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Potential bacterial core species associated with digital dermatitis in cattle herds identified by molecular profiling of interdigital skin samples

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Although treponemes are consistently identified in tissue from bovine digital dermatitis (DD) lesions, the definitive etiology of this debilitating polymicrobial disease is still unresolved. To study the microbiomes of 27 DD-infected and 10 healthy interdigital skin samples, we used a combination of different molecular methods. Deep sequencing of the 16S rRNA gene variable regions V1–V2 showed that Treponema, Mycoplasma, Fusobacterium and Porphyromonas were the genera best differentiating the DD samples from the controls. Additional deep sequencing analysis of the most abundant genus, Treponema, targeting another variable region of the 16S rRNA gene, V3–V4, identified 15 different phylotypes, among which Treponema phagedenis-like and Treponema refringens-like species were the most abundant. Although the presence of Treponema spp., Fusobacterium necrophorum and Porphyromonas levi was confirmed by fluorescence in situ hybridization (FISH), the results for Mycoplasma spp. were inconclusive. Extensive treponemal epidermal infiltration, constituting more than 90% of the total bacterial population, was observed in 24 of the 27 DD samples. F. necrophorum and P. levi were superficially located in the epidermal lesions and were present in only a subset of samples. RT-qPCR analysis showed that treponemes were also actively expressing a panel of virulence factors at the site of infection. Our results further support the hypothesis that species belonging to the genus Treponema are major pathogens of DD and also provide sufficient clues to motivate additional research into the role of M. fermentans, F. necrophorum and P. levi in the etiology of DD.

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1. Introduction

Bovine digital dermatitis (DD) is presently the most common cause of lesions associated with lameness in dairy cattle worldwide (Laven and Logue, 2006). This debilitating disease has a huge impact on animal welfare and productivity (Losinger, 2006). DD most probably has a bacterial etiology because no virus or fungus of any significance has been identified from the lesions (Krull et al., 2014). Likewise, the immunological response of afflicted cows and the positive effect of antibiotic treatment seem to support this notion (Demirkan et al., 1999). Various species of spirochetes have been cultivated from the affected lesions (Pringle et al., 2008; Evans et al., 2009); and with the advent of 16S rRNA gene sequencing and fluorescence in situ hybridization (FISH), it has been shown that a considerable number of Treponema phylotypes are present in DD lesions (Moter et al., 1998; Klitgaard et al., 2008; Rasmussen et al., 2012). Phylogenetically, the identified treponemes mainly belong to 4 major groups, Treponema phagedenis-like, Treponema denticola/Treponema pedis-like, Treponema medium/Treponema vincentii-like and Treponema refringens-like phylotypes (Yano et al., 2010; Santos et al., 2012; Klitgaard et al., 2013). These bacteria are consistently reported to be located deep within the affected tissue (in the front part of the lesion) and to outnumber other bacterial morphotypes. Furthermore, their presence is always associated with degenerative and necrotic changes of the infected tissue (Choi et al., 1997). These tissue-destructive properties have been reported to be enhanced by and to probably be dependent upon the presence of other microorganisms found in these polymicrobial infections (Edwards et al., 2003). However, it must be emphasized that Koch's postulates have not been fulfilled for DD, and the definitive etiology of the disease is therefore still unresolved.
In addition to treponemes, the range of taxa identified from DD lesions by bacteriological, histopathological, and molecular biological investigations include the genera Porphyromonas, Prevotella, Fusobacterium, Campylobacter, Bacteroides, Mycobacterium, Mycoplasma and Guggenheimella (Wyss et al., 2005; Santos et al., 2012; Krull et al., 2014). Many of these bacterial groups, however, seem to be primarily located in the superficial parts of the lesions, and their potential roles in the pathogenesis of DD are still unresolved. The bacterium Dichelobacter nodosus, the cause of bovine foot rot, is commonly seen invading the epidermis in DD lesions (Rasmussen et al., 2012).

The aim of the present study was to determine whether other bacteria, besides treponemes, are consistently associated with DD and to investigate whether we could link distinct phylotypes of Treponema to specific stages of DD lesion development as previously indicated (Krull et al., 2014; Zinicola et al., 2015). FISH analysis was applied to visualize and localize selected microbial taxa identified by deep sequencing of the 16S rRNA gene. Finally, the expression of a number of known or putative Treponema virulence genes in DD lesions was measured by reverse transcription real-time PCR (RT-qPCR). To date, only the immunological host gene response has been investigated for DD (Zuerner et al., 2007), and the active expression of candidate virulence genes would be a further indication that Treponema are actively involved in the etiology of this disease.

2. Materials and methods

2.1. Sample collection and preparation

All sample material was collected from a Danish cattle slaughterhouse. Tissue samples were collected from the hind feet of slaughtered Holstein-Friesian dairy cattle after routine slaughter procedures. The feet were gently rinsed with water and cleaned with a soft-haired brush to remove any gross organic debris, and the dorsal/posterior skin between the digits was classified as early (M1), acute ulcerative (M2), healing (M3) or chronic lesions (M4) according to Döpfer et al. (1997).

Tissue samples were collected with a sterile scalpel and transferred to a sterile Petri dish. The samples were subdivided and stored for histologic examination and DNA purification. The sample portions intended for 16S rRNA gene analysis were immediately transferred to a nucleic acid stabilization solution (RNALater®, Ambion, Austin TX) and stored at −20°C. Samples intended for histopathology analysis were fixed in 10% neutral buffered formalin solution, dehydrated, embedded in paraffin wax, sectioned (5-μm thick) and mounted on SuperFrost Plus slides (Menzel-Gläser, Braunschweig, Germany).

2.2. Histopathology

All tissue samples were stained with hematoxylin and eosin (H&E) for histopathological evaluation. The degree of epidermal damage and the inflammatory response of the dermis were scored from 0 to 2 according to Rasmussen et al. (2012), with minor modifications. A score of 0 was defined as normal epidermis or mild epidermal damage with some epithelial proliferation and hyperkeratosis, dermis being unaffected. Score 1 was defined as epithelial proliferation and acanthosis (with ballooning degeneration and mal-keratinization) while the inflammatory response was seen as mild to moderate increase in the number of lymphocytes and mononuclear cells. Score 2 was defined as severe epithelial proliferation, acanthosis and exudation, erosion or necrosis of the dermal papilla and moderate to severe infiltration with lymphocytes and/or mononuclear cells in dermis and perivascular dermatitis.

2.3. Fluorescent in situ hybridization (FISH)

The oligonucleotide probes used in this study are listed in Table S1 and include probes specific for Domain Bacterium, Treponema spp., Fusobacterium necrophorum, Dichelobacter nodosus, Porphyromonas levii, Mycoplasma spp., and Mycoplasma fermentans. Newly designed oligonucleotide probes were selected using the software ARB (http://www.arb-home.de). The oligonucleotide probes were 5’ labeled with fluorescein isothiocyanate (FITC) or the isothiocyanate derivative Cy3 (Eurofins MWG Operon, Ebersberg, Germany). The hybridization was carried out at 45°C with 100 μl of hybridization buffer (10 μl of 1 M Tris [pH 7.2], 18 μl of 5 M NaCl, 1 μl of 10% sodium dodecyl sulfate, 71 μl of H2O) and 500 ng of each probe for 16 h in a Sequenza slide rack (Thermo Shandon, Cheshire, United Kingdom). The sections were then washed three times in prewarmed (45°C) hybridization buffer for 9 min and subsequently washed three times in prewarmed (45°C) washing solution (10 μl of 1 M Tris [pH 7.2], 18 μl of 5 M NaCl, 72 μl of H2O). The sections were rinsed in water, air dried, and mounted in Vectashield (Vector Laboratories Inc., Burlingame, CA) for epifluorescence microscopy. An Axioimager M1 epifluorescence microscope equipped for epifluorescence with a 100-W HBO lamp and filter sets 43 and 38 was used to visualize Cy3 and FITC, respectively. Images were obtained using an AxioCam MRm version 3 FireWiremonochrome camera and AxioVision software version 4.5 (Carl Zeiss, Oberkochen, Germany).

2.4. Nucleotide extraction

DNA and RNA were extracted from the biopsies using an AllPrep® DNA/RNA/miRNA kit (Qiagen, Hilden, Germany). Portions (20 mg) of stabilized tissue were first homogenized in 600 μl of RTL plus buffer (included in the kit). A sterile 5-mm steel bead (Qiagen) was added, and samples were run two times on a TissueLyser II (Qiagen) at 20 Hz for 2–5 min per run. All the subsequent procedures were performed according to the protocols of the supplier. After RNA extraction, the material was additionally treated with TURBOTM DNase, according to the protocol provided by the manufacturer (Ambion), to ensure total DNA degradation in the RNA preparations. For the evaluation of DNA and RNA concentration and purity, samples were analyzed on a NanoDrop ND-1000 spectrophotometer ( Fisher Scientific, Wilmington, MA). DNA samples with A260/A280 ratios >1.5 were selected for further analysis. The RNA quality was estimated on an Agilent Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA).

2.5. Reverse transcription

Reverse transcription was performed using a QuantiTect Reverse Transcription kit according to the manufacturers instructions (Qiagen). The concentration and quality of cDNA samples were measured with a NanoDrop ND-1000 spectrophotometer (Fisher Scientific). All cDNA samples with A260/A280 ratios ≥1.8 were included in the downstream analysis.

2.6. Preparation of 16S rRNA gene amplicon libraries and sequencing

PCR amplification of DNA was accomplished with a universal bacterial primer set (Wilmotte et al., 1993) and a Treponema-specific primer set (Klitgaard et al., 2008) targeting the V1–V2 region and the V3–V4 hyper variable regions of the 16S rRNA gene (primer sequences listed in S1). Each sample was amplified with unique forward and reverse primers that included an added hexamer barcode at their 5’ ends. Amplification PCRs were performed in 50-μl reaction mixtures containing 5 μl of 10XPCR Gold Buffer (Applied Biosystems, Foster City, CA, USA) 1.5 mM
MgCl₂ solution (Applied Biosystems), 200 μM of each deoxynucleoside triphosphate (Amersham Biosciences, Piscataway, NJ), 0.4 μM of each specific primer, 2.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems), and 2 μL of template DNA. For both primer sets, thermal cycling using a T3 thermocycler (Biometram, Göttingen, Germany) was performed as follows: denaturation at 94 °C for 6 min; 30 cycles of denaturation at 94 °C for 45 s, annealing at 57 °C for 45 s, and extension at 72 °C for 90 s. A final elongation step of 10 min was followed by cooling to 4 °C. Positive (DNA) and negative (dH₂O) controls were included for each PCR setup. The DNA concentration and quality of the PCR amplicons from all samples were assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies Inc.) prior to high-throughput sequencing (data not shown). Equal amounts of all amplicons were pooled (final concentration between 3.8–4 μg) and purified with the Qiagen Mini Elute kit (Qiagen) according to the protocol of the manufacturer. The DNA was submitted to the National High-Throughput DNA Sequencing Centre at the University of Copenhagen, Denmark for sequencing on the Illumina MiSeq platform.

Sequences generated by Illumina MiSeq are available under the accession number SRP065522 in the NCBI Sequence Read Archive (SRA).

2.7. Sequence analysis

For both sets of sequences, the obtained reads were analyzed using the BION-meta software (http://box.com/bion). The demultiplexing step was performed according to the primer- and barcode sequences. Forward and reverse sequences were joined, allowing no gaps, a maximum mismatch percentage of 80% and an overlap length of minimum 35 bp. Next, the sequences were cleaned at both ends by the removal of bases of a quality less than 98%, which is equivalent to a Phred score of 17. Identical sequences were de-replicated into consensus sequences of 300–322 bp. Consensus sequences of at least 250 nucleotides in length were mapped into a table, according to the individual barcodes, and taxonomically classified against the Ribosomal Database Project database II (RDP II; http://rdp.msu.edu/index.jsp) using a word length of 8 and a match minimum of 90%. To allow for the comparison of relative abundance between samples, the number of reads for each barcode was normalized.

To elucidate the microbial patterns involved in DD-etiiology, multivariate analysis of the resulting microbial profiles was carried out by Analysis Of Similarities (ANOSIM) and Constrained Analysis of Principal coordinates (CAP) with Bray-Curtis distances constrained by histological score (Anderson and Willis, 2003). Genera identified by CAP as being associated with DD were further analyzed with the Kruskal-Wallis test in order to pinpoint differences in bacterial compositions between scores 1 and 2.

2.8. Real-time quantitative reverse transcriptase PCR (RT-qPCR)

Amplification, detection and real-time quantitative analysis were performed using the Rotor-Gene Q (Qiagen, Hilden, Germany). The sequences of the Treponema genes studied were obtained from GenBank, and the primers were designed in Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) (Rozen and Skaltsky, 2000). The nucleotide sequences of the primers used in this study are listed in Table S1. Each PCR was performed in a 25-μl reaction mixture containing 12.5 μl of QuantiTect SYBR Green PCR master mix (Qiagen), a primer concentration of 0.2 μM (a mix of primers targeting T. denticola, T. vincentii and T. phagedenis) and 200 ng of cDNA. Three replicates were included for each sample. The thermal cycling conditions were as follows: 15 min at 95 °C, followed by 40 cycles of 30 s at 94 °C, 20 s at 55 °C, and 20 s at 72 °C. Data collection was performed during each extension phase. Positive controls (DNA) and negative

Table 1
Summary of pathology and histology scores for the 37 bovine skin samples.

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<th>F. necrophorum</th>
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*a* for explanation of this score see in text.

*b* Due to the inconclusive results, the FISH data for M. fermentans were not included in the Table.
controls (distilled water) were included in each run. The control for DNA contamination was performed by RT-qPCR on the mRNA samples. Melting curve analysis was performed for each species of Treponema, which resulted in single product-specific melting curves for all primer sets.

For every gene, a standard curve comprising a pool of cDNA (5 points of 5 × dilutions) from all the samples was included in each run and was used to determine the amplification efficiency and the target concentration of the individual samples. The reference genes were tested in geNorm (GenEx 6; MultiD Analyses AB, Göteborg, Sweden), and the geometric mean of two internal reference genes, tpiA and tkt, was used to correct the raw values for the genes of interest (Vandesompele et al., 2002).

2.9. PCR of fermentans

All the included DNA samples and a subset of cDNA samples were subjected to selective detection of M. fermentans by the PCR amplification of a conserved 206 bp region of the insertion sequence-like element, IS1550, of M. fermentans (Wang et al., 1992) and a 414 bp region of the macrophage-activating lipopeptide gene (malp) (Afshar et al., 2008). The 206 bp region was detected by standard PCR with 2.5 U thermostable recombinant AmpliTaq DNA polymerase (Applied Biosystems), 5 μl of 10xPCR Buffer II (Applied Biosystems), 1.5 mM MgCl₂ Solution (Applied Biosystems), 100 μM of each deoxynucleoside triphosphate (Amersham Biosciences, Piscataway, NJ), 0.2 μM of each specific primer, and 0.3 μg template DNA in a 50 μl total volume. Thermal cycling was performed in a T3 thermocycler (Biometra, Göttingen, Germany) under the following conditions: denaturation at 94 °C for 3 min and 45 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 90 s. A final elongation step of 10 min was followed by cooling to 4 °C. For detection of the malp gene, we first amplified the entire MALP-404 lipoprotein coding sequence. From this 1373 bp PCR fragment, a second nested PCR was performed using primers targeting a 414 bp fragment. PCR and cycling conditions were performed according to the protocol provided by Afshar et al. (2008). For all PCR reactions, boiling lysate of M. fermentans (E-9) was used as a positive control. The PCR products were separated on a 2% E-gel (Invitrogen, Carlsbad, CA, USA), and the DNA was visualized by UV fluorescence. Two malp PCR products were sequenced on an ABI

Fig. 1. (A) Bovine digital dermatitis lesion (biopsy No. 13), characterized by acanthotic epidermis with degenerated ballooning keratinocytes, exocytosis, ulceration of dermal papillae, and bacterial infiltration. Hair follicle (star). The shading represents the area used for fluorescent in situ hybridization (FISH) on parallel sections in B and C, H&E. B) Demonstration of Porphyromonas levii organisms (orange arrows) in the superficial layers of the lesion, FISH. C) Demonstration of severe infiltration with Treponema spp.
3130 genetic analyzer (Applied Biosystems) using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems), according to the manufacturer’s instructions.

3. Results

3.1. Pathology and histology

Twenty-seven cases of DD with varying degrees of affected skin and 10 control cases were selected and sampled at a slaughterhouse. Four cases were grossly characterized by circumscribed ulcerations and hyperkeratosis with crust formation (M3). Fourteen cases showed circumscribed lesions dominated by significant hyperkeratosis and proliferation of the epidermis but without visible ulceration (M4). Nine cases showed only mild focal hyperkeratosis/proliferation of the epidermis (not definable according to Döpfer et al. (1997); thus, in this study, they were designated M5). Ten cases revealed no gross lesions. The scores are listed in Table 1.

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**Fig. 2.** Average relative abundance of taxonomic bacterial groups in control and DD lesions derived from 37 biopsies using 16S rRNA gene based metagenomics and V1–V2 general eubacterial primers. A) At the phylum level, B) at the family level (relative abundance of bacterial families that represented at least 1% of the bacterial reads acquired).
The samples in the present project originated from a slaughterhouse; we had no clinical anamnesis or information regarding the chronology of the lesions nor was it possible to pinpoint the infection stage from the histological examinations with certainty. Instead, the lesions were classified into groups based on histological criteria. The histology scores are presented in Table 1. Seventeen samples were histopathologically characterized by severe epithelial proliferation and acanthosis with exudation, erosion or necrosis of the dermal papilla (score 2) as illustrated in Fig. 1. Ten samples were characterized by epithelial proliferation and acanthosis with varying ballooning degeneration and malkeronization (score 1). All cases without gross lesions appeared within the normal range (score 0).

3.2. 16S rRNA sequencing

We sequenced 37 samples with the primers V1–V2 and 31 samples with the primers V3–V4. After de-multiplexing according to the sequences of the barcodes and primers, 5,047,554 and 3,235,052 sequences remained in the V1–V2 pool and V3–V4 pool, respectively. The 3’ and 5’ ends of these sequences were further trimmed according to quality. Sequences with quality below 98% were discarded. In total, 2,428,737 (V1–V2 pool) and 1,613,962 (V3–V4 pool) joined sequences were used for taxonomic classification, equivalent to average reads per sample of 65,641 and 52,063, respectively. Of these sequences, 27% of the V1–V2 pool and 76% of the V3–V4 pool were taxonomically classifiable according to the RDP II database (http://rdp.cme.msu.edu/).

3.3. Bacterial amplicons

The dataset included sequences of microbiota isolated from 37 bovine hoof skin samples, including 10 from macroscopically normal skin (controls) and 27 from visible lesions with variable morphology and lesion scores (Table 1). The consensus sequences were taxonomically assigned using the RDP II database. Diversity of the three histological groups was estimated by using a Shannon index (Hill et al., 2003). We observed a significant decrease in phylotype richness in the DD lesions (histology score 1 ($H = 1.02 \pm 0.43$) and 2 ($H = 1.17 \pm 0.73$)) when compared to normal skin (histology score 0 ($H = 3.53, \pm 0.23$)). The phyla in the control samples mainly belonged to Firmicutes, Actinobacteria, Proteobacteria and Bacteroidetes (Fig. 2A), together covering 15 families with an average relative abundance of more than 1%. Of these, only four

![Figure 3](image_url)
families, Corynebacteriaceae, Carnobacteriaceae, Ruminococcaceae, and Moraxellaceae, exceeded an average relative abundance of 5% (Fig. 2B).

The sequences originating from the 27 DD lesions could largely be ascribed to the phyla Spirochaetes, Tenericutes, Fusobacteria and Bacteroidetes (Fig. 2A). At the family level, only Spirochaetaceae (47%), Mycoplasmataceae (28%) Fusobacteriaceae (8%) and Porphyromonadaceae (7%) exceeded an average relative abundance of 5% (Fig. 2B). Within these families the main genera represented were Treponema, Mycoplasma, Fusobacterium and Porphyromonas. Fig. 3 shows the individual distribution of the identified amplicons at the genus or species level in the 27 DD-biopsies. In 15 of the samples, Treponema species accounted for over half of the total sequence population detected. The second most frequent bacterial species, M. fermentans, had an abundance of 20% in 16 samples.

Analysis of similarities (ANOSIM) revealed a significant difference in the microbial composition between samples from the control group (histology score 0) and the DD-biopsies (histology score 1 and 2), but not between the DD-biopsies with different histology scores. We used constrained analysis of principal coordinates (CAP) to probe the underlying microbial patterns associated with DD etiology on the genus level. In this analysis, 49.5% of the variation could be explained. In the first dimension, accounting for 47.8% of the variation, Treponema, Mycoplasma, Fusobacterium and Porphyromonas were the genera that best differentiated the DD samples from the controls. On the second dimension, explaining very little of the variation, i.e., 1.7%, we observed a limited separation of histological scores 1 and 2, with lesion 2 scores associated with higher abundance of Porphyromonas and Mycoplasma and lower abundance of Treponema and Fusobacterium. In the univariate analysis, however, only the distribution of Porphyromonas was significantly different between scores 1 and 2 (p < 0.01, Kruskal-Wallis, Fig. S1).

3.4. Fermentans-specific PCRs

The presence of M. fermentans in the biopsies was confirmed by two species-specific PCR assays targeting the IS1550 and malp genes of this bacterium. Whereas all controls were negative with the malp PCR, C2 and C7 were positive with the IS1550 PCR. The IS1550 PCR is extremely sensitive and should be able to detect one single copy of the gene (Wang et al., 1992). This result may explain why C2 and C7 tested positive; we did see M. fermentans amplicons in these samples, although in very low amounts (below <0.04% abundance). The PCR results for the DD biopsies are shown in Fig. 3. To determine whether IS1550 and malp were also expressed by M. fermentans in the DD lesions, the two PCR assays were likewise performed with cDNA as template for a subset of samples (no. 1, 5,

![Fig. 4. Relative abundance of Treponema phylotypes in the microbiota of bovine hoof skin samples sorted by the prevalence of T. phagedenis. Sequence amplicons were derived from 31 biopsy specimens by the use of Treponema-specific primers, V3–V4, and 16S rRNA gene-based metagenomics. C signifies samples with no visible lesions, the remaining samples all displayed macroscopic lesions.](image-url)
8, 9, 12, 18, 19, 17, 24, 25 and 27). No *M. fermentans* amplicons were detected from these samples, which indicated no or very limited expression of IS1550 and *malp* in the infected tissue.

3.5. *Treponema amplicons*

In contrast to the sequences generated with the V1–V2 general primers, with which only *T. denticola* could be determined to the species level, we were able to identify approximately 94% of the amplicons generated with the *Treponema*-specific primers to the species/phylotype level. *Treponema* phylotypes with a relative abundance of <1% were joined in a group named *Treponema* spp. In total, 15 different species/phylotypes of *Treponema* were identified from 31 samples. The number of phylotypes with a relative abundance of >1% in the individual samples ranged between 1 and 10, with an average of 5 per sample. Six of the control samples, C4–C9, were omitted from this part of the study as no PCR amplicons could be generated from these samples with the *Treponema*-specific primers.

*Fig. 4* illustrates the relative distribution of *Treponema* phylotypes in the individual samples. Very few DD-related treponemes were seen in control samples C1 and C2. The treponemes from the DD biopsies and control samples C3 and C10 were all homologous to species previously identified in DD lesions. The most common species was *T. phagedenis* (24%), followed by the *T. refringens*-like and not-yet-cultivable PN-20 (15%), PT1 (14%) and PT4 (10%). There appeared to be no obvious correlation between lesion score and the relative abundance of *Treponema* variants. Because *Treponema* specific sequences could be amplified from only a subset of the controls, no multivariate analysis was performed on the V3–V4 generated dataset.

3.6. *In situ hybridization*

Applying double in situ hybridization with the probes for Domain Bacterium and *Treponema* spp., severe and extensive treponemal epidermal infiltration, constituting more than 90% of the total bacterial population, was found in 24 of the 27 samples with scores 1 and 2 (*Table 1, Fig. 1C*). In two samples with score 1 (no. 10 and 21), no treponemes were observed, and only a moderate number of bacteria were found infiltrating the epidermis. One sample (no. 18 with score 1) was omitted from in situ detection due to damage during sectioning. Invasive bacteria were not detected in any of the ten score 0 samples (only scarce bacteria on the epidermal surface); thus, further in situ detection was not carried out. *D. nodosus* was identified in 12 samples (4 score 1 cases and 8 score 2 cases) within mal-keratinized epidermis, in areas free of treponemes and other bacteria, as single bacteria or in loose clusters with up to 50 bacteria as illustrated in *Fig. 1D*. *F. necrophorum* was detected as single bacteria together with other bacteria (including treponemes) superficially in the epidermal lesions of eight samples (3 score 1 cases and 5 score 2 cases). In one case, however, *F. necrophorum* was detected in a cluster with more than 100 bacteria. Similarly, *P. levii* was detected as single bacteria or in small clusters, together with other bacteria superficially in the lesions, in eight score 2 samples as illustrated in *Fig. 1B*. The probes for *Mycoplasma* spp. and *M. fermentans* were tested on various type strains, including a *M. fermentans* strain isolated from humans, and they revealed moderate fluorescence signals. When applied on the case samples, no specific signal (positive both for Domain Bacterium and *Mycoplasma* spp./*M. fermentans*) could be identified.

3.7. *RT-qPCR analysis of putative virulence genes*

To determine the metabolic activity of the treponemes in the DD lesions, *RT-qPCR* was performed on 27 of the samples from which we could extract treponemal RNA (C2 and C3 plus all the DD-biopsies, except samples no. 8 and 18, which were omitted due to low concentration and quality of RNA). The target genes included in the analysis comprised putative virulence determinants and reference genes for normalization (*Table 2*). Because *T. denticola*, *T. vincentii* and *T. phagedenis* display considerable variation in their genomic sequences, it was not possible to design primers that could amplify all three species simultaneously for any of the selected target genes. Consequently, we constructed primers targeting each species and pooled the primers for the *RT-qPCR*. The specificity, dynamic range and amplification efficiency of the primers were tested on DNA extracted from *T. denticola* (ATCC33520), *T. vincentii* (ATCC35580) and *T. phagedenis* (ATCC27087) (*Table S2 in the Supplemental Material*). The relative quantity of each sample was estimated using an internal standard curve. The quantification values were normalized to the geometric mean of the two reference genes *tkt* and *tpIA*. All points outside the dynamic range were excluded from the analysis.

Of the 27 samples included, no signal or a very weak signal was generated from eight samples (samples no. C1, C2, 7, 9, 15, 22, 26 and 27). Thus, 19 samples remained in the analysis. In the case of the controls, the negative result was most likely due to the lack of target cDNA because most of the treponemes in these samples, according to the sequencing results, were environmental species. In samples no. 7, 15, 26 and 27, the overall abundance of

### Table 2

<table>
<thead>
<tr>
<th>Gene name</th>
<th>GenBank locus no.</th>
<th>Possible function</th>
</tr>
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<tbody>
<tr>
<td><strong>Virulence genes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Msp</td>
<td>TDE_0405</td>
<td>Major outer sheath protein antigen</td>
</tr>
<tr>
<td>FlaA</td>
<td>TDE_1408</td>
<td>Flagellar filament outer layer protein</td>
</tr>
<tr>
<td>FlgE</td>
<td>TDE_1007</td>
<td>Flagellar basal body rod protein</td>
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<tr>
<td>HbpB</td>
<td>TDE_0205</td>
<td>Hemin-binding protein B, lipoprotein</td>
</tr>
<tr>
<td>MplA</td>
<td>TDE_2216</td>
<td>Galactose/methylgalactoside import ATP-binding protein</td>
</tr>
<tr>
<td>DnaK</td>
<td>TDE_0628</td>
<td>Chaperone protein (Hsp70)</td>
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<tr>
<td><strong>Reference genes</strong></td>
<td></td>
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<tr>
<td>TpiA*</td>
<td>TDE_1236</td>
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<td>Hex</td>
<td>TDE_2469</td>
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<td>Transketolase</td>
</tr>
<tr>
<td>Lnt</td>
<td>TDE_1708</td>
<td>Apolipoprotein N-acyltransferase</td>
</tr>
</tbody>
</table>

* Reference genes applied in the analysis for normalization.
The Firmicutes depicted main lesions. Expression mglA successfully reached the highest levels. Zinicola refringens studies had shown normal treponemes and the generality best differentiating the infected tissue samples from controls. Our results are therefore mostly in accordance with the observations by Krull et al. (2014), who have also found the families Spirochaetaceae and Mycoplasmataceae to be the main constituents of clinical lesions.

4. Discussion

In this study we used high throughput sequencing, FISH and expression analysis to examine the microbial communities of normal and DD-affected bovine hoof skin. The 16S rRNA gene studies all revealed substantial differences in the bacterial microbiota composition between healthy samples and DD lesions. Firmicutes, Actinobacteria, Proteobacteria, and Bacteroides, the four main phyla identified in normal foot skin in this study, were also identified in healthy skin in at least one of the previous sequencing studies (Yano et al., 2010; Santos et al., 2012; Knull et al., 2014; Zinicola et al., 2015). At the family level, Moraxellaceae, Corinebacteriaceae, Lachnospiraceae and Ruminococcaceae were the most abundant in healthy samples.

Most of the DD specimens in our investigation were from chronic lesions with areas of ulceration, corresponding to active lesions. We found Spirochaetes (47%) and Tenericutes (27.7%) to be the most ubiquitous bacterial phyla in the DD lesions, and very few Firmicutes (3.6%) were identified in these samples. So, contrarily to a number of previous studies (Santos et al., 2012; Zinicola et al., 2015), we did not find evidence suggesting a pathogenic role for Firmicutes in DD. In this study, when probing the underlying microbial patterns associated with DD etiology, we found that Treponema, Mycoplasma, Fusobacterium and Porphyromonas were the genera best differentiating the infected tissue samples from controls. Our results are therefore mostly in accordance with the observations by Krull et al. (2014), who have also found the families Spirochaetaceae and Mycoplasmataceae to be the main constituents of clinical lesions.

Except for the work of Krull et al. (2014), who have hypothesized that Mycoplasma spp. may be of importance in the transition from early lesion to active lesion, Mycoplasma has hitherto not received much attention as a candidate pathogen even though M. fermentans has previously been cultivated from the front of a DD lesion (Wyss et al., 2005) and identified by 16S rRNA gene sequencing (Santos et al., 2012; Zinicola et al., 2015). M. fermentans, which is a member of the phylum Tenericutes, was originally isolated from the human urogenital tract and has since been associated with a number of human diseases (Pitcher and Hilbous, 1998). In fact, it was first thought to be restricted to humans, but the species has since been isolated from genital lesions in sheep (Ayling et al., 2004). M. fermentans-specific PCR verified that this species was indeed present in the DD samples of the present study. However, the inconclusive results of the Mycoplasma-specific FISH analysis, combined with the negative results for 16S rRNA and mtp in the M. fermentans-specific RT-PCR, seem to imply that these bacteria are not very active in the lesions at the stages of the disease represented here.

Amplicons homologous to F. necrophorum and P. levii were the third and fourth most abundant variants in the DD biopsies,
respectively, and these species have previously been identified in DD lesions (Cruz et al., 2005; Wyss et al., 2005; Rasmussen et al., 2012). In this study, Porphyromonas was significantly associated with ulcerated lesions (stage 2). F. necrophorum and P. levii are mutually involved in the etiology of foot rot in ruminants (Walter and Mork, 2002; Zhou et al., 2009). Furthermore, antibodies against F. necrophorum and P. levii have been detected in dairy cattle with DD (Moe et al., 2010). However, both F. necrophorum and P. levii displayed much lower abundance and prevalence than Treponema and Mycoplasma spp. (Fig. 3), and the FISH results showed them to be mainly located in the superficial layers of the infected epidermis. Consequently, their possible roles in the pathogenesis of DD remain unclear. They may be actively involved in some specific stages of the disease – or they may merely represent a secondary colonization as previously indicated (Rasmussen et al., 2012; Krull et al., 2014). Another bacterium, D. nodosus, also suspected to be involved in the development of DD (Rasmussen et al., 2012) and recently shown to cause interdigital dermatitis in a challenge model (Knappe-Poindocker et al., 2015), was identified by FISH analysis in 12 of the samples but was absent or below the detection limit in the sequence data. Likewise, we did not observe Candidatus Amoebaphillus asiaticus to be part of the microbiomes of the present DD samples. This bacterium is among the most abundant species observed in both active and inactive lesions in the study by Zinicola et al. (2015).

Although very few Spirochaetes were identified in the control samples, this family was amply represented in the DD lesions. This observation was further supported by the FISH analysis, which showed Treponema spp. to be the dominating phyla in the deeper parts of the lesions, constituting approximately 90% of the infecting microbiota. Most of the DD specimens in our investigation were active lesions (score 2) that exhibited changes in both the epidermis and dermis layer and disruption of the basement membrane. As previously observed by our group and others (Evans et al., 2009; Klitgaard et al., 2013), there is a high diversity of Treponema species in the DD afflicted tissue, with T. phagedenis (including PT13) and T. refringens-like phylotypes (PT1–3, PT12 and PN–20) as the most abundant species (Fig. 4). Similar Treponema phylotypes have been detected in the deep sequencing studies by Krull et al. (2014) and Zinicola et al. (2015) both of which have reported temporal or stage changes in the treponemal composition of the lesions. Because most of the lesions in the present investigation were representative of variations over the same stage, we were not able to infer any predictions regarding microbial community changes over time. Moreover, the use of different lesion scoring systems in the metagenomics studies of DD conducted so far (Krull et al., 2014; Zinicola et al., 2015) illustrates how difficult it is to classify the stages of this disease, which in combination with variations in 16S rRNA gene sequence taxonomic classification methods make direct comparison between studies challenging with regard to lesion development over time.

To study the expression of selected potential Treponema virulence factors during infection, RT-qPCR was applied. In 17 of the samples, we detected expression of the major outer sheath protein antigen (msp), which is one of the principle virulence determinants of T. denticola (Fenno et al., 1997). Two genes encoding flagellar proteins were also actively expressed in 19 DD samples. Motility may be important for a bacterium’s ability to actively invade host tissue (Dashper et al., 2011). To survive in vivo, pathogenic bacteria have developed several mechanisms for acquiring iron in the host environment. The protein encoded by hbpB may contribute to hemin utilization by T. denticola (Xu et al., 2001) and was actively expressed by treponemes in this study. Along with the FISH analysis, these results further indicate that treponemes are actively involved in the pathology of DD.

5. Conclusion

We observed a clear distinction between the microbiota of healthy and DD-afflicted tissue. Treponema, Mycoplasma, Fusobacterium and Porphyromonas were the genera best differentiating the infected tissue samples from the controls. FISH analysis indicated that species belonging to the genus Treponema were the dominant bacteria in the deep part of the lesions, and RT-qPCR indicated that they were actively expressing putative virulence genes. F. necrophorum and P. levii were mainly observed in the superficial parts of the epidermis by FISH. Although the presence of M. fermentans was confirmed by species-specific PCR, it could not be detected by FISH or by RT-PCR. Although the involvement of a variety of Treponema species in DD lesion development seems well supported, the roles of other bacterial genera are still unclear. For example, it seems puzzling that M. fermentans appeared to be highly abundant in the infected tissue, yet appeared to be relatively inactive. F. necrophorum and P. levii may be secondary invaders, or they could also play important roles at specific stages of infection. To further clarify this notion, metatranscriptomic studies of DD microbiota during infection are presently ongoing in our group.

Conflict of interest

None of the authors have any conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j. vetmic.2016.03.003.

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