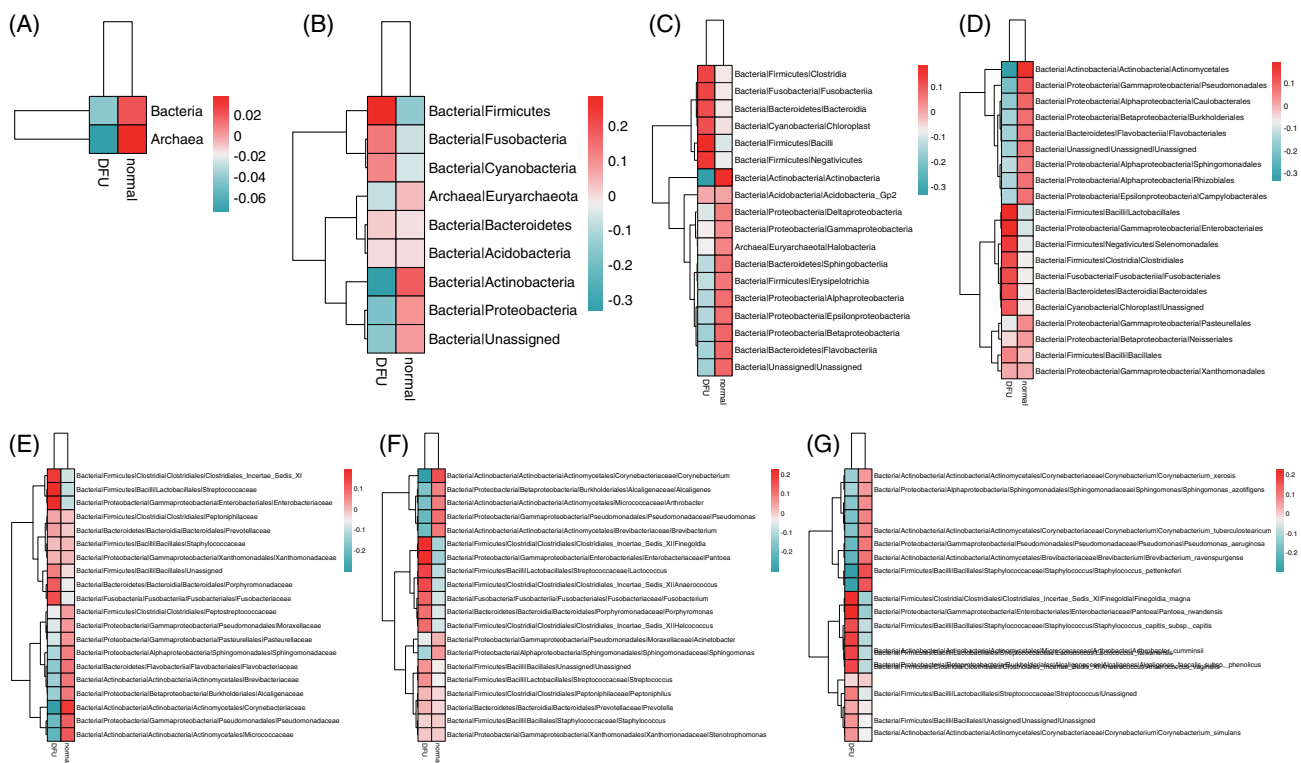
FIGURE 2 Significant differences in specie abundance between diabetic foot ulcer (DFU) and normal healthy samples. A, Kingdom levels of gut microbiota regarding specie abundance. B, Phylum levels of gut microbiota regarding specie abundance. C, Class levels of gut microbiota regarding specie abundance. D, Order levels of gut microbiota regarding specie abundance. E, Family levels of gut microbiota regarding specie abundance. F, Genus levels of gut microbiota regarding specie abundance. G, Species levels of gut microbiota regarding specie abundance

Schleiferi and *Streptococcus agalactiae* had notably higher abundance in DFU samples than in normal healthy samples (Figure 2G; Figure S1G).

Taken together, significant differences are presented in both species abundance and composition between DFU and normal healthy samples at the kingdom, phylum, class, order, family, genus and species levels.

3.3 | Seven differentially expressed microbiota in DFU samples

LEfSe software analysis showed noticeably higher abundance of *Bacteroidaceae*, *Prevotellaceae*, *Streptococcaceae*, *Lactobacillales*, *Bacilli*, *Veillonellaceae* and *Selenomonadales* in DFU samples than that in normal control samples (Figure 3A,B).

3.4 | *Streptococci* and *Bacillus* might participate in the pathogenesis of DFUs through modulation of the insulin pathway

Subsequently, we performed KEGG enrichment analysis of gut microbiota using the PICRUSt2 software. As

shown in Figure 4A,B, these gut microbiota were mainly enriched in carbon fixation in photosynthetic organisms, starch and sucrose metabolism, insulin signalling pathway, D-alanine metabolism, Huntington's disease, homologous recombination, peptidoglycan biosynthesis, biosynthesis of ansamycins, ribosome, Novobiocin biosynthesis and aminoacyl-tRNA biosynthesis. Among the aforementioned items, only the insulin signalling pathway has been reported to correlate with the repair diabetic wounds.[27] The contribution of various differentially expressed bacteria to the insulin pathway was further analysed using the PICRUSt2 software. The results displayed in Figure 4C demonstrated that *Streptococcus* and *Bacillus* were mainly involved in the insulin pathway. Hence, *Streptococcus* and *Bacillus* might be implicated in the pathogenesis of DFUs via regulating the insulin pathway.

3.5 | Gut microbiota in DFUs are susceptible to sulphur cycling

We further predicted the microbial community function using the FAPROTAX software. As demonstrated in Figure 5A, the gut microbiota in DFU samples
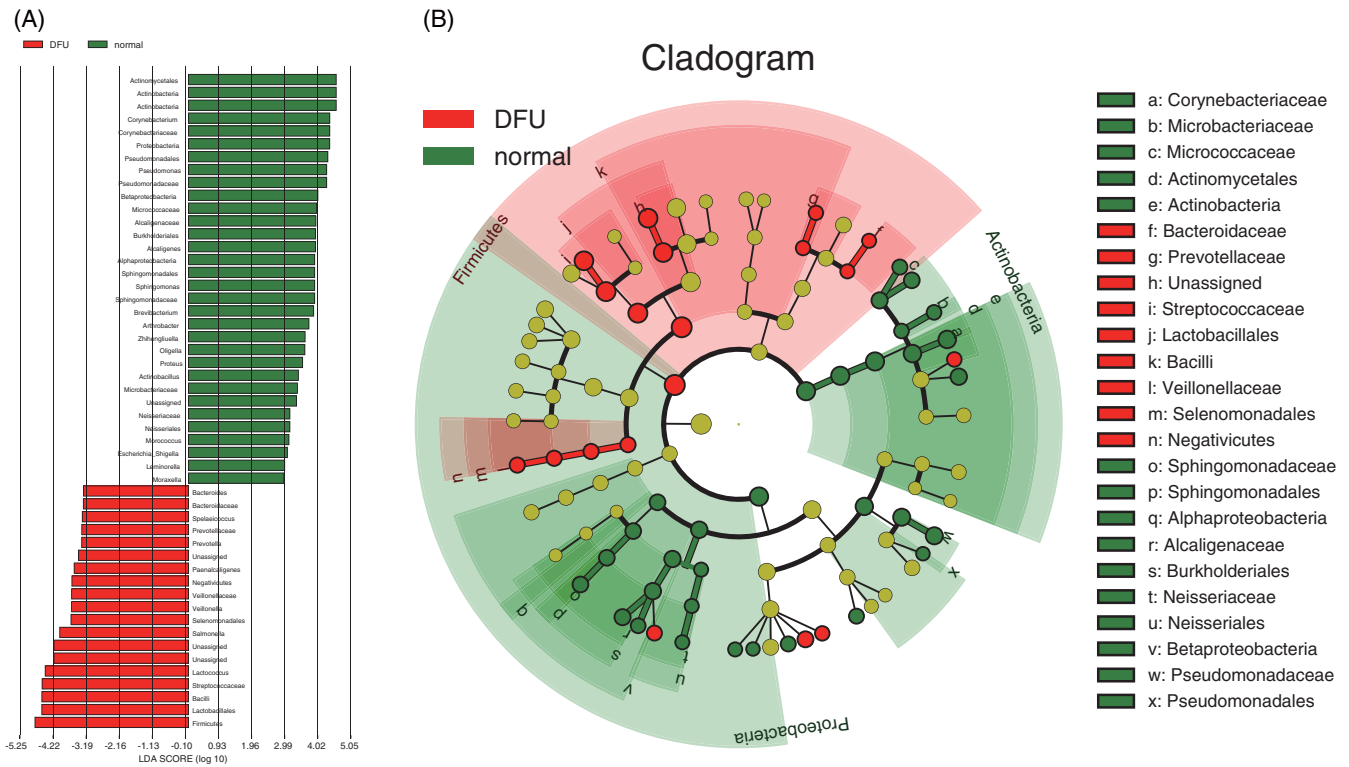
FIGURE 3 Seven differentially expressed microbiota in (DFU) samples. A, The histogram depicting linear discriminant analysis (LDA) distribution of species abundance in DFU and normal healthy samples. B, The classification evolving branch diagram of species abundance in DFU and normal healthy samples
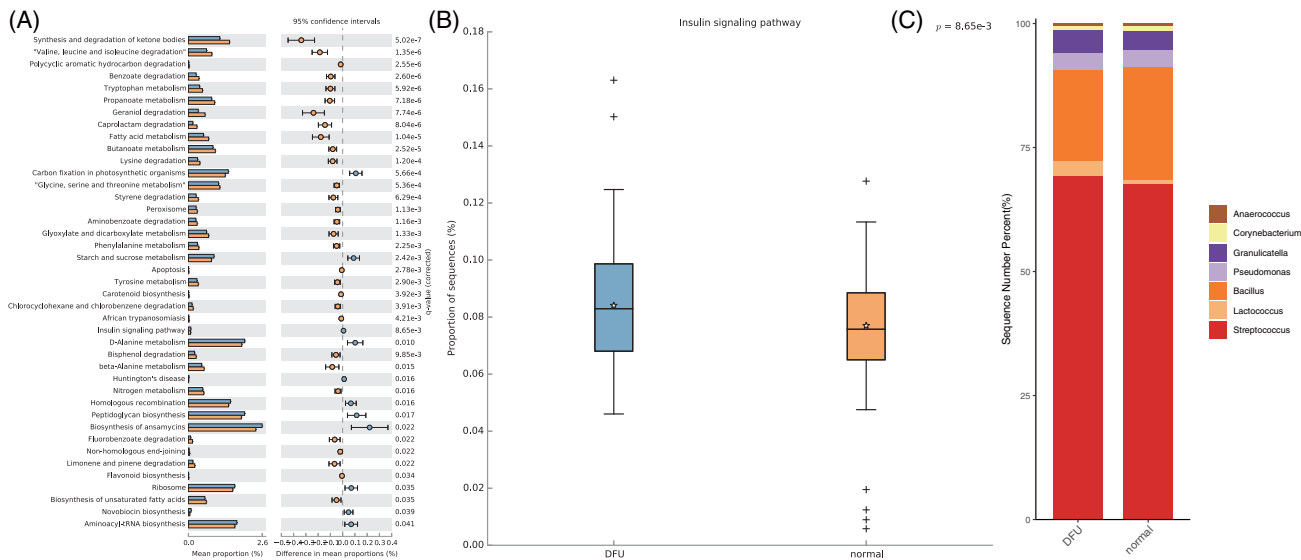
FIGURE 4 *Streptococcus* and Bacillus may participate in the pathogenesis of diabetic foot ulcers (DFUs) through controlling the insulin pathway. A, KEGG function analysis of gut microbiota in DFU and normal healthy samples. B, The difference in the insulin signalling pathway between DFU and normal healthy samples. C, The contribution of the differentially expressed microbiota to the insulin pathway

and normal healthy samples were mainly related to the cycling of sulphur elements. The gut microbiota in DFU samples are principally engaged in in thiosulphate respiration, sulphite respiration and respiration of sulphur compounds (Figure 5B-D). The above-mentioned results suggest the susceptibility of gut microbiota related to DFUs to the cycling of sulphur elements.

(A)

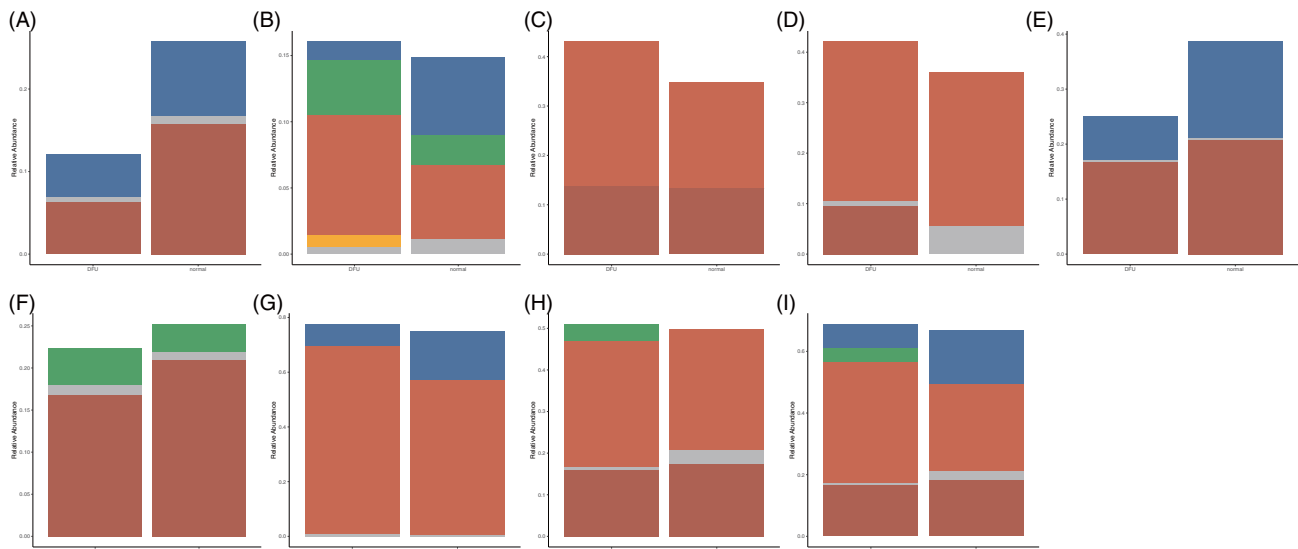**(B)**

(C)

(D)
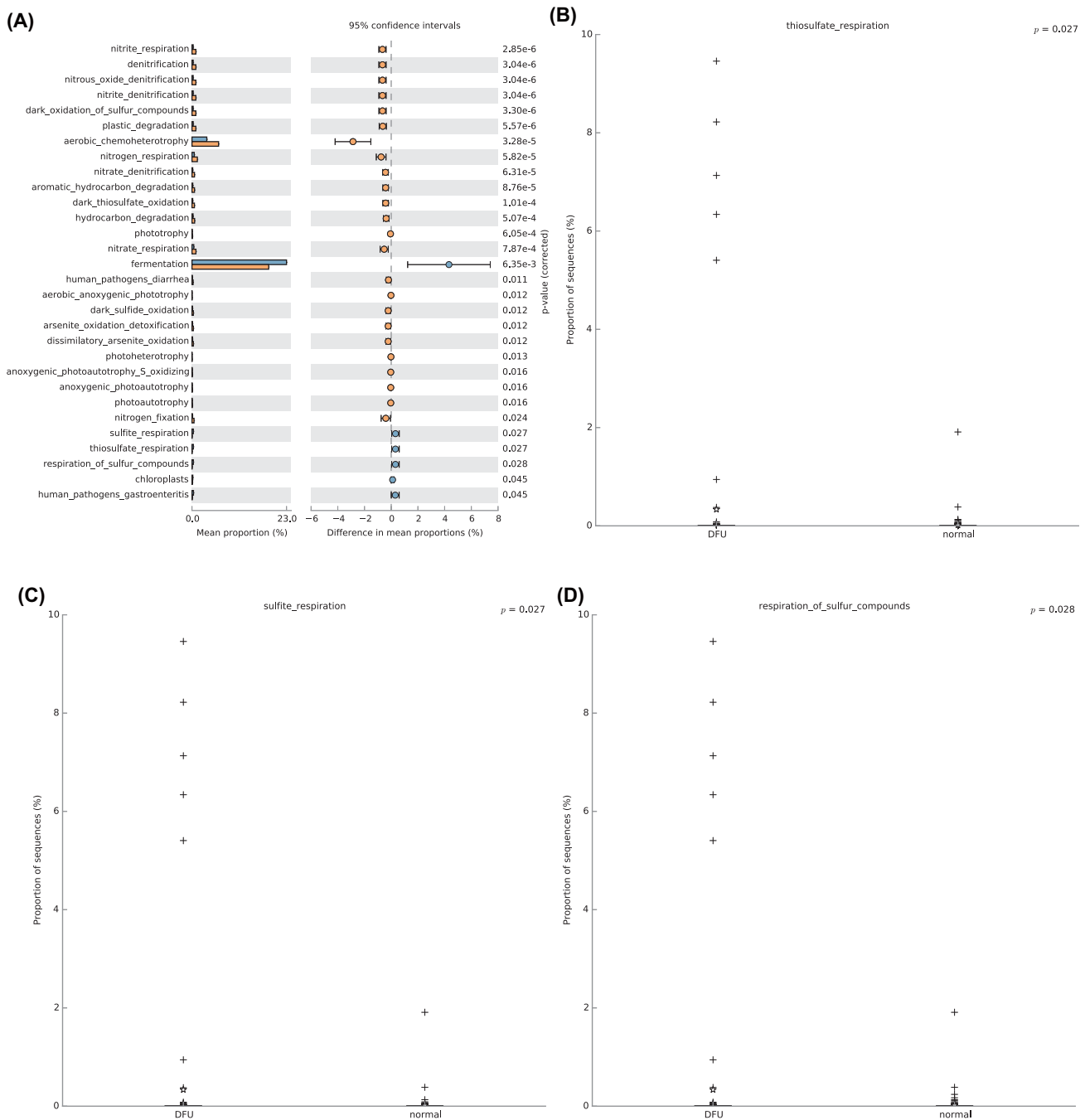
FIGURE 5 Ecological functions of gut microbiota in diabetic foot ulcers (DFUs). A, Prediction of the ecological functions of gut microbiota in DFU and normal healthy samples. B, The difference in thiosulphate respiration between DFU and normal healthy samples. C, The difference in sulphite respiration in DFU and normal healthy samples. D, The difference in respiration of sulphur compounds in DFU and normal healthy samples

3.6 | Gut microbiota in DFUs showed more pathogenic potential

Furthermore, we predicted phenotypes of gut microbiota through the BUGBASE software. As shown in Figure 6A-I, DFUs were mainly involved in anaerobic, biofilm formation, Gram-positive bacteria, pathogenic potential and oxidative stress tolerance.

3.7 | *Streptococcus* was closely related to the occurrence of DFUs

We analysed the correlations between gut microbiota of DFU samples and normal healthy samples. As illustrated in Figure 7A, correlations existed in between every two microbiota that included *Staphylococcus*, *Lactococcus*, *Corynebacterium*, *Streptococcus*, *Anaerococcus*, *Brevibacterium*,

FIGURE 6 Pathogenic potential of gut microbiota in diabetic foot ulcers (DFUs). A, Aerobic. B, Anaerobic. C, Contains mobile elements. D, Facultatively anaerobic. E, Forms biofilms. F, Gram-negative. G, Gram-positive. H, Potentially pathogenic. I, Stress tolerant
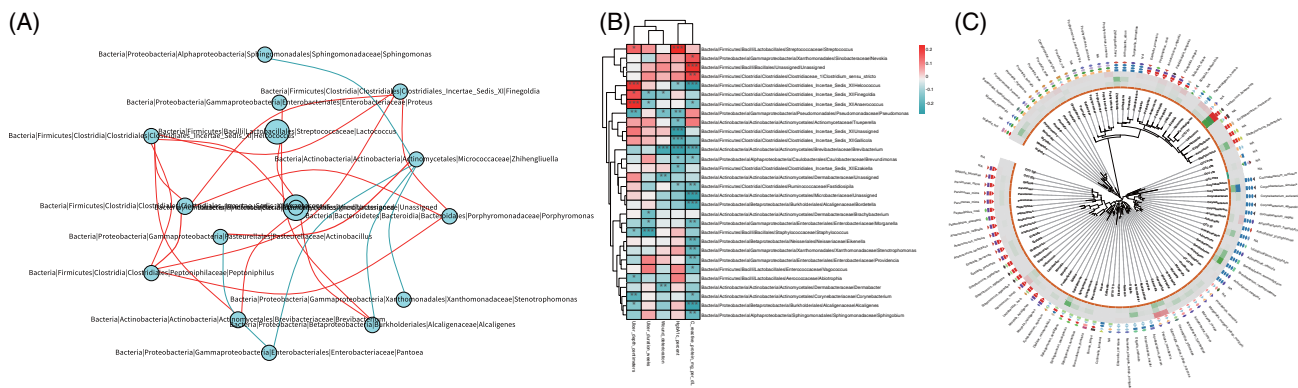
FIGURE 7 Correlations of gut microbiota with the occurrence of diabetic foot ulcers (DFUs). A, Correlation analysis of different microbiota between DFU and normal healthy samples. B, The correlations between microbiota and clinical indicators in DFU samples and normal healthy samples. C, The phylogenetic tree of gut microbiota in DFU samples and normal healthy samples

Acinetobacter, Alcaligenes, Porphyromonas, Finegoldia, Sphingomonas, Helcococcus, Arthrobacter, Peptoniphilus, Pantoea, Stenotrophomonas, Fusobacterium, Prevotella, Salmonella, Peptostreptococcus, Zhihengliuella, Proteus, Actinobacillus, Weeksella, Oligella, Bacteroides and *Veillonella*.

We further analysed the correlations between the microbiota and clinical indicators (HgbA1c percent, ulcer duration weeks, ulcer depth centimetres, wound deterioration and C reactive protein mg per dL). *Streptococcus* showed a strong positive correlation with these clinical indicators (Figure 7B). In addition, we constructed a phylogenetic tree of these differentially expressed microbiota, which also showed high abundance of *Streptococcus* in DFU samples (Figure 7C).

4 | DISCUSSION

DFUs pose a steadily growing worldwide burden, the outcomes of which rely strongly in social determinants of health; effective therapy for DFUs is complicated that requires huge expenditure of resources and large cost to the medical system.[28] In the current study, we set out to explore the significance of microbiota in DFUs, and our results mainly revealed high abundance of *Streptococcus* in the DFUs and its contribution to the pathology of DFUs through the insulin pathway.

Initially, our study found considerably higher abundance of gut microbiota in DFU samples in comparison to that in normal healthy ones, with notable differences found at the species abundance levels and compositions.
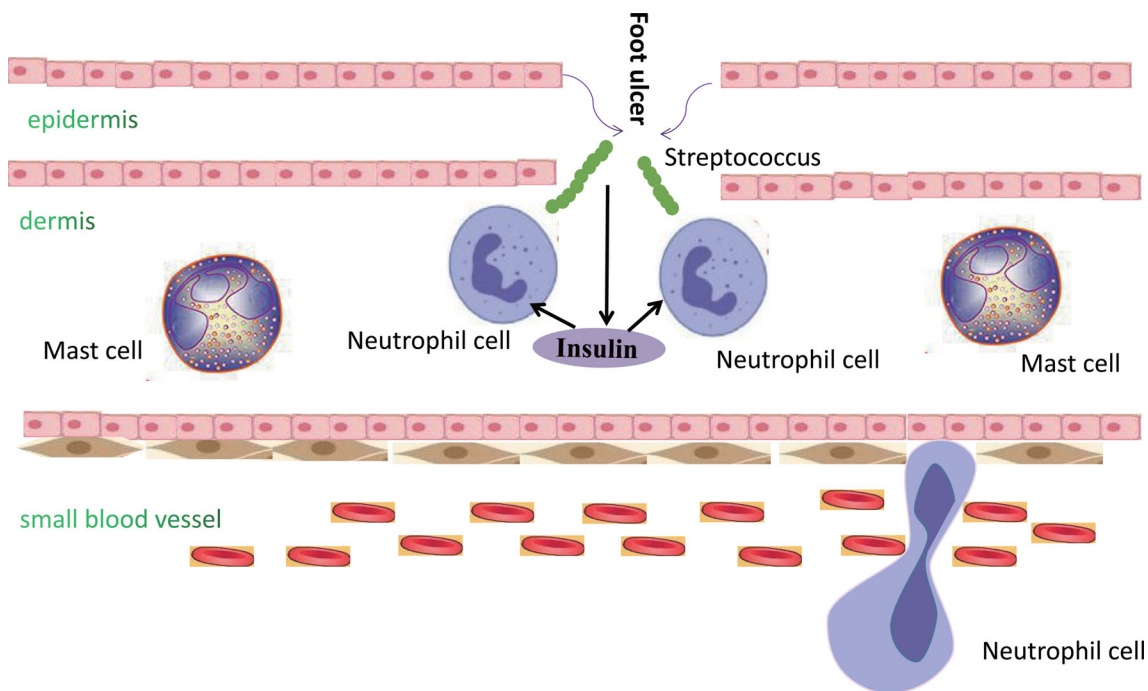
FIGURE 8 The molecular mechanism depicting the role of *Streptococcus* in diabetic foot ulcers (DFUs). *Streptococcus* may participate in the pathogenesis of DFUs through mediating the insulin pathway

More specifically, based on the results from our LEfSe analysis, *Bacteroidaceae*, *Prevotellaceae*, Streptococcaceae, *Lactobacillales*, *Bacilli*, *Veillonellaceae* and *Selenomonadales* showed high abundance in DFUs, with susceptibility to sulphur cycling. The interaction between sulphur cycling and intestinal microbiome has been highlighted in a previous study that sulphate-reducing bacteria principally support hydrogen sulphide generation and heterotrophic bacteria-evoked enzymatic desulphhydration of cysteine can also lead to the generation of hydrogen sulphide.[29] Amino acids-containing sulphur has been illustrated to have potential to interfere diabetes and its relevant complications.[30] An increasing number of reports have highlighted the significance of gut microbiota in diabetic complications including DFUs. Of note, wound microbial communities and the degree of their pathogenicity can determine the healing and non-healing nature of DFUs.[31] The microbial communities play an important role in DFUs that affect the complications ranging from superficial cellulitis to chronic osteomyelitis or even gangrenous extremity lower limb amputations.[32] Intestinal microbiota imbalance could affect diabetic nephropathy-induced kidney injuries by activating the intrarenal renin–angiotensin system.[33] Intriguingly, from the results of metagenome sequencing in a study by Zou et al., the microbial diversity and the abundance of the microbial functional genes were higher in diabetic foot osteomyelitis specimens than in post-traumatic foot osteomyelitis specimens, with *Firmicutes*, *Prevotellaceae* and *Prevotella* being the most abundant microbes.[34]

In addition, we revealed in the current study that *Streptococci* and *Bacillus* may participate in the development of DFUs through insulin pathway. Furthermore, we demonstrated the close relation of *Streptococcus* to the occurrence of DFUs. Strikingly, *Streptococcus agalactiae* was observed in shorter duration DFUs.[35] Moreover, *Streptococcus* spp. contributes to positive association of Firmicutes with the relative abundance and ulcer duration.[36] Besides, according to analyses of microbial community conducted by Gardner et al., poor glycaemic control in DFU clusters is associated with the richness in *Streptococcus*.[14] Previous studies have also found the involvement of *Bacillus* in diabetes and DFUs. For instance, Gram-negative *bacillus Escherichia coli* can be frequently detected in DFUs.[37] Additionally, *Bacteriocin* from *Bacillus subtilis* is suggested to be a potential drug against the bacterial pathogens in DFUs.[38] Although these findings have provided information for the significance of *Streptococci* and *Bacillus* in DFUs, the pathways related their significance are yet to be identified. It is known that the insulin pathway is a pivotal regulator in the development of diabetes.[39] Additionally, endothelial insulin/IGF signalling could regulate diabetes-related impaired wound healing through affecting neo-angiogenesis.[40] Intriguingly, an existing study has unfolded that the reduced abundance of genera *Streptococcus* by berberine could aid in alleviating insulin resistance.[41] The insulin receptor signalling mediated by extracellular SQSTM1 could mediate *Streptococcus pneumoniae*-induced septic death in mice.[42]

Taken together, the results obtained in the study demonstrated that high abundance of *Streptococcus* in DFU samples along with its functional significance in the pathogenesis of DFUs which may be associated with the insulin pathway (Figure 8). This finding may provide a novel direction for understanding the pathogenesis of DFUs. However, further studies are required for validation in cell models, animal experiments and clinical trials.

REFERENCES

1. Grennan D. Diabetic foot ulcers. *JAMA*. 2019;321(1):114. doi:10.1001/jama.2018.18323
2. Chang M, Nguyen TT. Strategy for treatment of infected diabetic foot ulcers. *Acc Chem Res*. 2021;54(5):1080-1093. doi:10.1021/acs.accounts.0c00864
3. Bandyk DF. The diabetic foot: pathophysiology, evaluation, and treatment. *Semin Vasc Surg*. 2018;31(2–4):43-48. doi:10.1053/j.semvascsurg.2019.02.001
4. Pehde CE, Bennett J, Kingston M. Orthoplastic approach for surgical treatment of diabetic foot ulcers. *Clin Podiatr Med Surg*. 2020;37(2):215-230. doi:10.1016/j.cpm.2019.12.001
5. Rice JB, Desai U, Cummings AK, Birnbaum HG, Skornicki M, Parsons NB. Burden of diabetic foot ulcers for medicare and private insurers. *Diabetes Care*. 2014;37(3):651-658. doi:10.2337/dc13-2176
6. Jeffcoate WJ, Vileikyte L, Boyko EJ, Armstrong DG, Boulton AJM. Current challenges and opportunities in the prevention and Management of Diabetic Foot Ulcers. *Diabetes Care*. 2018;41(4):645-652. doi:10.2337/dc17-1836
7. Everett E, Mathioudakis N. Update on management of diabetic foot ulcers. *Ann N Y Acad Sci*. 2018;1411(1):153-165. doi:10.1111/nyas.13569
8. Fan P, Nelson CD, Driver JD, Elzo MA, Penagaricano F, Jeong KC. Host genetics exerts lifelong effects upon hindgut microbiota and its association with bovine growth and immunity. *ISME J*. 2021;15(8):2306-2321. doi:10.1038/s41396-021-00925-x
9. Saad MJ, Santos A, Prada PO. Linking gut microbiota and inflammation to obesity and insulin resistance. *Physiology (Bethesda)*. 2016;31(4):283-293. doi:10.1152/physiol.00041.2015
10. Sadeghpour Heravi F, Zakrzewski M, Vickery K, GA D, Hu H. Bacterial diversity of diabetic foot ulcers: current status and future Prospectives. *J Clin Med*. 2019;8(11):1935.
11. Kalan LR, Meisel JS, Loesche MA, et al. Strain- and species-level variation in the microbiome of diabetic wounds is associated with clinical outcomes and therapeutic efficacy. *Cell Host Microbe*. 2019; 25(5):641-655 e645. doi:10.1016/j.chom.2019.03.006
12. Xu Z, Hsia HC. The impact of microbial communities on wound healing: a review. *Ann Plast Surg*. 2018;81(1):113-123. doi:10.1097/SAP.0000000000001450
13. Naderpoor N, Mousa A, Gomez-Arango LF, Barrett HL, Dekker Nitert M, de Courten B. Faecal microbiota are related to insulin sensitivity and secretion in overweight or obese adults. *J Clin Med*. 2019;8(4):452.
14. Gardner SE, Hillis SL, Heilmann K, Segre JA, Grice EA. The neuropathic diabetic foot ulcer microbiome is associated with clinical factors. *Diabetes*. 2013;62(3):923-930. doi:10.2337/db12-0771
15. Jnana A, Muthuraman V, Varghese VK, et al. Microbial community distribution and Core microbiome in successive wound grades of individuals with diabetic foot ulcers. *Appl Environ Microbiol*. 2020;86(6):e02608–e02619.
16. Lipsky BA, Richard JL, Lavigne JP. Diabetic foot ulcer microbiome: one small step for molecular microbiology ... One giant leap for understanding diabetic foot ulcers? *Diabetes*. 2013; 62(3):679-681. doi:10.2337/db12-1325
17. Price LB, Liu CM, Melendez JH, et al. Community analysis of chronic wound bacteria using 16S rRNA gene-based pyrosequencing: impact of diabetes and antibiotics on chronic wound microbiota. *PLoS ONE*. 2009;4(7):e6462. doi:10.1371/journal.pone.0006462
18. Kong Y, Liu H, Li W, Wang D. Intestine-specific activity of insulin signaling pathway in response to microgravity stress in Caenorhabditis elegans. *Biochem Biophys Res Commun*. 2019; 517(2):278-284. doi:10.1016/j.bbrc.2019.07.067
19. Sadagurski M, White MF. Integrating metabolism and longevity through insulin and IGF1 signaling. *Endocrinol Metab Clin N Am*. 2013;42(1):127-148. doi:10.1016/j.ecl.2012.11.008
20. Wang XQ, Lee S, Wilson H, et al. Ganglioside GM3 depletion reverses impaired wound healing in diabetic mice by activating IGF-1 and insulin receptors. *J Invest Dermatol*. 2014;134(5): 1446-1455. doi:10.1038/jid.2013.532
21. Bolyen E, Rideout JR, Dillon MR, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol*. 2019;37(8):852-857. doi:10.1038/s41587-019-0209-9
22. Lozupone C, Lladser ME, Knights D, Stombaugh J, Knight R. UniFrac: an effective distance metric for microbial community comparison. *ISME J*. 2011;5(2):169-172. doi:10.1038/ismej.2010.133
23. Kanehisa M, Sato Y, Kawashima M, Furumichi M, Tanabe M. KEGG as a reference resource for gene and protein annotation. *Nucleic Acids Res*. 2016;44(D1):D457-D462. doi:10.1093/nar/gkv1070
24. Douglas GM, Maffei VJ, Zaneveld JR, et al. PICRUSt2 for prediction of metagenome functions. *Nat Biotechnol*. 2020;38(6): 685-688. doi:10.1038/s41587-020-0548-6
25. Parks DH, Tyson GW, Hugenholtz P, Beiko RG. STAMP: statistical analysis of taxonomic and functional profiles. *Bioinformatics*. 2014;30(21):3123-3124. doi:10.1093/bioinformatics/btu494
26. Louca S, Parfrey LW, Doebeli M. Decoupling function and taxonomy in the global ocean microbiome. *Science*. 2016; 353(6305):1272-1277. doi:10.1126/science.aaf4507
27. Dam DHM, Wang XQ, Sheu S, et al. Ganglioside GM3 mediates glucose-induced suppression of IGF-1 receptor-Rac1 activation to inhibit keratinocyte motility. *J Invest Dermatol*. 2017; 137(2):440-448. doi:10.1016/j.jid.2016.09.028
28. Sorber R, Abularrage CJ. Diabetic foot ulcers: epidemiology and the role of multidisciplinary care teams. *Semin Vasc Surg*. 2021;34(1):47-53. doi:10.1053/j.semvascsurg.2021.02.006

29. Barton LL, Ritz NL, Fauque GD, Lin HC. Sulfur cycling and the intestinal microbiome. *Dig Dis Sci.* 2017;62(9):2241-2257. doi:10.1007/s10620-017-4689-5

30. Manna P, Das J, Sil PC. Role of sulfur containing amino acids as an adjuvant therapy in the prevention of diabetes and its associated complications. *Curr Diabetes Rev.* 2013;9(3):237-248. doi:10.2174/1573399811309030005

31. Noor S, Zubair M, Ahmad J. Diabetic foot ulcer–a review on pathophysiology, classification and microbial etiology. *Diabetes Metab Syndr.* 2015;9(3):192-199. doi:10.1016/j.dsx.2015.04.007

32. Pitocco D, Spanu T, Di Leo M, et al. Diabetic foot infections: a comprehensive overview. *Eur Rev Med Pharmacol Sci.* 2019; 23(2 Suppl):26-37. doi:10.26355/eurrev_201904_17471

33. Lu CC, Hu ZB, Wang R, et al. Gut microbiota dysbiosis-induced activation of the intrarenal renin-angiotensin system is involved in kidney injuries in rat diabetic nephropathy. *Acta Pharmacol Sin.* 2020;41(8):1111-1118. doi:10.1038/s41401-019-0326-5

34. Zou M, Cai Y, Hu P, et al. Analysis of the composition and functions of the microbiome in diabetic foot osteomyelitis based on 16S rRNA and metagenome sequencing technology. *Diabetes.* 2020;69(11):2423-2439. doi:10.2337/db20-0503

35. Malone M, Johani K, Jensen SO, et al. Next generation DNA sequencing of tissues from infected diabetic foot ulcers. *EBioMedicine.* 2017;21:142-149. doi:10.1016/j.ebiom.2017.06.026

36. Candel Gonzalez FJ, Alramadan M, Matesanz M, et al. Infections in diabetic foot ulcers. *Eur J Intern Med.* 2003;14(5): 341-343. doi:10.1016/s0953-6205(03)00107-9

37. Wang P, Jiang S, Li Y, et al. Virus-like mesoporous silica-coated plasmonic ag nanocube with strong bacteria adhesion for diabetic wound ulcer healing. *Nanomedicine.* 2021;34:102381.

38. Joseph B, Dhas B, Hena V, Raj J. Bacteriocin from Bacillus subtilis as a novel drug against diabetic foot ulcer bacterial pathogens. *Asian Pac J Trop Biomed.* 2013;3(12):942-946. doi:10.1016/S2221-1691(13)60183-5

39. Rains JL, Jain SK. Oxidative stress, insulin signaling, and diabetes. *Free Radic Biol Med.* 2011;50(5):567-575. doi:10.1016/j.freeradbiomed.2010.12.006

40. Aghdam SY, Eming SA, Willenborg S, et al. Vascular endothelial insulin/IGF-1 signaling controls skin wound vascularization. *Biochem Biophys Res Commun.* 2012;421(2):197-202. doi:10.1016/j.bbrc.2012.03.134

41. Yue SJ, Liu J, Wang AT, et al. Berberine alleviates insulin resistance by reducing peripheral branched-chain amino acids. *Am J Physiol Endocrinol Metab.* 2019;316(1):E73-E85. doi:10.1152/ajpendo.00256.2018

42. Zhou B, Liu J, Zeng L, et al. Extracellular SQSTM1 mediates bacterial septic death in mice through insulin receptor signalling. *Nat Microbiol.* 2020;5(12):1576-1587. doi:10.1038/s41564-020-00795-7

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.