Ovine pedomics- the first study of the ovine foot 16S rRNA based microbiome

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Keywords: footrot, microbiome, sheep

Running title: Ovine pedomics

Abstract

We report the first study of the bacterial microbiome of ovine interdigital skin based on 16S rRNA by pyrosequencing and conventional cloning with Sanger-sequencing. Three flocks were selected, one a flock with no signs of footrot or interdigital dermatitis, a second flock with interdigital dermatitis alone and a third flock with both interdigital dermatitis and footrot. The sheep were classified as having either healthy interdigital skin (H), interdigital dermatitis (ID) or virulent footrot (VFR). The ovine interdigital skin bacterial community varied significantly by flock and clinical condition. The diversity and richness of operational taxonomic units was greater in tissue from sheep with ID than H or VFR affected sheep. Actinobacteria, Bacteriodetes, Firmicutes and Proteobacteria were the most abundant phyla comprising 25 genera. *Peptostreptococcus*, *Corynebacterium* and *Staphylococcus* were associated with H, ID and VFR respectively. Sequences of *Dichelobacter nodosus*, the causal agent of ovine footrot, were not amplified due to mismatches in the 16S rRNA universal forward primer (27F). A specific real time PCR assay was used to demonstrate the presence of *D. nodosus* which was detected in all samples including the flock with no signs of ID or VFR. Sheep with ID had significantly higher numbers of *D. nodosus* (10^4-10^9 cells/g tissue) than those with H or VFR feet.

Introduction

Dichelobacter nodosus, a Gram negative bacterium, causes footrot in small ruminants. The first clinical sign of footrot is interdigital dermatitis (ID), in certain environments, and with some strains of *D. nodosus*, separation of the hoof horn from the sensitive tissue can arise causing virulent footrot (VFR) (Beveridge, 1941). Footrot, both ID and VFR, is responsible for over 90% of lameness in sheep in the UK (Kaler and Green, 2008), and it is one of the most important causes of poor welfare and economic loss to the sheep industry in the world. Several taxa other than *D. nodosus* have been linked to footrot; this disease can be considered as a polymicrobial disease with opportunistic colonizers contributing to increased severity and / or persistence of the disease (Beveridge, 1941; Stewart, 1989; Billington *et al.,* 1996). The bacterial species associated with footrot are *Fusobacterium necrophorum* (Beveridge, 1941; Robert and Egerton, 1969;), *Arcanobacterium pyogenes* (Lavín *et al.,* 2004) and *Treponema* (Beveridge, 1941; Egerton *et al.,* 1969; Naylor *et al.,* 1998; Colligham *et al.,* 2000; Dhawi *et al.,* 2005). The structure of the total bacterial community and how this differs between healthy and diseased sheep is unknown.

In microscopic examination of samples from foot lesions cocci, corynebacteria and other rod-shape microorganisms were abundant near the surface of the skin and in lesions (Beveridge, 1941; Egerton *et al.,* 1969). However, *D. nodosus* and *Treponema* spp. were present in small numbers and less frequently present compared with *F. necrophorum*, but all were present in the deeper parts of the tissue (Beveridge, 1941). Aerobic and anaerobic cultivation of bacteria from diseased feet have also revealed the presence of other microorganisms including *Bacteroides* spp., *Porphyromonas* spp., *Prevotella* spp., *Peptostreptococcus* spp., *Clostridium* spp among others (Berveridge 1941; Moore *et al.,* 2005). Cultivation of bacteria from affected goats showed that the major taxa were *D. nodosus, Peptostreptococcus, Megasphaera* and *Fusobacterium* (Piriz-Duran *et al.,* 1990).

The aim of the current study was to investigate the microbial community of the interdigital skin of sheep comparing individuals with healthy feet (H), interdigital dermatitis (ID) or virulent footrot (VFR). Sheep were selected from three flocks with and without footrot to test the hypothesis that the structure of the bacterial community varies by clinical condition of the sheep and flock.

Materials and Methods

Source of tissue samples

Three geographically separated farms located in the South West of England were selected for the study. Flock A (20 Badger Faced Welsh Mountain sheep) had had no clinical cases of footrot or interdigital dermatitis for the past 10 years, VFR had been eradicated by a combination of culling and use of parenteral oxytetracycline (Kaler *et al.*, 2010a). Sheep were not foot-trimmed. Flock B (100 Wiltshire Horn sheep) had sheep with ID but no VFR. Affected sheep were sprayed with oxytetracycline or copper sulphate spray and there was no policy for culling lame sheep. Sheep were foot-trimmed once a year. Flock C (200 Suffolk cross mule sheep) had sheep with ID and VFR. Affected sheep were sprayed with oxytetracycline and necrotic material was trimmed away. Ewes were also routinely foot-trimmed once a year. There was no culling policy.

The sheep were selected from the three flocks as follows; flock A three sheep with healthy feet (H), flock B three sheep with H feet and two with ID feet and flock C two sheep with healthy feet, three with ID and two with VFR (Table 1). Healthy feet were without clinical abnormality, feet with ID had irritation present in the red interdigital space, with or without a white/grey pasty scum and loss of hair in the interdigital space and virulent footrot (VFR) presented as separation of horn from the underlying tissue with or without interdigital dermatitis. All four feet of all sheep were examined *post mortem* and feaces / grass were

removed aseptically to expose the interdigital skin for sampling. Tissue samples were taken from the interdigital skin using a sterile 0.5 cm core borer (0.8 cm depth). All material was stored at -80^{o}C. All tissue samples from flocks A and B were collected in summer 2008; tissue samples for flock C were collected in summer 2008 (H1C, H2C, H3C, VFR1C), spring (ID1C, ID2C, ID3C) and summer 2009 (VFR2C). The DNA from all feet was pooled per sheep for all analyses with exception of the qPCR assays where *D. nodosus* cell number was quantified in each foot separately.

Bacterial DNA extraction from tissue

Tissue samples (130-160 mg) were treated with 10 mg/ml collagenase (Collagenase NB 4G, SERVA,) in 0.05 M TES/ 0.36 mM $CaCl_2$ pH 7.5 at 37^{o}C for 5-7 h to release the microbial cell. The supernatant was centrifuged at 15,871 × g for 15 min and the pellet formed was used to extract DNA using MagMAX™ Express Magnetic Particle Processors (AMBION, Applied Biosystems, Inc.) according to the manufacturer's recommendations. DNA was eluted into 60 µl of elution buffer (10 mM Tris-HCL pH 8).

16S rRNA PCR amplification for library construction

All PCR amplifications were carried out using PCR-Promega master mix (Promega). All PCR reactions had a final volume of 50 µl containing 25 µl Master mix (50 units/ml of *Taq* DNA polymerase supplied in a reaction buffer (pH 8.5), 400 µM each dNTP, 3 mM MgCl2), 10 µM of each primer, 2.5 µl of DMSO (Dimethyl Sulfoxide, Fisher Scientific), 2 µl BSA (bovine serum albumin 10 mg/ml, SIGMA) and 1-3 µl of template DNA (50-100 ng) were performed using the following conditions: 1 cycle of 95^{o}C for 2 min, 35 cycles of 95^{o}C for 1 min, 55^{o}C for 1 min and 72^{o}C for 2 min with a final extension of 72^{o}C for 10 min.

Detection of *D. nodosus* by 16S rRNA specific PCR

To test the reliability of *D. nodosus* extraction, PCR reactions were performed using a direct or a nested PCR approach. All DNA samples were screened for the presence of *D. nodosus* using the specific primers for the 16S rRNA gene (*Cc* 5'-TCGGTACCGAGTATTTCTACCCAACACCT-'3 and *Ac* 5'-CGGGGTTATGTAGCTTGC-'3) (La Fontaine *et al.,* 1993) at 60^{o}C annealing temperature for direct detection of *D. nodosus*. In some cases to increase sensitivity, a nested PCR was used comprising a round of PCR using universal 16S rRNA primers (27F and 1525R) (Lane, 1991; Baker *et al.,* 2003) at 55^{o}C instead of 60^{o}C annealing temperature followed by a second round of PCR using *D. nodosus* 16S rRNA specific primers. Strain VCS1703A (Prof. Julian I. Rood, Monash University, Australia) was used as positive control and sterile water as negative control.

PCR libraries based construction, Sanger sequencing and data analysis

For PCR clone libraries, 16S rRNA genes were amplified from the total community DNA from feet tissue (see above) using primers 27F and 1525R at 55^{o}C. All amplicons were gel purified (QIAquick Gel Extraction Kit, Qiagen UK) and cloned into the pGEM-T Easy vector system (Promega, London, UK) according to the manufacturer's recommendations. A minimum of 100 colonies per ligation were recovered and grown and the plasmid DNA purified (QIAprep Spin Miniprep Kit, Qiagen UK) and sequenced using the 27F primer on an ABI PRISM 3130xl Genetic Analyser (Applied Biosystems).

For phylogenetic analyses, all sequences from each library were edited, aligned and trimmed with SeqMan II (Lasergene 6). Sequences were aligned using the NAST alignment tool on the greengenes website (http://greengenes.lbl.gov) (DeSantis *et al.,* 2006a, b). For taxonomic classification, nearest-neighbour, diversity indices (Shannon and Simpson 1-D) and richness estimates (Chao1 richness), sequences were grouped into Operational Taxonomic

Units (OTUs) by the furthest-neighbour algorithm using DOTUR (Schloss and Handelsman, 2005) at a 97% similarity cut off.

Pyrosequencing and data analysis

Pyrosequencing was performed using bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) similar to that described previously (Dowd *et al.,* 2008). bTEFAP was based upon the Titanium sequencing platform rather than FLX (Roche Indianapolis, IN USA). The average sequence length was 405 bp with range of 300-500 bp. A single step reaction was utilized with 30 cycles of PCR to reduce chimera formation.

Raw sequence data were edited using a series of custom Perl and Bioperl scripts which performed the following initial steps: trimming of pyrosequencing tag sequences, screening for presence of PCR primers, length screening, and removal of sequences with one or more ambiguous base calls. BLASTN was run locally with default parameters using type strains from Release 102 of the Silva SSU rRNA database to determine the identity of sequences (Preusse *et al.,* 2007). Sequences were clustered into operational taxonomic units (OTUs) using CD-HIT (Li and Godzik 2006). Summary analyses of OTU frequency distributions, including rarefaction curves and CCA, were performed in R (R Development Core Team, 2009) automated with a series of scripts in the R language. Output from CD-HIT was converted to mothur format (Schloss *et al.,* 2009) with Perl, and community similarity trees and Venn diagrams were constructed in mothur. Sequences were aligned against a template alignment from the Silva rRNA database project for phylogenetic analysis (Preusse *et al.,* 2007) using the mothur alignment package. Trees were built with maximum-likelihood and neighbour-joining algorithms in ARB (Ludwing *et al.,* 2004) using a 75% homology filter. Phylogenetic clustering was assessed with UniFrac (Lozupone and Knight 2005) which uses both branch-length and position to compare actual phylogenies to a null model of randomly

permuted sites. To estimate the level of richness and diversity (the efficiency of new OTUs sampling recovery at 97% similarity cut off), rarefraction curves were created for condition and flock.

Quantitative PCR (qPCR) of *D. nodosus*

Quantification of *D. nodosus* in samples, standards and no template controls (sterile water) was done in triplicate using Applied Biosystems 7500 Fast real-time PCR system. The RNA polymerase sigma-70 factor gene (*rpoD*; single copy number in *D. nodosus* genome) was used as a target with a thermal cycle profile of 1 cycle at 50^{o}C for 2 min, 1 cycle at 95^{o}C for 10 min, 40 cycles at 95^{o}C for 15 sec and the final stage at 55^{o}C for 1 min. Each reaction contained 12.5 µl of Taqman universal PCR master mix (Promega) (50 units/ml of *Taq* DNA polymerase supplied in a proprietary reaction buffer pH 8.5, 400 µM of each: dATP, dGTP, dCTP, dTTP, 3 mM MgCl2), 0.9 µM of each primer (*rpoDF* and *rpoDR*) (Table 2), 0.25 µM of Taqman (5' 6-carboxyfluorescein-tetramethyl-6-carboxyrhodamine 3') (Table 2), 2.5 µl of a 10 mg/ml bovine serum albumin (BSA) solution, 1 µl of template DNA and nuclease free water in a total 25 µl reaction. Analytical specificity of *rpoD* against *D. nodosus* was performed experimentally using DNA from other bacterial species found in the hoof, soil and farm animal faeces including *Fusobacterium necrophorum*, *Arcanobacterium pyogenes*, *Streptomyces spp. Streptococcus spp. Mycobacterium bovis*, *Pseudonomas putida*, *E. coli* laboratory strains. A database search also indicted that these primers were specific for the *rpoD* target gene form *D. nodosus* as predicted from the assay. These DNA extracts were also spiked with *D. nodosus* DNA which produced amplification. DNA dilutions of 1:10 and 1:100 were used to investigate potential inhibitors of the reaction. The *rpoD* copy number in the unknown sample was estimated based on the standard curve using *D. nodosus* VSC1703A as template.

201 **Denaturing gradient gel electrophoresis (DGGE) analysis**

202 To profile the total bacterial community by DGGE, the V3 region of the 16S rRNA
203 gene between positions 341 and 534 (*Escherichia coli* numbering) was amplified by PCR
204 with primers P2 and P3 (Muyzer *et al.,* 1993). DGGE was done using the DCode mutation
205 detection system (Bio-Rad, Hertfordshire, United Kingdom) with 20-60% denaturing gradient
206 gels (Muyzer *et al.,* 1993). PCR products (400-500 ng) were loaded into 12% acrylamide gels
207 and run at 60 V for 16.5 h at a constant temperature of 60^{o}C in 0.5 × TAE buffer (40mM Tris-
208 acetate and 1mM EDTA, pH8.0). The gels were stained for 20 min in 1 × TAE containing
209 ethidium bromide (0.5 mg/l) then de-stained for 20 min in Milli-Q water. The gels were then
210 visualised and photographed using the Gene flash UV imager (Syngene Bio imaging).
211 Selected DGGE bands were cut from the gel and were re-amplified by PCR, and cleaned up
212 (QIAquick, Qiagen) prior to sequencing. All sequences were edited using the DNAstar
213 SeqMan II sequence analysis package (Lasergene, Inc., Madison, Wis.) and the best matches
214 determined with BLASTN (http://www.ncbi.nlm.nih.gov/Genbank/index.html). The DGGE
215 band positions and intensities were determined with the GelCompar II software (Applied
216 Maths, Austin, TX, USA). The similarity matrix was calculated based on Jaccard's coefficient
217 and a dendrogram was created using a UPGM algorithm in GelCompar software.
218
219 **Results and discussion**
220 *Comparison of the bacterial diversity of the ovine foot by pyrosequencing and clone library*
221 *Sanger sequencing*
222 A total of 61,708 sequences with a length of 350 to 535 nucleotides were generated
223 from pyrosequencing and 1130 sequences from clone-libraries for all flocks and conditions. A
224 total of 25,672, 25,083 and 10,953 sequences that passed all quality control screens were
225 detected in H, ID, and VFR samples respectively which corresponded to 6,009 from flock A,

15,301 from flock B and 40,398 from flock C respectively. The bacterial community structure was different between flocks, which might have been attributable to location or breed, but was mostly driven by the disease status of the sheep (Figure 1a). The sequences were clustered into two groups (Figure 1b) overall and independent of the sequencing approach used; the bacterial populations present in healthy sheep were more similar than those in diseased sheep. The bacterial populations from healthy sheep from flocks A, B and C (Cluster 1) were unique for each flock but the bacterial populations for diseased sheep in flocks B and C were more similar, irrespective of flock of origin (Cluster 2). These results were in agreement with the CCA analyses (Figure 1a). This suggests that flocks / farms / breeds have a unique population structure that differs from each other based on the proportion of the bacterial consortium. However, the population structure becomes more similar within diseased sheep and distinct from healthy sheep. There was a high sequence richness in flock C where there were more OTUs recovered, possibly because all clinical conditions H, ID and VFR were in this flock, or possibly because more sheep were sampled (Figure 2a). ID had the highest richness of OTUs sampled in flock C. The rarefaction curves were stable at 2000 sequence reads for H sheep from flocks A and B compared with 5000 - 6000 for ID and VFR suggesting that to recover additional OTUs for animal samples with ID would require more sampling than for H animals and less sampling for VFR animals (Figure 2a). A core population was shared between disease and healthy sheep (Figure 2b); however because each condition had its own distinctive and unique population; we next investigated these differences in more detail.

Comparison of the microbial communities and taxonomic classification

The phylogenetic distribution of 717 representative sequences for each OTU was significantly different among conditions based on the calculation of the UniFrac distance metric between communities ($p<0.002$) and Parsimony tests ($p<0.01$) (Figure 3a), indicating

that the bacterial population was not randomly distributed but clustered by clinical condition. Sequences most closely related to *Staphylococcus* were associated with disease (Figure 3b, supplementary Figure 1), and phylotypes most closely related to *Macrococcus* and *Micrococcus* were associated with disease but were ubiquitous across all conditions (Figure 3b, supplementary Figure 1). Sequences classified as *Corynebacterium* were significantly associated with the ID condition (Figure 4a). However, when considered by OTU classification, the most abundant OTUs associated with disease (ID or VFR) were most-closely related to *Macrococcus, Micrococcus,* and *Staphylococcus* (Figure 3a, supplementary Figure 1). Phylotypes most closely related to *Peptostreptococcus* were associated with H sheep (Figure 3c, supplementary Figure 1).

These 717 sequences were taxonomically assigned and distributed in 25 genera with 4 mayor phyla (Figure 4a). Firmicutes followed by Actinobacteria, Proteobacteria and Bacteriodetes were the most abundant phyla (Figure 4b). Firmicutes was the most diverse phylum and *Macrococcus, Corynebacterium, Peptostreptococcus, Staphylococcus, Escherichia* and *Streptococcus* were the predominant genera. Actinobacteria were represented by 12 genera with a significant difference between ID and H, and ID and VFR (Figure 4b). *Peptostreptococcus* (20% in H), *Corynebacterium* (32% in ID) and *Staphylococcus* (12% in VFR) had significantly different populations at the genus level by condition (Figure 4a) suggesting that these populations might be associated with each condition. The majority of sequences were Firmicutes with a percentage range from 40% for ID, 75% for VFR and 80% for H (Figure 4b). The taxonomic identity of some members of Firmicutes showed 92-94% sequence similarity to the database indicating that there might be novel, uncultured species.

The presence and high abundance of *Micrococcus* and *Macrococcus* in all conditions and independent of the sequencing approach used in all the flocks was not surprising. These aerobic bacteria have frequently been isolated from human (Kocur *et al.,* 2006) and animal

(Kloos *et al.,* 1998; Chin and Watts, 1992) skin and might be considered part of the normal microflora of the skin in both human and animal hosts. *Corynebacterium* is a non-motile, facultative anaerobic bacterium widely distributed in nature. They were significantly more abundant in animals with ID in the pyrosequencing data in the current study, suggesting that although a common inhabitant of moist sites in the human skin (Grice *et al.,* 2008; Grice *et al.,* 2009) they may have an association with ID. *Corynebacterium* has been reported to be abundant near the surface of the interdigital skin of sheep and in footrot lesions by others (Beveridge, 1941; Egerton *et al.,* 1969). The *Peptostreptococcus* population was significant higher in H sheep and is widely distributed in humans and animals. It is found in the upper respiratory tract, gingiva, gut, and urogenital tract; these bacteria are opportunistic pathogens that can cause a wide spectrum of local and systemic disease (Conrads *et al.,* 1997; Murdoch, 1998). The presence of *Staphylococcus* is unsurprising as this group commonly colonise human skin and nasal cavities. *S. epidermidis* is one of the major inhabitants of the human skin and mucosa representing 90% of the aerobic flora (Cogen *et al.,* 2008).

In the clone libraries the sequences were assigned to three main phyla, Actinobacteria, Firmicutes and Proteobacteria with 27 genera (data not shown). *Macrococcus* (Firmicutes) was the most abundant genus for all conditions, however, H sheep had a greater proportion of their population in this genus (35.6%) compared with ID (15.9%) and VFR (13.1%). *Streptococcus*, *Facklamia* and *Abiotrophia* (Firmicutes) were also abundant in VFR at >5% of the population. Although both sequencing approaches produced similar results, pyrosequencing produced a clearly higher resolution of bacterial diversity yielding 3.5 orders of magnitude more taxa.

Detection of Fusobacterium necrophorum, Arcanobacterium pyogenes and Treponema species

Sanger sequencing clone libraries did not show the presence of *Fusobacterium.* These bacteria were detected in the pyrosequencing data (Figure 4a). We confirmed the findings by using nested PCR. There were 8 phylotypes of *Fusobacterium* detected from sheep in flocks B (ID sheep) and C (H and ID sheep). These sequences showed 98% and 95% sequence similarity to *Fusobacterium necrophorum subsp. funduliforme* and *Fusobacterium gonidoformans* respectively. *Fusobacterium necrophorum* is a Gram-negative, non-spore-forming anaerobe. It has been strongly associated with ID and VFR (Robert and Egerton, 1969; Bennett *et al.*, 2009). In a recent analysis from a longitudinal study (Witcomb *et al.*, submitted), *F. necrophorum* was monitored in H, ID and VFR sheep and there was no difference in *F. necrophorum* load between feet with H, ID or VFR (except in some of the sheep with VFR). These authors suggested that *F. necrophorum* plays a role in persistence and / or severity of disease once the VFR lesion has developed. *F. necrophorum* is a normal inhabitant of the alimentary tract of animals (Langworth, 1977) and is detected in faecal (Tadepalli *et al.*, 2009) and oral (Zaura *et al.*, 2009) material. It is also associated with abscesses in sheep feet (Nagaraja *et al.*, 2005; Zhou *et al.*, 2009).

There were 9 phylotypes of *Arcanobacterium* detected in all three flocks and with all clinical presentations. The phylotype from flock B sheep with ID had 99% sequence similarities to *A. pyogenes* isolated from cows with endometritis and resistant to antimicrobial resistance gene *TetW* (Liu *et al.*, 2009) whereas phylotypes from H sheep in flocks A and C showed 98% to *A. pluranimalium*, isolated from dog skin and deep lung abscesses (Lawson *et al.*, 2001). *Arcanobacterium pyogenes* is a Gram positive, non-motile, non-spore forming facultative anaerobe. It is a short, rod-shaped bacterium and a common inhabitant of the mucous membranes of ruminants, pigs and other domestic animals (Carter and Chengappa, 1991). It is an opportunistic pathogen causing diseases in dairy and beef cattle and swine (Jost and Billington, 2005), and foot diseases in domestic and wild animals (Davies *et al.*, 1999;

326 Lavín *et al.,* 2004). This bacterium has been isolated from necrotic disease caused by *F.*
327 *necrophorum* (Chrino-Trejo *et al.,* 2003; Jones *et al.,* 2004; Nagaraja *et al.,* 2005), however,
328 there is no clear evidence of its association with footrot in sheep. *A. pluranimalium* is a new
329 species of *Arcanobacterium* recently described (Lawson *et al.,* 2001). It has been isolated
330 from the spleen of a dead harbour porpoise, from a lung abscess from a dead fallow deer and
331 from a pyoderma in a dog (Ulbegi-Mohyla *et al.,* 2010). It has never been found or described
332 in other hosts.

333 *Treponema* spp. were detected in only one sheep with ID from flock C. There were
334 two sequences and they had 94 and 99% similarity to uncultured *Treponema* phylotypes from
335 samples from cattle with digital dermatitis and from animal faecal samples (Klitgaard *et al.,*
336 2008; Ley *et al.,* 2008). *Treponema* are often free living but are linked to contagious ovine
337 digital dermatitis (CODD) and bovine digital dermatitis (BDD) in sheep and cattle
338 respectively (Demirkan *et al.,* 2006; Evans *et al.,* 2008; Evans *et al.,* 2009; Collinghan *et al.,*
339 2000; Moore *et al.,* 2005; Sayers *et al.,* 2009). Several species have been associated to CODD
340 and DD including *Treponema phagedenis*-like and *Treponema medium/Treponema vincentii*-
341 like, *Treponema medium/Treponema vincentii*-like, *Treponema phagedenis*-like, and
342 *Treponema denticola/Treponema putidum*-like (Sayers *et al.,* 2009). *Fusobacterium*,
343 *Treponema* and *Arcanobacteria* were not detected in the clone libraries but only in the
344 pyrosequencing data in the flock with footrot history suggesting that due to the low
345 prevalence may not be associated with VFR.

346 The difference in the structure of the bacterial community by farm might be linked to
347 factors such as different breeds which might have differing susceptibility to disease; footrot
348 has low heritability (Emery *et al.,* 1984; Skerman and Moorhouse, 1987; Escayg *et al.,* 1997)
349 or location with e.g. varying soil types or climate. In addition, management factors such as
350 use of antibiotics, hoof horn trimming and culling diseased sheep (Howell-Jones *et al.,* 2005;

Green *et al.*, 2007) might have affected the bacterial community. The managements used in these flocks is unknown, however, antibiotics or physical damage to the interdigital skin may alter the microbial community structure on the skin, increasing or decreasing (Kaler *et al.*, 2010a; 2010b) the incidence of disease.

Profiling bacterial community by DGGE

A comparative analysis of the profile of the total bacterial community assessed by DGGE (Figure 5) showed a visual, qualitative analysis of the predominant bacterial populations across flocks and conditions that were confirmed by deep sequencing and analyses of clone libraries. DGGE banding patterns from H sheep from flocks A and B were clustered independently from samples from sheep with ID and VFR from flocks B and C, suggesting that the latter samples shared a similar bacterial population. Unique and common DGGE bands were selected and a total of 31 DGGE bands were extracted, purified and sequenced covering all flocks and feet conditions. These bands were identified (95-99% sequence similarities) to *Corynebacterium sp.* and *Actinobacterium sp.* (DGGE-1 and DGGE-7), *Arcanobacterium sp.* (DGGE-2), *Macrococcus spp* (DGGE-3), *gamma Proteobacteria* (DGGE-4), uncultured *Actinobacterium sp.*, (DGGE-5), two uncultured *Bacillus* spp. (DGGE-6 and DGGE-8) and swine faecal bacterium (DGGE-9). One of the advantages of using DGGE for comparative analysis of parallel samples is the low cost and fast visual interpretation. The DGGE band position of *Macrococcus* sp. was similar to that of a DDGE of *D. nodosus*, which might be one of the reasons why *D. nodosus* was not isolated from the bands extracted from the gel.

Detection and quantification of D. nodosus in feet

375 *D. nodosus* were difficult to amplify and detect using bacterial community 16S rRNA
376 libraries. This can be explained by either primer mismatches or low abundance or both.
377 Bacterial community analyses of environmental samples relied on PCR amplification of the
378 16S rRNA gene using universal primers, targeting the variable regions (Lane, 1991). The *D.*
379 *nodosus* 16S rRNA gene does not amplify at 60^{o}C using the 27F and 1525R primers because
380 of two continuous mismatches at the 5' end of the 16S rRNA in *D. nodosus*
381 (AGAGTTTGAT**TC**TGGCTCAG) of the 27F primer (AGAGTTTGAT**CM**TGGCTCAG)
382 (Lane, 1991) that prevent amplification at 60^{o}C. PCR amplification was observed at 50-55^{o}C
383 and confirmed by sequencing. Amplification of *D. nodosus* occurred in all samples from all
384 sheep in all flocks by direct or nested PCR. Amplicons were confirmed by sequencing.
385 Variable or failed amplification occurred when direct amplification of 16S rRNA specific to
386 *D. nodosus* was performed because the cell number may was below the detection limit. This
387 variability in the amplification was solved by using nested PCR. The variable regions V1–V9,
388 of the 16S rRNA genes (rDNAs) have been used for species identification (Lane, 1991;
389 Weisburg *et al.,* 1991) but also to assess bacterial diversity in several habitats for the past 17
390 years. However, the use of the universal primer single mismatch 27F has recently been
391 criticised for the its amplification efficiency (Frank *et al.,* 2008; Galkiewicz *et al.,* 2008) and a
392 new 27F priming-binding site has been suggested that can accommodate mismatching
393 allowing minimal loss of efficiently and without compromising specificity with the reduction
394 of annealing temperature.
395 *D. nodosus* was also not detected in the 61,709 sequences produced by
396 pyrosequencing; however analysis of the sequence reads removed during quality control
397 checks revealed that *D. nodosus* sequences were amplified but removed due to sequence
398 errors or mis-priming when using bacterial universal primers (see material and methods).

We developed a quantitative real time PCR (qPCR) platform based on the presence of the RNA polymerase sigma-70 factor gene (*rpoD*) (single copy in the genome) in order to enumerate *D. nodosus* in individual feet and assess differences between across flock and condition. The absolute quantification of *D. nodosus rpoD* in clinical tissue DNA samples for individual feet from 15 sheep (60 foot samples) is shown in Figure 6a. As we mention above, *D. nodosus* was PCR detected in all feet sampled by direct or nested PCR for all three flocks, however, *D. nodosus* was quantifiable by qPCR in only 8 (25 feet) out of 15 sheep (60 feet) with a variable number depending on the clinical condition. This may be because cell numbers in these samples were below the detection limit (10^3 cell/g). The quantity of *D. nodosus* was significantly higher in sheep from flock B than from flock A based on the Mann-Whitney test (Figure 6b). The numbers of *rpoD* copies per gram of tissue sample ranged from 10^3 to 10^9. In H feet, *D. nodosus* was detected at 10^3 to 10^5 cell/g tissue in flock B but was not quantifiable in H feet from flocks A or C. In feet with ID, *D. nodosus* was detected with values from 10^4 - 10^9 for samples from flock B and flock C. *D. nodosus* numbers were significantly higher in feet with ID than in healthy feet across all flocks (Mann-Whitney P <0.01). *D. nodosus* was not quantifiable in VFR feet, but detected by PCR. These results are similar to those of Witcomb *et al.,* (submitted) who also reported an increase in abundance before the development of VFR and a reduction once a sheep had VFR. The absence or lower numbers of *D. nodosus* in cases of VFR may be because the organism is deeper in the tissue of the foot (Egerton *et al.,* 1969), has sloughed off in necrotic tissue or because other bacteria dominate once VFR has occurred. Although *D. nodosus* cell load is critically important (Witcomb *et al.,* unpublished) there are other factors that may contribute with the development/clinical presentation of the diseases as described above.

Conclusions

We present the first study of the ovine foot 16S rRNA based microbiome. Our results show that independent of the sequencing approached used, the bacterial community structure, diversity and abundance differ by clinical condition with some variation between sheep and flock. Pyrosequencing produced a higher resolution of the taxa present in the skin compared with clone libraries. We identified bacterial populations that were associated with healthy (*Peptostreptococcus*), ID (*Corynebacterium*) and VFR (*Staphylococcus*) affected sheep. Although the three genera are common inhabitants of human and animal skin, we envisage that extrinsic and intrinsic factors might affect their niche, producing an imbalance in the bacterial community from healthy to diseased feet. *D. nodosus* was present in all samples independent of the clinical condition but the number peaked in ID.

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Acknowledgment

This research was supported by the Biotechnology and Biological Sciences Research Council UK (BBRSC) under the Combating Endemic Diseases of Farmed Animals for Sustainability (CEDFAS) initiative Grant No. BBE01870X1.

Figure legends

Figure 1 OTU-based similarity among communities considered by disease condition and flock using canonical correspondance analysis (a) and Jaccard similarity clustering (b) based on observed richness of taxa defined at a 97% similarity cutoff as described in the methods. Panel (a) superimposes two separate ordinations; pyrosequenced samples (representing 61,708 sequences) are shown in black while Sanger-sequenced clone libraries (representing 1,130 sequences) are shown in blue in (a) and the right-hand side of (b). Scale bars in (b) represent 5% dissimilarity.

Figure 2 Rarefaction curves (a) and the number of shared and unique OTUs among the three disease conditions (b) Venn diagram of the number of shared and unique OTUs among the three disease conditions for sequences from pyrosequences for OTUs defined at a 97% similarity cutoff. The legend in (a) shows the number of OTUs observed and the Shannon diversity index, H'. A total of 717 OTUs were recovered.

Figure 3 a) Phylogenetic positions of 717 representative sequences for each OTU defined at a 3% cutoff as described in the text. Disease conditions are shown on the outer ring (white, healthy; grey, ID; black, VFR). Significant phylogenetic clustering of disease conditions was indicated by the UniFrac test ($p<0.002$) and Parsimony tests ($p<0.01$) as described in the text. b) Phylogenetic positions of signature taxa for diseased condition (red) and taxa defined as ubiquitous (blue) in both healthy and diseased animals. c) Phylogenetic position of signature taxa for the healthy condition (green). Signature taxa are as shown in supplementary Figure 1. Trees in (b) and (c) include a non-redundant list of the best matches to each sequence as defined by the Silva project (Release 102);

trees were constructed using the PhyML maximum-likelihood algorithm as fully described in the methods.

Supplementary Figure 1 Distributions of each of the 717 clusters (OTUs) defined at a 3% cut off according to the proportion of sequences representing each disease condition. Signature taxa were defined based on the relative proportions of each condition (red, diseased; blue, ubiquitous; green, healthy).

Figure 4 Summary of taxonomic classifications at the genus and phylum levels for sequences obtained from pyrosequencing for each of the three disease conditions H (healthy), ID (interdigital dermatitis), and VFR (virulent footrot) at the genus (a) and phylum (b) levels. Y axes represent proportion of sequences for each disease condition. Taxonomic classifications are based on EMBL taxonomy and were obtained by blastn with default parameters against a customized database of all type strains from the Silva SSU Ref database (Release 102) as described in the methods. Significant differences ($p<0.05$, Chi-square test) between disease conditions for any given taxon are indicated by * symbols above bars; number of symbols corresponds to number of significant pairwise differences.

Figure 5. Denaturing gradient gel electrophoresis (DGGE) gel of 16S rRNA genes and UPGM (Jaccard's coefficient) dendrogram from interdigital skin tissue sample DNA. All feet were clinically scored as healthy (H), with interdigital dermatitis (ID) and with virulent footrot (VFR) sheep for flock A (H1A, H2A, H3A), flock B (H1B, H2B, H3B, ID1B, ID2B) and flock C (H1C, H2C, H3C, ID1C, ID2C, ID3C, VFR1C, VFR2C). M; molecular weight marker; DN = *Dichelobacter nodosus*. DGGE bands 1-9.

Figure 6. Quantitative PCR (qPCR) of the RNA polymerase sigma-70 factor gene (*rpoD*) from interdigital skin biopsy tissue DNA of 15 animals from flocks A, B and C. (5a) *D. nodosus rpoD* was quantified in all feet clinically scored as healthy (h), with interdigital dermatitis (id) and with virulent footrot (vfr) for H, ID and VFR diagnosed sheep for flock A (1A, 2A, 3A), flock B (1B, 2B, 3B) and flock C (1C, 2C). Samples were taking from all feet as follows; LH left hind, RH right hind, RF right front, LF left front. † Not quantitative, detected by 16S rRNA PCR *D. nodosus* specific but below detection limit. (5b) Mann-Whitney test for comparison of *D. nodosus* numbers prevalence based on clinical condition in feet across flocks for healthy (H) and feet with interdigital dermatitis (ID). Calibration standards generated a curve line R^2=0.99 with a -3.70 slope and a Ct range of 17-27.

742 **Tables**

743 **Table 1. Flock code by clinical condition of feet and flock**

Flock	Healthy (H)	Interdigital dermatitis (ID)	Virulent footrot (VFR)
Flock A			
sheep 1	**H1A**	**-**	**-**
	LF h		
	RF h		
	RH h		
	LH h		
sheep 2	**H2A**	**-**	**-**
	LF h		
	RF h		
	RH h		
	LH h		
sheep 3	**H3A**	**-**	**-**
	LF h		
	RF h		

	RH h		
	LH h		
Flock B			
sheep 1	**H1B**	**ID1B**	**-**
	LF h	**LF id**	
	RF h	**RF h**	
	RH h	**RH h**	
	LH h	**LH h**	
sheep 2	**H2B**	**ID2B**	**-**
	LF h	**LF id**	
	RF h	**RF h**	
	RH h	**RH id**	
	LH h	**LH id**	
sheep 3	**H3B**	**NA**	**-**
	LF h		
	RF h		
	RH h		
	LH h		

Flock C			
sheep 1	**H1C**	**ID1C**	**VFR1C**
	LF h	**LF id**	**LF vfr**
	RF h	**RF id**	**RF vfr**
	RH h	**RH id**	**LH vfr**
	LH h	**LH id**	**RH vfr**
sheep 2	**H2C**	**ID2C**	**VFR2C**
	LF h	**LF h**	**LF vfr**
	RF h	**RF h**	**RF h**
	RH h	**LH id**	**LH h**
	LH h	**RH h**	**RH h**
sheep 3	**NA**	**ID3C**	**NA**
		LF id	
		RF id	
		LH id	
		RH id	

744

745 The sheep were classified as having either healthy interdigital skin (H), interdigital
746 dermatitis (ID) or virulent footrot (VFR). The feet were classified as foot without

abnormality (h), having interdigital dermatitis (id) inflamed interdigital space, white/grey pasty scum hair loss or having hoof horn separation (separation of horn from underlying tissue with or without interdigital dermatitis) (vfr). NA, Not available

772 **Table 2** Primers and Taqman probes used

Primer name	**Sequence (5'- 3')**
Cc	TCGGTACCGAGTATTTCTACCCAACACCT
Ac	CGGGGTTATGTAGCTTGC
16S rRNA-27F	AGAGTTTGATCMTGGCTCAG
16S rRNA-1525R	AAGGAGGTGWTCCARCC
P2	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGG
	GGGCCTACGGGAGGCAGCA
P3	ATTACCGCGGCTGCTGG
rpoDF	gCTCCCATTTCgCgCATAT
rpoDR	CTgATgCAgAAgTCggTAgAACA
Taqman rpoD	6FAM1 CATTCTTACCggKCg-BBQ2

773

774 16FAM reporter, 2BBQ BlackBerry Quencher, K=A/T/C