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Microbiota analysis of environmental slurry and its potential role as a reservoir of bovine digital dermatitis pathogens

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Running Head: Microbiota analysis of slurry from dairy herds.

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At present, very little information exists regarding what role the environmental slurry may play as an infection reservoir and/or route of transmission for bovine digital dermatitis (DD), a disease which is a global problem in dairy herds. To investigate, if DD-related bacteria belong to the indigenous microbiota of the dairy herd environment, we used deep amplicon sequencing of the 16S rRNA gene in 135 slurry samples collected from different sites in 22 dairy farms, with and without DD-infected cows. Both the general bacterial populations as well as digital dermatitis-associated *Treponema* were targeted in this study. The results revealed significant differences in the bacterial communities between the herds, with only 12 bacterial taxa shared across at least 80% of all the individual samples. These differences in the herd microbiota appeared to reflect mainly between-herd variation. Not surprisingly, the slurry was dominated by ubiquitous gastrointestinal bacteria, such as *Ruminococcaceae* and *Lachnospiraceae*. Despite the low relative abundance of spirochetes, which ranged from 0 to 0.6%, we were able to detect small amounts of bacterial DNA from DD-associated treponemes in the slurry. However, the DD-associated *Treponema* spp. were only detected in samples from herds with reported problems of DD. These data indicate that treponemes involved in the pathogenesis of DD are not part of the normal environmental microflora in dairy herds without clinical DD and, consequently, that slurry is not a primary reservoir of infection.
IMPORTANCE

Bovine digital dermatitis (DD), a dermal disease which causes lameness in dairy cattle, is a serious problem worldwide. To control this disease, the infection reservoirs and transmission routes of DD pathogens need to be clarified. The dairy herd slurry may be a possible pathogen reservoir of DD-associated bacteria. The rationale for the present study was, therefore, to examine whether DD-associated bacteria are always present in slurry or if they are only found in DD-afflicted herds. The results strongly indicated that DD *Treponema* are not part of the indigenous slurry and, therefore, do not comprise an infection reservoir in healthy herds. This study applied next-generation sequencing technology to decipher the microbial compositions of environmental slurry of dairy herds with and without digital dermatitis.

INTRODUCTION

Bovine Digital dermatitis (DD) is an inflammation of the skin around the digits and the main cause of lameness in cattle (1). This disease is one of the most widespread and costliest problems in modern dairy farms (2). Members of the genus *Treponema* in particular, along with other bacteria, such as *Mycoplasma*, *Fusobacterium*, *Porphyromonas* and *Dichelobacter*, are identified in the DD lesions and are rarely associated with healthy skin from the feet of cattle (3–5).

Disrupting the chain of transmission may be an effective way to prevent the spread of DD, but, presently, the infection reservoirs and transmission routes of DD-associated bacteria are still unclear. Cattle produce ample amounts of slurry which is a mixture of feces and urine, along with bedding, microorganisms, wastewater and other secretions (e.g. from the nose, vagina and mammary glands).
Slurry harbors a wide variety of unknown microorganisms, non-pathogenic as well as potentially pathogenic, which all the animals of the herds are exposed to daily and, therefore, might be a potent means of spreading DD and other bovine diseases.

DD-related spirochetes have been identified from various parts of the gastrointestinal tract. Evans et al. (6) found evidence of DD-associated treponemes in the oral cavity and rectal tissue of dairy cows on DD-affected farms. Meanwhile, Zinicola et al. (7) found DD treponemes to be ubiquitously present in rumen and fecal microbiomes. While these findings indicate that slurry and feces could be a potential reservoir of DD bacteria, DD-associated bacteria have proven hard to find in the environment outside the lesion areas (6). However, in a previous study, we have demonstrated that it is possible to isolate small amounts of DNA from *Treponema* spp. associated with DD pathogenesis from the environment of herds with DD problems through a targeted deep-sequencing approach (5). Still, since only herds with DD problems have been investigated using this method, it is still unknown whether bacteria associated with DD are an indigenous part of the slurry microbiota or only present in infected herds.

Most metagenomics studies in ruminants have focused on the phylogenetic structure of the microbial communities in the rumen or in cattle feces (8–10). Few studies have applied next-generation sequencing technologies to the slurry in dairy herds (5). Consequently, there is very limited knowledge of the microbial composition of the environmental slurry in the cows’ local habitat. Here, we investigated which—potentially pathogenic—bacteria the cow is exposed to in its local environment and if these bacteria are ubiquitous in the dairy herds. Furthermore, we tested the possible influence of the management, geographic locality, breed, floor type, bedding, sample type and DD status on the bacterial composition in the stable. We used general bacterial primers to estimate the phylogenetic composition and relative abundance of the slurry microbiota at family and genus levels. As the slurry...
content of treponemes potentially could be relatively rare (5, 6), we specifically targeted this genus with primers know to include the DD-associated treponemes. These primers amplify a 322 bp region of the 16S rRNA gene which we have previously shown is well suited to classify the DD-associated treponemes at the species level (11), since these primers do not amplify non-treponeme DNA.

MATERIALS AND METHODS

Sample collection and preparation  Environmental slurry samples were collected from 22 Danish farms at different geographical locations in Zealand (n = 6), Funen (n = 2) and Jutland (n = 14). The criteria for selecting a farm were 1) a positive response to take part in the study (emails were sent out to most Danish dairy farmers) and 2) from these positive responses we selected a subset of farms based on their geographical locations which allowed us to do the sampling within three days. With a few exceptions, six samples were collected from each herd (n = 138). For each herd, we noted the following variables, when possible: management (conventional vs. organic farming), geographic locality (Sealand, Funen or Jutland), breed (Holstein, Jersey, other), floor type (slated or firm), bedding (sand or mat), sample type (sock, floor, floor near drinking facility, floor under winging cow brush) and DD status of the herd (“no clinical DD observed,” “clinical DD observed” or “no information on DD status in herd avaible”) (Table 1). Herds were considered as having clinical DD when these included cows with visible lesions, mainly M2 according to the scoring systems by Döpfer et al. (12). The clinical DD status of the herds were based on reports from the herd owners.

In each herd, two boot polypropylene sock samples (Abena, Aabenraa, Denmark) were collected by walking the common area of the stable with socks on both feet. Slurry samples (2 × 4) were collected on April 3, 2017.
from different locations on the floor with a wooden spatula: two random samples, one sample from the floor of the drinking area and one sample from below the winging cow brush. The drinking and winging cow brush area were assumed to be highly accessed zones frequented by the entire herd. Samples were immediately transferred to RNA_later stabilization solution (Ambion, Austin, TX, USA). After being kept at 4°C for 24 h, according to the manufacturer’s instructions, the samples were stored at −20°C until use.

Bacterial DNA was extracted from slurry samples using the Maxwell 16 LEV Blood DNA Kit and the Maxwell 16 AS1290 instrument (Promega, Wisconsin, USA). Portions (200 mg) of slurry were first resuspended in 200 µl 25mg/ml lysozyme solution (20 mM Tris-HCl, pH 8, 2mM EDTA, 1.2% TritonX added lysozyme) and subsequently heated for 30 min at 37°C to break down bacterial cell walls and improve DNA extraction efficiency. A sterile 5-mm stainless steel bead (Qiagen, Hilden, Germany) and 350µl lysis buffer (Maxwell 16 LEV Blood DNA Kit) were added into each reaction, which was then bead-beated in a TissueLyser (Qiagen) at 20 Hz for 4 min. Next, 20 µl of proteinase K was added, and the samples were incubated for 1 h at 56°C. All subsequent steps were performed according to the protocol provided in the Maxwell 16 LEV Blood DNA Kit. The concentrations and purity of the samples were evaluated using a Nanodrop 1000 spectrophotometer (Fisher Scientific, Wilmington, MA), and only samples with A260/A280 ratios of >1.5 were used in further analyses.

Preparation of 16S rRNA gene amplicon libraries and sequencing. PCR amplification of DNA was accomplished with a universal bacterial primer set, F- 5’ AGAGTTTGATCCTGGCTCAG 3’ and R- 5’ CTGCTGCCTYCCGTA 3’ (13), and a Treponema-specific primer set, F- 5’ GGGAGGCAGCAGCTAAGAA 3’ and R- 5’ ATCTACAGATTCACCCCTA 3’ (14), targeting the V1–V2 region and the V3–V4 hyper variable regions of the 16S rRNA gene, respectively.
The Treponema-specific primers have been shown to cross-react with the majority of treponemes hitherto identified in DD lesions (14). 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. Santa Clara, CA) 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 manufacturer’s protocol. The DNA was submitted to the National High-Throughput DNA Sequencing Centre at the University of Copenhagen, Denmark for sequencing on the Illumina HiSeq™ platform.

The sequences generated by Illumina HiSeq are available under the accession number SUB2135215 in the NCBI Sequence Read Archive (SRA).
Sequence analysis. For both sets of sequences, the obtained reads were analyzed using the BION-meta software (http://box.com/bion). BION is a supported semi-commercial open-source package for microbial community analysis of 16S rRNA and other reference genes (manuscript under preparation). The major advantage of this program is that where all other packages classify mostly to genus, BION does it mostly to species. The de-multiplexing step was performed according to the primer and barcode sequences. Forward and reverse sequences were joined allowing no gaps, a maximum mismatch percentage of 85% and a minimum overlap length of 20 base pairs (bp). Next, the sequences were cleaned at both ends through the removal of bases of a quality less than 99%, 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.cme.msu.edu/index.jsp), using a word length of 8 and a match minimum of 80%. To allow for the comparison of relative abundance between samples for barplots, the number of reads for each barcode was normalized. To explore the unclassified treponemes further, chimera-filtered sequences were clustered at 97% using VSEARCH (15) similarity within each sample, and command line BLAST with the Nucleotide Collection (nt) database was used to classify the reads. Due to computational limitations stemming from the size of the nt database, only clusters > 100 sequences were used.

The sequences were analysed for associations with herd, management, geographic locality, breed, floor type, bedding, sample type and DD status with the DEseq2-package in R (16), which normalizes the read counts and fits the data using a negative binomial distribution, followed by a likelihood ratio test.
Non-metric multidimensional scaling was used to search for multivariate patterns in the data across 165 independent variables.

RESULTS AND DISCUSSION

DD is a polymicrobial disease, where *Treponema phagedenis*-like, *Treponema denticola/Treponema pedis*-like, *Treponema medium/Treponema vincentii*-like and *Treponema refringens*-like phylotypes are the most prevalent species found in the lesions (4, 17–19). However, it still remains to be answered where these treponemes come from and how the disease might spread between animals. A possible reservoir of the microbes associated with this disease is the cow’s gastrointestinal tract (6, 7), in which case the slurry may be a potential vehicle of transmission for DD pathogens in the dairy herd environment. Evans et al. did not find any evidence of DD treponemes in dairy cow feces and environmental slurry by conventional PCR (6). Since then, however, we have been able to detect small amounts of DNA from DD-associated *Treponema* species in slurry through a targeted deep-sequencing approach (5). Although, it must be noted that all the samples in that study came from DD-infected farms.

Therefore, in the present study, we sequenced samples from randomly selected dairy farms with and without a history of DD problems. The aim was to clarify what bacteria the cows are exposed to daily from the environmental slurry and, in particular, if treponemes and other DD-associated bacteria, such as *Fusobacterium necrophorum, Porphyromonas levii* and *Dichelobacter nodosus*, are indigenous to this material. Additionally, we tested if specific environmental variables influenced the composition of the slurry microbiota.
We sequenced a 310 bp region of the 16S rRNA gene of 135 slurry samples (3 of the 138 samples were negative) from 22 dairy herds, with primers targeting general bacteria (V1–V2 region) and the *Treponema*-group, specifically (V3–V4 region). After de-multiplexing according to the sequences of the barcodes and primers, 7,216,000 and 20,099,832 sequences remained in the general bacterial pool and the *Treponema*-group pool, respectively. The 3' and 5' ends of these sequences were further trimmed, as sequences with quality below 99% were discarded. In total, 1,991,550 (general bacterial pool) and 6,485,538 (*Treponema*-group pool) joined sequences were used for taxonomic classification, equivalent to average reads per sample of 65,641 and 52,063, respectively. Of these sequences, 74% of the general bacterial pool and 92% of the *Treponema*-group pool were taxonomically classifiable to family and genus level, respectively, according to the RDPII database (http://rdp.cme.msu.edu/index.jsp).

We further investigated the unclassified *Treponema* reads by clustering the unclassified sequences at 97% similarity and using BLAST with the nt-database, which revealed several large clusters in each sample that matched (between 80 and 98%) uncultured and unclassified ruminant treponemes, the most frequently observed being an uncultured bacterium clone KO1 aai43a12 identified by Ley et al. (20). Using exact de-replication did not change this conclusion, nor did using any other databases.

A core group of bacterial families was identified with an abundance of ≥ 0.5% in at least 80% of the herds. Shared taxa spanned the families *Prevotellaceae, Bacteroidaceae, Porphyromonadaceae, Rikenellaceae, Aerococcaceae, Ruminococaceae, Lachnospiraceae, Erysipelotrichaceae* and *Corynebacteriaceae*, together with unclassified groups of *Bacteroidetes, Firmicutes, Bacteroidia* and *Clostridia*. The most abundant taxa included *Ruminococaceae, Aerococcaceae* and *Lachnospiraceae*.
Most of these families are ubiquitously present in bovine rumen material or feces (8, 10, 21). In previous deep-sequencing metagenomic studies (4, 19), Corynebacteriaceae, Ruminococcaceae, Carnobacteriaceae and Lachnospiraceae were also present in relatively high abundances in interdigital skin samples from the healthy feet of dairy cattle. Although the family Porphyromonadaceae to which P. levii belongs was among the most abundant taxa identified, the members of this family could not be determined to the species level. Meanwhile, sequences representing the family Spirochaetaceae and Fusobacteriaceae had a relative abundance below 1% and the family Cardiobacteriaceae, which includes the DD-associated pathogen D. nodosus, was not represented among the amplicons sequenced with the general bacterial primers.

Analysis by non-metric multidimensional scaling revealed no underlying multivariate patterns. We also tested if the variables herd, management, geographic locality, breed, floor type, bedding, sample type and DD status had any effect on the bacterial composition of the samples (at family level). The importance of each individual variable was tested separately. Not surprisingly, “Herd” was the variable which corresponded to the largest part of the difference in bacterial composition between samples. Figure 2 shows the families with abundances that were significantly associated with DD status (DD vs. no DD). The most interesting of these families was the Actinomycetaceae, which was almost 14 times more abundant in DD herds compared to herds with no DD. This family was also significantly more abundant in herds with firm floors and mats, compared to herds with slated floors and herds with sand in the boxes. The Actinomycetaceae were mainly comprised of members of the genus Trueperella, but we were not able to classify these to the species level. Based on the current information, it is difficult to determine if members of the Actinomycetaceae are relevant to DD. Trueperella is not usually associated with DD; however, one species from this genus, Trueperella pyogenes, has been implicated...
in infectious conditions manifesting in lameness in sheep and goat populations (22, 23). Other bacterial families with significantly higher abundance in DD herds, such as *Staphylococcaceae*, *Aerococcaceae* and *Corynebacteriaceae*, are usually associated with the skin microbiota of healthy feet (4, 19) and, thus, are most likely of no importance to the development of DD.

*Spirochaetaceae* are natural inhabitants of the bovine rumen (24) and include commensal species as *Treponema bryantii* and *Treponema saccharophilum*, both of which have been isolated from the rumen of cows (25, 26). These and other commensal gastrointestinal (GI) treponemes belong to another phylogenetic clade than the DD-associated *Treponema* spp. (27). Although spirochetes are part of the normal GI microbial community, they appear to be less common in the slurry. The results from the general bacterial primers showed that members of the phylum *Spirochaetes* constituted only a very small fraction of the total bacterial amplicons, with relative abundances between 0 and 0.6%. This result is in good accordance with the study of Shanks et al. (10), which observed an overall abundance of 0.54% for *Spirochaetes* in cattle fecal microbiomes.

Despite the low spirochete abundance in the slurry, we were able to amplify DNA reads from this genus from 99% of the samples with the use of *Treponema*-specific primers. The majority of these amplicons could only be determined to genus level and most likely belonged to the non-pathogenic environmental members of the genus. Many of the unclassified *Treponema* reads resembled a not-yet-cultivated ruminant clone, *Treponema KO1_aai43a12*, which was isolated from red kangaroo feces (20). Meanwhile, DD-associated treponemal species, homologous to *T. refringens*, *T. phagedenis*, *T. medium* and *T. denticola*, were present in samples from dairy farms with DD or unknown status, though with very low abundances, constituting between 0 and 0.6% of the *Treponema*-specific amplicons (Fig. 3). These pathogenic bacteria were significantly associated with DD-status ($p < 0.001$).
Besides the DD-associated species, we also identified the commensals *T. bryantii* and *T. berlinense* (26, 28).

Conclusion: We identified only a few bacterial families from the slurry microbiota, such as the *Actinomycetaceae*, which might be associated with the DD status of the herds. In addition, DNA amplicons from DD-associated bacteria, such as *P. levii* and *D. nodosus*, were not detectable in the slurry samples tested in the present study. Spirochetes appear to make up a very small part of the slurry microbiota in dairy herds, and DD-associated treponemes an even smaller fraction. Still, with the use of a targeted deep-sequencing approach, it is possible to detect these minute amounts of bacterial DNA from DD treponemes, but only from herds with DD problems. Possibly, the amplified DD *Treponema* DNA originated from bacteria sloughed off from the DD lesions. All in all, the results do not indicate that the environmental slurry is primary reservoir for DD-related treponemes. This leaves short-term persistence in slurry, direct skin-to-skin transmission from infected to uninfected feet or transmission via hoof-trimming implements as the most plausible routes of infection for DD treponemes (6, 29).

**ACKNOWLEDGEMENTS**

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REFERENCES


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NN: Not known

**Figure 1.** The relative abundances of the most highly represented bacterial taxa (at the family level, when possible) in the individual slurry samples from the 22 dairy farms included in the study.

**Figure 2.** A forest plot of the families significantly associated with DD status, according to the DESeq2 analysis. Values are log2-fold differences, and bars denote the standard error of the log fold change.

**Figure 3.** The abundance of DD-associated *Treponema* spp. (except for *T. berlinense*, which is presently not associated with DD) in the slurry samples from dairy farms with no known problems of DD (Negative), dairy farms with DD-infected cows (Positive) and dairy farms with unknown status (No info).