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A metagenomics approach to characterize the footrot microbiome in Merino sheep

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ABSTRACT

In the Portuguese Alentejo region, Merino sheep breed is the most common breed, reared for the production of meat, dairy, and wool. Footrot is responsible for lameness, decreased animal welfare, and higher production losses, generating a negative economic impact. The disease is caused by *Dichelobacter nodosus* that interacts with the sheep foot microbiome, to date largely uncharacterized. In fact, *Dichelobacter nodosus* is not able to induce footrot by itself being required the presence of a second pathogen known as *Fusobacterium necrophorum*. To understand and characterize the footrot microbiome dynamics of different footrot lesion scores, a whole metagenome sequencing (WMGS) approach was used. Foot tissue samples were collected from 212 animals with different degrees of footrot lesion scores, ranging from 0 to 5. Distinct bacterial communities were associated with feet with different footrot scores identifying a total of 63 phyla and 504 families. As the severity of footrot infection increases the microorganisms' diversity decreases triggering a shift in the composition of the microbiome from a dominant gram-positive in mild stages to a dominant gram-negative in the severe stages. Several species previously associated with footrot and other polymicrobial diseases affecting the epidermis and provoking inflammatory responses such as *Treponema* spp., *Staphylococcus* spp., *Streptococcus* spp. and *Campylobacter* spp. were identified proliferating along with the lesions' severity. Although these bacteria are not able to initiate footrot, several evidences have been described supporting their association with the severity and incidence increase of footrot lesions caused by *Dichelobacter nodosus* and *Fusobacterium necrophorum*. Further investigation is required to establish the roles of particular taxa and identify which of them play a role in the disease process and which are opportunistic pathogens.

1. Introduction

Ovine footrot is a contagious disease caused primarily by *Dichelobacter nodosus (D. nodosus)*, an anaerobic gram-negative bacterium (Beveridge, 1941), being the main cause of lameness affecting sheep and other livestock animals worldwide (Zanolari et al., 2021). Footrot affects the interdigital skin and hooves, being a welfare and economic concern for the wool, milk and meat industries. Footrot disease is classified in two different clinical presentations: Interdigital Dermatitis (ID) which is characterized by the inflammation of the interdigital epidermis,

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including or not the underrunning footrot, and the severe form of the disease, denoted as Severe Footrot (SFR). In SFR, the separation of the hoof horn from the sensitive underlying tissue occurs, resulting in necrotizing lesions of the interdigital skin accompanied by a characteristic fetid odor leading to lameness (Zanolari et al., 2021).

Footrot is a multi-factorial, polymicrobial and complex disease which depends on different factors such as host susceptibility, farm management, environmental conditions, virulence of *D. nodosus* (which is known to be conferred by the presence of the aprV2 gene, coding for the thermostable AprV2 protein) and the presence of co-infecting bacteria like *Fusobacterium necrophorum* (*F. necrophorum)* (Zanolari et al., 2021). *Fusobacterium necrophorum* is another anaerobic bacterium which is known to be involved in the persistence and severity of footrot development, playing a role as an opportunistic, secondary pathogen. The synergistic relationship between *D. nodosus* and other microorganisms such as *F. necrophorum* is not clear (Zanolari et al., 2021). The bacterial community diversity observed in the sheep hooves' with footrot makes the identification of the different prevalence of taxa and its contribution to the development and expression of footrot a challenging task.

Since the mid-20th century most of the available information regarding the bacterial etiology of the ovine footrot was obtained from classical microbiological techniques, a labour-intensive cultured-based approach, which limitations are associated to a limited number of bacteria than can be cultured, and histological sections observation (Beveridge, 1941; Egerton et al., 1969). However, rapid advances in the next-generation sequencing (NGS) field as based marker-gene (16 S rDNA gene) and whole metagenome sequencing (WMGS) has enabled new insights in the research of polymicrobial diseases like ovine footrot (Calvo-Bado et al., 2011; Maboni et al., 2017; McPherson et al., 2019; Clifton et al., 2022). Despite 16 S rDNA gene sequencing is able to provide rapid information about the taxonomic composition of microbial communities, the main disadvantage of this technique is the limited amount of information produced, for instance, about metabolic pathways and functional capabilities. WMGS overcomes these limitations being able to obtain deeper insights about the functional capabilities, metabolic pathways, novel genes, host-microbiota interactions and co-evolution, offering a great specificity of identification and representation of diversity in the microbiomes (Durazzi et al., 2021). To our knowledge, the etiology of ovine footrot in the Portuguese Merino breeds and crossbreed has not yet been investigated using WMGS. However, several studies of footrot affecting different breeds of sheep have been conducted using other approaches in Australia and United Kingdom (Calvo-Bado et al., 2011; Maboni et al., 2017; McPherson et al., 2019; Clifton et al., 2022). Moreover, the role of bacterial diversity, its load and how that differs between the healthy and footrot-affected sheep feet remains unclear.

In this context, the aim of this study was to characterize the bacterial communities present on the feet of healthy and footrot-affected Merino sheep, grouped by footrot score, using WMGS. With this approach, it was intended to determine which prevalence of the different bacteria are represented in each footrot score lesions and could contribute to the development of the disease on these sheep breeds in Portugal.

2. Material and methods

a. Sample collection, DNA extraction, and sequencing

Interdigital skin punch biopsies were collected, under local anesthesia (Lidocaine, Anestesin®), from 212 sheep using disposable sterile Biopsy Punches (6 mm diameter) within seventeen flocks of White Merino and Black Merino breeds and Merino crossbreed, from different geographical locations in the Portuguese Alentejo region (Supplementary Table S1). Those flocks were randomly selected and examined between January 2017 and June 2018 for clinical diagnosis of footrot infection. Following the Modified Egerton System (scores from 0 to 5) the sheep feet lesions were scored and registered for each animal. The scores 1 and 2 correspond to ID and the scores 3, 4 and 5 to SFR. Samples were immediately frozen with liquid nitrogen and kept at − 20 ºC until being processed. Total DNA was extracted, from up to 25 mg of each 212 biopsy samples, using QIAamp® cador® Pathogen Mini Kit + T2 pretreatment (Qiagen, Hilden, Germany, Cat No. 50214) according to manufacturer instructions. DNA quality and quantity were assessed using a UV–visible spectrophotometer (Nanovue, Biochrom). DNA was sent to BGI (Shenzhen, China) for paired-end library construction and then the libraries were subjected to 2 x 100 bp sequencing using WMGS strategy on the BGISeq-500 platform.

b. Pre-processing

Prior to sequence analysis, the quality of the paired-end reads was evaluated with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and then pre-processed using Trimmomatic v.0.38 (Bolger et al., 2014) in order to trim/remove low quality reads. Only the reads with a minimum quality of 12 and a minimum read length of 80 bp, screened over a sliding window of 10 bp, were kept. The pre-processed reads were then mapped against the sheep genome (NCBI: GCF_002742125.1) to remove the DNA from the host using BWA mem (v.0.7.15) with default parameters (Li and Durbin, 2009). The mapped reads were filtered out and the remaining reads were used for further analyses.

c. Taxonomy assignment and difference abundance analyses

The same set of samples used in this work was used in a previous study to identify via qPCR presence or absence of *D. nodosus* (Albuquerque et al., 2022). Hence, samples where *D. nodosus* was not identified either by qPCR or metagenomics sequencing data quantification were considered as outgroup (OG), representing the healthy control group. The samples of the remaining flocks were divided between two groups, one with ID (footrot lesion score 0 and 1) was classified as the group of no footrot infection severe signs (NFIS) and the other with the remaining samples with higher footrot lesion scores (FIS – footrot infection severe signs). Thus, the dataset was divided into three categories: i) OG samples (no footrot infection), ii) samples with NFIS and iii) samples with FIS.

The microbiome taxonomic classification of each sample was done using Kraken v.2 (Wood et al., 2019) with default parameters based on the lowest common ancestor (LCA) approach. Its partner tool Bracken (Bayesian Reestimation of Abundance with KrakEN) (Lu et al., 2017) was applied at the species level to estimate abundances. The Kraken database was built comprising the complete genomes of Refseq for the bacterial, archaeal and viral domains, along with the human genome and a collection of known core element vectors (downloaded on August 2020) while the Bracken database was built for a read length of 80 bp (the minimum read length allowed in the pre-processing step). The abundances obtained were then used to perform the difference abundance analyses with the edgeR package from Bioconductor (R v.4.2) (Robinson et al., 2009). For this analysis, as the number of samples with a footrot score of 5 was too low (only two samples), those were removed from the set to avoid noise in the statistical analyses. All samples belonging to the same footrot score were considered as biological replicates as well as all samples belonging to the outgroup (Supplementary Table S2). Taxa with low abundances were filtered out using the *filterByExp* function implemented in edgeR with default parameters. By default, this function set the minimum number of samples per condition-group, with at least 10–15 counts each, as the 70% of the smallest condition-group sample size. Hence, the larger number of biological replicates the more restrictive the filtering is. Then, a Trimmed Mean of M-values (TMM) normalization was applied. Over the normalized taxa, the test for differential expression was performed applying the GLMs method. Two different strategies were followed to

analyze the data. The first strategy was based on the comparison of (1) NFIS vs OG, (2) NFIS vs FIS and (3) OG vs and footrot infection (FI: NFIS+FIS). The second strategy was based on a pairwise comparison between all the different footrot scores (FS: 0–4). At the end, in both strategies, only species with differences in abundance with a log fold change (logFC) $\geq |2|$ and a false discovery rate (FDR) ≤ 0.05 were considered significant. Additionally, to identify species clusters with similar abundance profile within the different footrot scores, a *k*-means clustering analysis was performed (number of clusters = 10) using functions from the CummeRbund package of R (Goff et al., 2013).

Within-sample (alpha) diversity was assessed as Shannon's diversity index while the between-sample (beta) diversity was estimated based on multidimensional scaling (MDS) plot.

3. Results

a. Pre-processing

Out of 13.2 billion raw reads, 12.9 billion (97.2%) passed the Trimmomatic quality control criteria. After removing from this set those reads belonging to the host DNA (sheep) a total of 114.5 million reads remained (0.9%) which were used for downstream analyses.

b. Taxonomy assignment

Kraken2 was applied over the pre-processed reads of each sample for the taxonomy assignment resulting in 5126 species taxonomically identified. The taxonomic assignment revealed a percentage of classified reads per sample ranging from 8.5% to 66.6%, being most of them below 20%. The low percentage of classified reads obtained is expected when working with non-targeted genomics sequencing data. The taxonomic classification also revealed the presence of 63 phyla and 504 families. At the phylum level, the set of the dominant phyla was quite similar among the different footrot scores, but the percentage of reads assigned to each differed as the footrot score increase (Fig. 1A). The phylum *Actinobacteria* was the most dominant in samples from OG and FS1 (41.88% and 34.73%), the *Proteobacteria* in FS0, FS2 and FS3 samples (34.99%, 31.17% and 25.20%), *Fusobacteria* in FS4 samples (36.94%) and *Bacteroides* in FS5 samples (31.1%). At family level, the set of dominant families among the different footrot scores was different due to the change of the percentage of reads assigned to each family as the footrot score increase. Fig. 1B represents the set of dominant families obtained

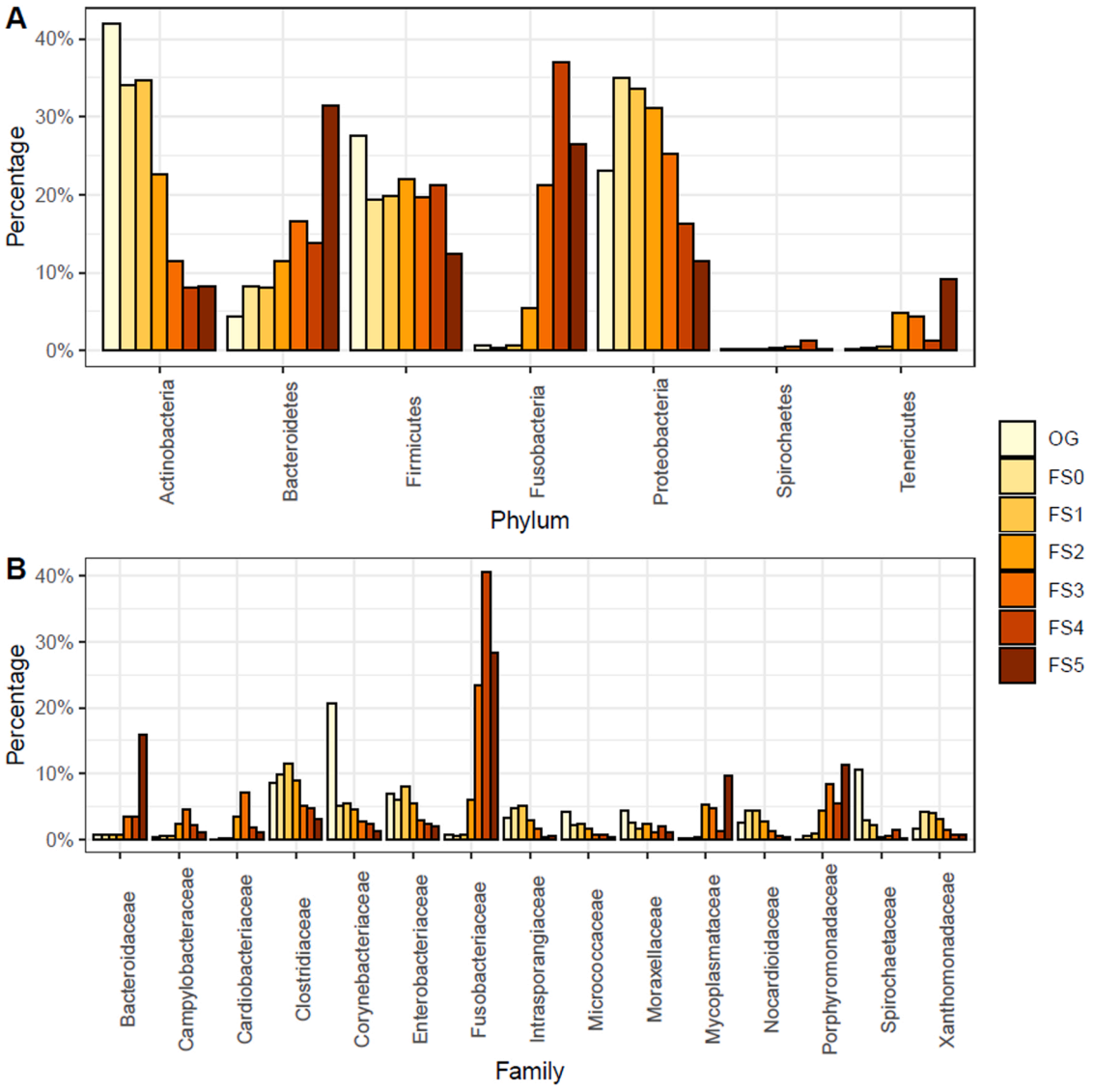

Fig. 1. Prevalence of the most dominant phyla (A) and families (B) in the footrot microbiome among the different footrot scores. All values are in percentage terms. FS: Footrot Score, OG: Out Group.

from merging the most representative families from each footrot score. The family *Corynebacteriaceae* (20.74%) was the most dominant in OG samples. In samples FS0, FS1 and FS2 the most abundant family was the *Clostridiaceae* (9.78%, 11.42% and 8.87%). Family *Fusobacteriaceae* was the second most abundant in FS2 samples (6.01%) and the most abundant in FS3, FS4 and FS5 samples (23.38%, 40.69% and 28.41%). Additionally, in FS5 samples the second most abundant family, with much lower representation in the other samples, was the family *Bacteroidaceae* (15.81%).

c. Overall difference abundance analyses

The taxonomic classification was followed by the abundance estimation of the taxa identified using Braken. As mention before, a total of 5126 species were taxonomically identified in the whole set of samples but only 869 passed the filtering of the taxa with low abundances represented in all samples of at least one group of replicates. Those taxa were then used for the differential abundance analyses, and also to assess alpha and beta diversities. The Shannon's index showed that as the severity of footrot infection increases, the microorganisms' diversity of footrot microbiota tends to decrease (Supplementary Fig. S1). Additionally, it can be observed that microorganisms' diversity of the OG and FIS samples was lower than the diversity observed in the first stages of the footrot (NFIS) infection (Supplementary Fig. S1B). However, after applying an analyses of variance (ANOVA) no significant differences were observed between diversity means between categories. Regarding beta-diversity, the MDS plot suggest that OG and NFIS samples were different than FIS samples although also no significant differences were observed (Supplementary Fig. S2).

In order to identify differences in species abundances between the three categories, pairwise comparisons between them were performed (Supplementary Fig. S3). When comparing samples with footrot infection versus OG samples (FI vs OG), 186 species with significant differences in their abundances were identified (Supplementary Table S3). From those, 146 species were found with significantly increased abundances in samples with footrot infection. These included *Mycoplasma fermentans* (logFC 11.6), *D. nodosus* (logFC 8.2), *Treponema phagedenis* (logFC 8.06), *Porphyromonas asaccharolytica* (logFC 7.6), *Treponema pedis* (logFC 4.5) and *F. necrophorum* (logFC 3.4), all species known to cause various foot diseases in sheep (Maboni et al., 2017; McPherson et al., 2019; Duncan et al., 2021; Clifton et al., 2022). Moreover, 10 species of *Planococcus* spp., nine species of *Corynebacterium* spp., eight species of *Acinetobacter* spp. and five species of *Staphylococcus* spp., among others, were identified with significantly decreased abundances in samples with footrot infection. Differences in the microbiome between the mild and severe stages of footrot infection (NFIS vs FIS) were also assessed resulting in 128 species with significant differences (Supplementary Table S4). Among the species found with significantly increased abundances in severe stages of footrot infection were *Streptococcus* spp. (n = 13), *Campylobacter* spp. (n = 13), *Fusobacterium* spp. (n = 8), *Prevotella* spp. (n = 7) and *Treponema* spp. (n = 4). The species found with significantly increased abundances in mild stages of footrot infection were *Brevundimonas* spp. (n = 5) *Streptomyces* spp. (n = 2) and *Staphylococcus* spp. (n = 2) among others. Finally, 175 species were found with significant differences between mild stages of footrot and OG samples (no NFIS vs OG) while between severe stages of footrot and OG samples (FIS vs OG) were found 219 species (Supplementary Table S5 and S6, respectively). In mild stages of footrot infection, *Psychrobacter* spp. (n = 9), *Brevundimonas* spp. (n = 8), *Marinobacter* spp. (n = 6) and *Sphingomonas* spp. (n = 5) were the main species found with significantly increased abundances while in severe stages those were *Streptococcus* spp. (n = 10), *Psychrobacter* spp. (n = 8) and *Fusobacterium* spp. (n = 8). In both comparisons, the main species in OG samples with significantly more abundances were *Planococcus* spp., *Acinetobacter* spp. and *Corynebacterium* spp. Table 1 summarizes the top 10 genera found more abundant in each sample category for all the comparisons performed.

d. Differences in the microbiome between different footrot infection stages

In the pairwise comparison between different footrot stages a total of 281 species were found significantly more abundant in at least one of the comparisons made (Supplementary Table S7, Supplementary Fig. S4). The higher number of species with significant differences in their abundances was found when comparing samples of FS0 and FS1 against the ones of FS4 (n = 218 and 185, respectively). In contrast, the lower number of species (n = 24) was found when comparing samples of FS0 against the ones of FS1, both considered as samples with NFIS. The two main relevant species associated with footrot disease, *D. nodosus and F. necrophorum* were found within all the species with significant differences in abundances. Additionally, other pathogens previously identified as important in other polymicrobial diseases such as CODD (Contagions Ovine Digital Dermatitis) and in footrot were also found with significant differences: *Campylobacter* spp. (n = 15), *Streptococcus*

Table 1
Top 10 genera more represented with significantly differences of abundances in each sample group per each pairwise comparison performed.

FI vs OG		NFIS vs FIS		NFIS vs OG		FIS vs OG	
↑FI	↑OG	↑NFIS	↑FIS	↑NFIS	↑OG	↑FIS	↑OG
Streptococcus(10 spp.)	*Planococcus*(10 spp.)	*Brevundimonas*(5 spp.)	*Streptococcus*(13 spp.)	*Psychrobacter*(9 spp.)	*Planococcus*(10 spp.)	*Streptococcus*(10 spp.)	*Planococcus*(10 spp.)
Psychrobacter(8 spp.)	*Corynebacterium*(9 spp.)	*Streptomyces*(2 spp.)	*Campylobacter*(13 spp.)	*Brevundimonas*(8 spp.)	*Acinetobacter*(10 spp.)	*Psychrobacter*(8 spp.)	*Corynebacterium*(9 spp.)
Fusobacterium(8 spp.)	*Acinetobacter*(8 spp.)	*Staphylococcus*(2 spp.)	*Fusobacterium*(8 spp.)	*Marinobacter*(6 spp.)	*Corynebacterium*(8 spp.)	*Fusobacterium*(8 spp.)	*Acinetobacter*(8 spp.)
Campylobacter(6 spp.)	*Staphylococcus*(5 spp.)	*Brevibacterium*(1 spp.)	*Prevotella*(7 spp.)	*Acidovorax*(6 spp.)	*Staphylococcus*(6 spp.)	*Campylobacter*(6 spp.)	*Staphylococcus*(5 spp.)
*Marinobacter*5 spp.)	*Tessaracoccus*(1 spp.)	*Sphingomonas*(1 spp.)	*Bacteroides*(6 spp.)	*Sphingomonas*(5 spp.)	*Campylobacter*(3 spp.)	*Marinobacter*(5 spp.)	*Tessaracoccus*(1 spp.)
Treponema(4 spp.)	*Shigella*(1 spp.)	*Sphingobium*(1 spp.)	*Treponema*(4 spp.)	*Xanthomonas*(4 spp.)	*Tessaracoccus*(1 spp.)	*Treponema*(4 spp.)	*Shigella*(1 spp.)
Porphyromonas(4 spp.)	*Pseudomonas*(1 spp.)	*Rhizobium*(1 spp)	*Porphyromonas*(4 spp.)	*Hydrogenophaga*(4 spp.)	*Shigella*(1 spp.)	*Porphyromonas*(4 spp.)	*Pseudomonas*(1 spp.)
Acidovorax(4 spp.)	*Pradoshia*(1 spp.)	*Pseudomonas*(1 spp.)	*Peptoniphilus*(3 spp.)	*Chryseobacterium*(4 spp.)	*Jeotgalibaca*(1 spp.)	*Acidovorax*(4 spp.)	*Pradoshia*(1 spp.)
Xanthomonas(3 spp.)	*Kocuria*(1 spp.)	*Plantactinospora*(1 spp.)	*Tannerella*(2 spp.)	*Campylobacter*(4 spp.)	*Dolosigranulum*(1 spp.)	*Xanthomonas*(3 spp.)	*Kocuria*(1 spp.)
Variovorax(3 spp.)	*Jeotgalibaca*(1 spp.)	*Planococcus*(1 spp.)	*Mycoplasma*(2 spp.)	*Variovorax*(3 spp.)	*Aerococcus*(1 spp.)	*Variovorax*(3 spp.)	*Jeotgalibaca*(1 spp.)

FIS: Footrot Infection severe Signs; NFIS: No Footrot Infection severe Signs; FI: Footrot Infection; OG: Outgroup.

spp. (n = 14), *Prevotella* spp. (n = 10), *Psychrobacter* spp. (n = 8), *Clostridium* spp. (n = 6), *Treponema* ssp. (n = 4), *Porphyromonas* spp. (n = 4), *Mycoplasma* spp. (n = 2) and *Gemela* spp. (n = 2). (Maboni et al., 2017; Gelasakis and Bossis, 2019).

In order to identify species with similar patterns of abundances among the different stages of footrot infection, all the species found with significant differences in their abundances in pairwise comparisons were clustered based on their abundance profile among different footrot stages into 10 clusters (Fig. 2). The number of species per cluster are 11, 61, 13, 25, 39, 42, 22, 37, 19 and 12, respectively from cluster 1–10). To get more details about which species is found in which cluster please see Supplementary Table S8.

Out of the 10 clusters obtained, five were selected for further discussion due their abundance profile (Clusters 1, 2, 7, 8 and 10, Fig. 2). The species within these clusters proliferate along the footrot infection process, with slightly differences of abundance between FS0 and FS1, and FS3 and FS4 stages. Among these species were included *D. nodosus*, *F. necrophorum* and diverse *Treponema*, *Staphylococcus*, *Streptococcus* and *Campylobacter* species.

4. Discussion

The main aim of this study was to characterize the bacterial communities present on the feet of healthy and footrot-affected Merino and Merino-related sheep and to identify the changes of the bacterial community over the different stages of footrot infection. The results of the taxonomic classification showed that footrot infection seemed to cause a shift in the composition of the microbiome as severity of the lesion (score) increases from a dominant gram-positive in mild stages of footrot infection to a dominant gram-negative in the severe stages (Fig. 3). This

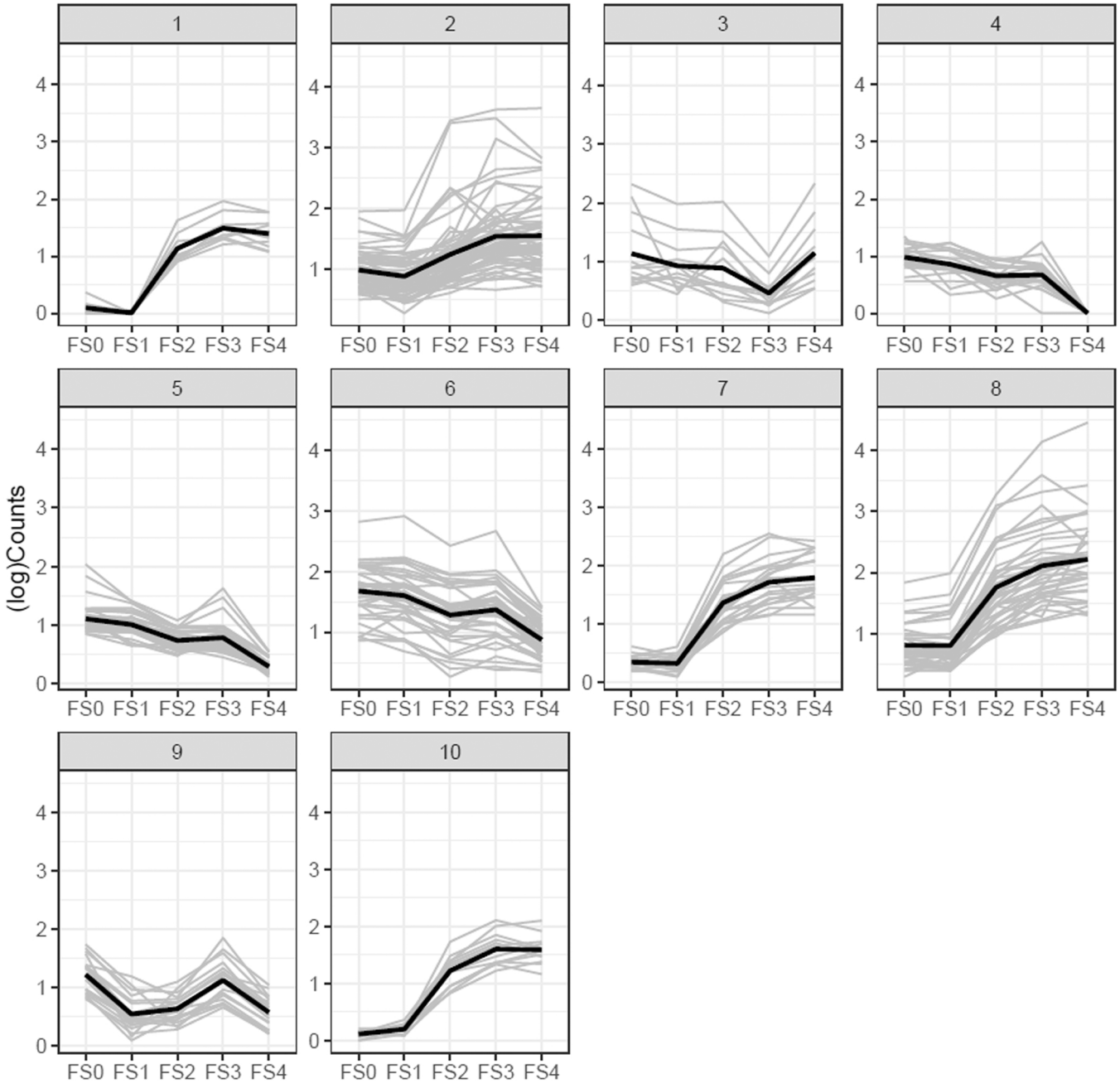

Fig. 2. k-means clustering analysis of species with significant differences in their abundance in the pairwise comparison (k = 10). The grey lines represent mean abundance profile (log10) for each species across the footrot scores. The black line represents the mean abundance profile observed in each cluster. FS: Footrot Score.

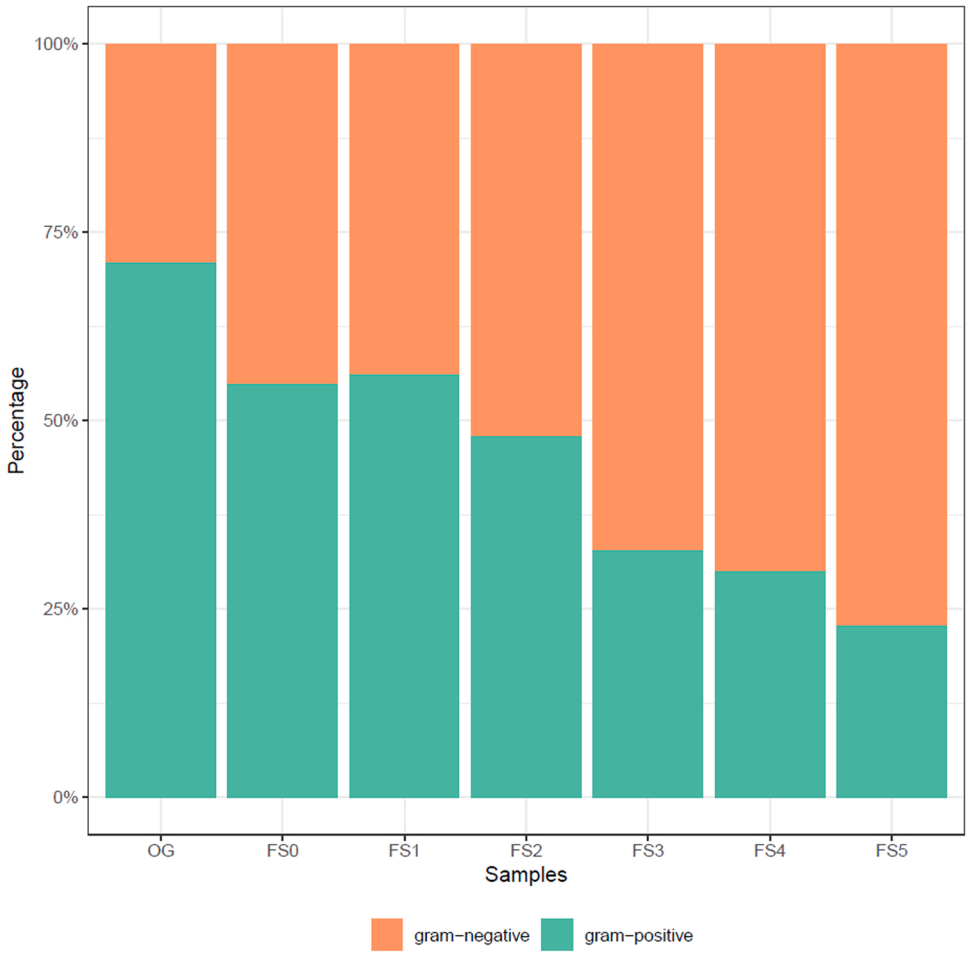

Fig. 3. The dominant phyla obtained from merging the most represented phyla (≥ 1%)) from each footrot score and with a clear definition of their gram definition were used to display the percentage of gram-positive vs gram-negative phyla as long the footrot infection disease aggravates.OG: Ourgroup; FS: Footrot Score.

shift has been also observed in previous studies of footrot as well as other polymicrobial diseases being directly associated with the evolution of the disease from the healthy to the disease stage (Zanolari et al., 2021). Additionally, the footrot microbiome showed a diminished diversity as the footrot infection aggravates (Supplementary Fig. S1) which is accompanied by the increased abundances of *D. nodosus* along with other species such as *Mycoplasma fermentans*, *F. necrophorum*, *P. asaccharolytica*, *Ezakiella massiliensis*, *Treponema* ssp. and *Staphylococcus* spp., *Streptococcus* spp. and *Campylobacter* spp. Several studies have addressed the role of *F. necrophorum*, which is known to colonize, under environmental predisposition, the epidermis facilitating other taxa proliferation along with its own due to the associated necrosis and anaerobiosis (Zanolari et al., 2021). The results obtained in this study reflects the proliferation of *F. necrophorum*, as the diseases intensifies, its abundances increase (Cluster 8, Figure2). However, other taxa following a similar proliferation profile have been identified suggesting that they may be significant to the footrot infection process.

Species from genus *Porphyromonas* have been previously associated with the pathogenesis of footrot in sheep and were related with higher levels of inflammation (Maboni et al., 2017; McPherson et al., 2019). In this study, were identified *P. asaccharolytica*, *P. cangingivalis*, *P. crevioricanis* and *P. gingivalis* with significant increased abundances along the different disease severity stages (Clusters 2 and 8, Fig. 2, Supplementary Table S8). *Porphyromonas asaccharolytica*, a synergistic wound pathogen, was also recently found associated to footrot infection (Blanchard et al., 2021). It is known that *P. gingivalis*, a keystone pathogen for periodontitis in humans, promotes the dysbiosis of the microbiome and triggers the host inflammatory response, dysbiosis that was also described to occur in footrot (Kaler et al., 2010; Maekawa et al., 2014; McPherson et al., 2019). Regarding *P. cangingivalis* and *P. crevioricanis*, both have been also associated to periodontitis and inflammatory conditions in ovine, being the latest also associated with bovine interdigital necrobacillosis (Sweeney et al., 2009; Borsanelli et al., 2017).

Different microbiological studies have associated the *Treponema* spp. with other claw diseases such as CODD and bovine digital dermatitis (BDD). Recently, it has been suggested that CODD and footrot could be different stages of the same diseases due to the similarities of the bacteriological and epidemiological features (Duncan et al., 2021). Three main *Treponema* species are known to be associated with CODD and BDD, *T. medium*, *T. phagedenis* and *T. pedis*. The latest two together with *T. denticola* and *T. putidum*, both associated to CODD and BDD lesions, were found in this work sharing a similar proliferation profile with *D. nodosus* and *F. necrophorum* (Sayers et al., 2009; Mamuad et al., 2020; Caddey, 2021). Hence, these results are highly congruent with what has previously been identified (Maboni et al., 2017; Blanchard et al., 2021; Duncan et al., 2021).

In previous studies, *Staphylococcus aureus* and *Staphylococcus epidermidis*, were significantly associated with footrot (Calvo-Bado et al., 2011; Anto et al., 2014). The *S. aureus* and *S. epidermidis*, present at the skin commensal flora in human and livestock animals, and considered as important opportunistic pathogens, are known as etiological agents of bovine, ovine and caprine mastitis leading to an inflammatory response of the mammary gland (Watts, 1988). Moreover, it has been reported that *S. aureus* can cause necrotic/staphylococcal dermatitis in sheep and also was found to be present in CODD (Duncan et al., 2014). In

accordance to this, the results obtained in this study showed that both, *S. aureus* and *S. epidermidis*, seems to take profit of the ideal environmental conditions of the disease to proliferate (Cluster 2, Fig. 2, Supplementary Table S8). However, it is important to note that, besides the competition existing between *S. aureus* and *S. epidermidis*, being the first one the most aggressive, virulent and abundant, both found a way to survive and spread based on different colonization approaches (Massey et al., 2006; Cheung et al., 2010). Further, other *Staphylococcus* spp. were found with significant differences in their abundance along the progression of the disease. In general, the proliferation of *Staphylococcus* species seems to be inhibited and diminished in the mild stages of the disease, which agrees with the interspecies competition, whereas, suddenly, in the severe stages (from FS3 to FS4) their abundance increase (Cluster 2 and 3, Fig. 2). In contrast, when we compared OG samples against NFIS, FIS and FI samples, most of these *Staphylococcus* species were always found more significantly abundant in OG samples (Supplementary Tables S3, S5 and S6). These results are in accordance with other studies (Maboni et al., 2017; McPherson et al., 2019; Blanchard et al., 2021). Hence, the comparison of the different footrot score, regarding *Staphylococcus* species, was able to clarify their abundance profile, in which abundance seems to increase at the very late stages. Another species also found associated with footrot infection was *Streptococcus pyogenes* (Calvo-Bado et al., 2011; Anto et al., 2014) which is widely known for causing diverse diseases in humans, including skin infections such as necrotizing fasciitis which destroys the tissue and has a rapid disease progression (Stevens and Bryant, 2016). *Streptococcus pyogenes* was found in the same cluster than *D. nodosus* and *F. necrophorum*. Other *Streptococcus* species were found significantly differentiated in their abundances along footrot infection, most of them with an increasing abundance profile (Clusters 1, 2, 7, 8 and 10, Fig. 2, Supplementary Table S8). Hence, the *Staphylococcus* spp. and *Streptococcus* spp. seem to be associated to the severity of the disease triggering an inflammatory response and damaging the skin of the hoof.

The species *Trueperella pyogenes* (*T. pyogenes*) was found in Cluster 8 together with *D. nododus* and *F. necrophorum* (Fig. 2, Supplementary Table S8). *Trueperella pyogenes*, which was formerly known as *Arcanobacterium pyogenes,* belongs to the commensal flora of skin and mucous membranes of animals, yet is also known as an important opportunistic pathogen, being an etiological agent of diverse animal infections, including footrot in sheep (Calvo-Bado et al., 2011; Wani et al., 2015). Our results are then in accordance with previous associations with lameness and footrot in animals such as sheep and goats (Calvo-Bado et al., 2011; Wani et al., 2015).

Little is known about the *Ezakiella* genus although it has been described as commensal flora of human and animals. It was first identified in 2015 from a human fecal sample in Peru – *Ezakiella peruensis* (Patel et al., 2015). Later in 2017 a new *Ezakiella* species, *Ezakiella massiliensis* (*E. massiliensis*), was isolated from the human vagina (Diop et al., 2017). More recently, in 2019, the species *Bacteroides coagulans*, was proposed to be classified as *Ezakilella coagulans*. (García-López et al., 2019). Hence, only three species of this genus are known. Recently, *E. massiliensis* was found with higher prevalence in samples with BDD (Caddey, 2021). Additionally, in this study, *E. massiliensis* was grouped in the same cluster with *D. nodosus* and *F. necrophorum* (Cluster 8, Fig. 2, Supplementary Table S8). Further studies are necessary in order to assess the role of this bacteria in footrot.

Regarding *Campylobacter* spp., in this study were identified 15 *Campylobacter* species with significant differences in their abundances. The clustering analysis showed that, in general, their abundances increased along with the lesions' severity, although with different abundance profiles, and therefore integrated in different clusters (Clusters 1,2,7,8 and 10, Fig. 2). This trend is in accordance with the shift, already mentioned, from dominant gram-positive species in mild stages of footrot infection to dominant gram-negative species in more severe stages, and with the results of (McPherson et al., 2019) where the genus *Campylobacter* was found more abundant in samples recovered from animals with footrot. The species of *Campylobacter* constitute a highly biologically diverse group of organisms, some of which are well-known as causative agents of clinical illness in animals and humans, whereas many other members of the genus appear to be commensals in the intestinal tract or lack clearly established associations with overt disease (Sahin et al., 2017). *Campylobacter* species are frequenctly present in the farm environment, which makes their presence in the interdigital tissues an expected result. In sheep, *Campylobacter fetus* subsp. *fetus* and *Campylobacter jejuni* subsp. *jejuni* are the major *Campylobacter* species associated with sheep abortion outbreaks (Sahin et al., 2017). The development of microscopic lesions, triggering an inflammatory response in pregnant sheep, leads to abortion. Additionally, species such as *C. jejuni* were also associated to digital dermatitis in cattle (Refaai et al., 2013). The role of *Campylobacter* species in footrot is still unknown, but the abundance profile found in this study might suggest some kind of relevance that must be taken into account in further studies.

Finally, the methodology applied, WMGS, has some inherent limitations associated with in-tissue samples such as skin biopsies where the total DNA yield contains a high level of host contamination, usually accounting for more than 99%. However, the taxa resolution achieved is higher than the one obtained with 16 S rRNA gene, allowing to perform taxonomic classifications at the species level. While WMGS screens the complete genomic DNA, 16 S rRNA gene taxonomic composition is limited and influenced by selected primers and targeted variable regions, introducing bias due to the poor taxa resolution between bacteria (genus level). Therefore, this study complements the previous studies of the ovine footrot microbiome performed using 16 S rRNA gene (Calvo-Bado et al., 2011; Maboni et al., 2017; McPherson et al., 2019; Clifton et al., 2022) providing higher resolution on the bacterial taxonomic classification. Hence, we were able to identify a variety of bacterial key species spanning multiple genera, most of which were previously identified, that are consistently associated with footrot infection.

5. Conclusions

This is the first study of the ovine footrot whole metagenome sequencing-based microbiome. The metagenomics analyses identified differences in the bacterial composition between different severity stages of footrot as well as the abundance profile of different bacteria along the disease progression. Based on improved resolution provided by this methodology *D. nodosus*, *M. fermentans*, *F. necrophorum*, *P. asaccharolytica*, *E. massiliensis*, *T. pyogenes, Treponema* ssp., *Campylobacter* spp., *Staphylococcus* spp., *Streptococcus* spp. and other species were identified as particularly abundant in the microbiome of samples from animals with footrot infection. Their abundance profile along the disease indicated that they proliferate as the diseases aggravates, being key species that differentiated mild and severe footrot lesion stages. Further analysis on the individual species of *Campylobacter* and *E. massilensis*, as well as other species with similar abundance profiles, is necessary to further understand their roles in footrot. Although these bacteria are not able to initiate footrot, several evidences had been described supporting that they are associated to the increase of the severity of footrot lesions caused by *D. nodosus* and *F. necrophorum.* Overall, our findings provided significant information to better understand disease pathogenesis and provides evidences to focus primarily on the potential footrot pathogens identified. Last but not least, the knowledge of the microbiome present in mild and severe forms of footrot, compared with healthy sheep claws, represents an important contribution for the improvement of therapeutic and prophylactic measures that are crucial for controlling the disease and improve animal welfare.

Ethical approval

This study was approved by the ethics committee for animal

experimentation ORBEA-U Évora, Portugal (ID: GD/20467/2021/P1).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The metagenomics sequencing data generated under the scope of this work has been deposited at the NCBI in the Short Read Achieve (SRA) databases under the bio project number PRJNA933156.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2023.109745.

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