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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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ABSTRACT

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).

60 Slurry harbors a wide variety of unknown microorganisms, non-pathogenic as well as potentially
61 pathogenic, which all the animals of the herds are exposed to daily and, therefore, might be a potent
62 means of spreading DD and other bovine diseases.

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

73 Most metagenomics studies in ruminants have focused on the phylogenetic structure of the microbial
74 communities in the rumen or in cattle feces (8–10). Few studies have applied next-generation
75 sequencing technologies to the slurry in dairy herds (5). Consequently, there is very limited knowledge
76 of the microbial composition of the environmental slurry in the cows' local habitat. Here, we
77 investigated which—potentially pathogenic—bacteria the cow is exposed to in its local environment
78 and if these bacteria are ubiquitous in the dairy herds. Furthermore, we tested the possible influence of
79 the management, geographic locality, breed, floor type, bedding, sample type and DD status on the
80 bacterial composition in the stable. We used general bacterial primers to estimate the phylogenetic
81 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

103 from different locations on the floor with a wooden spatula: two random samples, one sample from the
104 floor of the drinking area and one sample from below the winging cow brush. The drinking and
105 winging cow brush area were assumed to be highly accessed zones frequented by the entire herd.
106 Samples were immediately transferred to RNA*later* stabilization solution (Ambion, Austin, TX, USA).
107 After being kept at 4°C for 24 h, according to the manufacturer's instructions, the samples were stored
108 at −20°C until use.

109 Bacterial DNA was extracted from slurry samples using the Maxwell 16 LEV Blood DNA Kit and the
110 Maxwell 16 AS1290 instrument (Promega, Wisconsin, USA). Portions (200 mg) of slurry were first
111 resuspended in 200 µl 25mg/ml lysozyme solution (20 mM Tris-HCl, pH 8, 2mM EDTA, 1.2%
112 TritonX added lysozyme) and subsequently heated for 30 min at 37°C to break down bacterial cell
113 walls and improve DNA extraction efficiency. A sterile 5-mm stainless steel bead (Qiagen, Hilden,
114 Germany) and 350µl lysis buffer (Maxwell 16 LEV Blood DNA Kit) were added into each reaction,
115 which was then bead-beated in a TissueLyser (Qiagen) at 20 Hz for 4 min. Next, 20 µl of proteinase K
116 was added, and the samples were incubated for 1 h at 56°C. All subsequent steps were performed
117 according to the protocol provided in the Maxwell 16 LEV Blood DNA Kit. The concentrations and
118 purity of the samples were evaluated using a Nanodrop 1000 spectrophotometer (Fisher Scientific,
119 Wilmington, MA), and only samples with A_{260}/A_{280} ratios of >1.5 were used in further analyses.

120 **Preparation of 16S rRNA gene amplicon libraries and sequencing.** PCR amplification of DNA was
121 accomplished with a universal bacterial primer set, F- 5' AGAGTTTGATCCTGGCTCAG 3' and R- 5'
122 CTGCTGCCTYCCGTA 3' (13), and a *Treponema*-specific primer set, F- 5'
123 GGGAGGCAGCAGCTAAGAA 3' and R- 5'ATCTACAGATTCCACCCCTA 3' (14), targeting the
124 V1–V2 region and the V3–V4 hyper variable regions of the 16S rRNA gene**,** respectively.

125 The *Treponema*-specific primers have been shown to cross-react with the majority of treponemes
126 hitherto identified in DD lesions (14). Each sample was amplified with unique forward and reverse
127 primers that included an added hexamer barcode at their 5′ ends. Amplification PCRs were performed
128 in 50-µl reaction mixtures containing 5 µl of 10xPCR Gold Buffer (Applied Biosystems, Foster City,
129 CA, USA) 1.5 mM $MgCl_2$ solution (Applied Biosystems), 200 µM of each deoxynucleoside
130 triphosphate (Amersham Biosciences, Piscataway, NJ), 0.4 µM of each specific primer, 2.5 U of
131 AmpliTaq Gold DNA polymerase (Applied Biosystems), and 2 µl of template DNA. For both primer
132 sets, thermal cycling using a T3 thermocycler (Biometram, Göttingen, Germany) was performed as
133 follows: denaturation at 94 °C for 6 min; 30 cycles of denaturation at 94 °C for 45 s, annealing at 57 °C
134 for 45 s, and extension at 72 °C for 90 s. A final elongation step of 10 min was followed by cooling to
135 4 °C. Positive (DNA) and negative (dH_2O) controls were included for each PCR setup. The DNA
136 concentration and quality of the PCR amplicons from all samples were assessed with an Agilent 2100
137 Bioanalyzer (Agilent Technologies Inc. Santa Clara, CA) prior to high-throughput sequencing (data not
138 shown). Equal amounts of all amplicons were pooled (final concentration between 3.8–4 µg) and
139 purified with the Qiagen Mini Elute kit (Qiagen) according to the manufacturer's protocol. The DNA
140 was submitted to the National High-Throughput DNA Sequencing Centre at the University of
141 Copenhagen, Denmark for sequencing on the Illumina HiSeq™ platform.

142 The sequences generated by Illumina HiSeq are available under the accession number SUB2135215

143 in the NCBI Sequence Read Archive (SRA).

144 **Sequence analysis.** For both sets of sequences, the obtained reads were analyzed using the BION-meta
145 software (http://box.com/bion). BION is a supported semi-commercial open-source package for
146 microbial community analysis of 16S rRNA and other reference genes (manuscript under preparation).
147 The major advantage of this program is that where all other packages classify mostly to genus, BION
148 does it mostly to species. The de-multiplexing step was performed according to the primer and barcode
149 sequences. Forward and reverse sequences were joined allowing no gaps, a maximum mismatch
150 percentage of 85% and a minimum overlap length of 20 base pairs (bp). Next, the sequences were
151 cleaned at both ends through the removal of bases of a quality less than 99%, which is equivalent to a
152 Phred score of 17. Identical sequences were de-replicated into consensus sequences of 300–322 bp.
153 Consensus sequences of at least 250 nucleotides in length were mapped into a table, according to the
154 individual barcodes, and taxonomically classified against the Ribosomal Database Project database II
155 (RDP II; http://rdp.cme.msu.edu/index.jsp), using a word length of 8 and a match minimum of 80%. To
156 allow for the comparison of relative abundance between samples for barplots, the number of reads for
157 each barcode was normalized.

158 To explore the unclassified treponemes further, chimera-filtered sequences were clustered at 97% using
159 VSEARCH (15) similarity within each sample, and command line BLAST with the Nucleotide
160 Collection (nt) database was used to classify the reads. Due to computational limitations stemming
161 from the size of the nt database, only clusters > 100 sequences were used.

162 The sequences were analysed for assocations with herd, management, geographic locality, breed, floor
163 type, bedding, sample type and DD status with the DEseq2-package in R (16), which normalizes the
164 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 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 specfic 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 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*, *Ruminococcaceae*, *Lachnospiraceae*, *Erysipelotrichaceae* and *Corynebacteriaceae,* together with unclassified groups of *Bacteroidetes, Firmicutes*, *Bacteroidia* and *Clostridia*. The most abundant taxa included *Ruminoccocaceae, Aerococcaceae* and *Lachnospiraceae*

208 (Fig. 1). Most of these families are ubiquitously present in bovine rumen material or feces (8, 10, 21).
209 In previous deep-sequencing metagenomic studies (4, 19), *Corynebacteriaceae*, *Ruminococcaceae,*
210 *Carnobacteriaceae* and *Lachnospiraceae* were also present in relatively high abundances in interdigital
211 skin samples from the healthy feet of dairy cattle.

212 Although the family *Porphyromonadaceae* to which *P. levii* belongs was among the most abundant
213 taxa identified, the members of this family could not be determined to the species level. Meanwhile,
214 sequences representing the family *Spirochaetaceae* and *Fusobacteriaceae* had a relative abundance
215 below 1% and the family *Cardiobacteriaceae*, which includes the DD-associated pathogen *D. nodosus,*
216 was not represented among the amplicons sequenced with the general bacterial primers.

217 Analysis by non-metric multidimensional scaling revealed no underlying multivariate patterns. We also
218 tested if the variables herd, management, geographic locality, breed, floor type, bedding, sample type
219 and DD status had any effect on the bacterial composition of the samples (at family level). The
220 importance of each individual variable was tested separately. Not surprisingly, "Herd" was the variable
221 which corresponded to the largest part of the difference in bacterial composition between samples.
222 Figure 2 shows the families with abundances that were significantly associated with DD status (DD vs.
223 no DD). The most interesting of these families was the *Actinomycetaceae*, which was almost 14 times
224 more abundant in DD herds compared to herds with no DD. This family was also significantly more
225 abundant in herds with firm floors and mats, compared to herds with slated floors and herds with sand
226 in the boxes. The *Actinomycetaceae* were mainly comprised of members of the genus *Trueperella*, but
227 we were not able to classify these to the species level. Based on the current information, it is difficult to
228 determine if members of the *Actinomycetaceae* are relevant to DD. *Trueperella* is not usually
229 associated with DD; however, one species from this genus, *Truperella pyogenes*, has been implicated

230 in infectious conditions manifesting in lameness in sheep and goat populations (22, 23). Other bacterial
231 families with significantly higher abundance in DD herds, such as *Staphylococcaceae*, *Aerococcaceae*
232 and *Corynebacteriaceae*, are usually associated with the skin microbiota of healthy feet (4, 19) and,
233 thus, are most likely of no importance to the development of DD.

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

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

252 Besides the DD-associated species, we also identified the commensals *T. bryantii* and *T. berlinense*
253 (26, 28).

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

265

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351 **Table 1. Herd variables.**

Herd	Location	Management	Breed	Floor type	Bedding	DD positive	No. of samples
A	Zealand	Organic	NN	NN	NN	No	15
B	Zealand	Conventional	Holstein	Firm	Mat	Yes	6
C	Zealand	Conventional	Holstein	Slated	Mat	Yes	6
D	Zealand	Conventional	Holstein	Slated	NN	Yes	6
E	Zealand	Conventional	Holstein	Firm	Mat	Yes	6
F	Zealand	Conventional	Holstein	Slated	Mat	Yes	6
G	Jutland	Conventional	Holstein	Slated	Sand	No	6
H	Jutland	Conventional	Jersey	Slated	Mat	Yes	6
L	Jutland	Conventional	Holstein	Firm	Mat	Yes	6
M	Jutland	Conventional	Holstein	Slated	Mat	Yes	6
N	Jutland	Conventional	Holstein	Firm	Sand	No	6
O	Jutland	Organic	Holstein	Slated	Sand	No	6
P	Jutland	Conventional	Holstein	Slated	Mat	Yes	6
Q	Jutland	Conventional	Holstein	Slated	Mat	Yes	6
R	Jutland	Organic	Jersey	Slated	Mat	Yes	6
S	Jutland	Organic	Holstein	Slated	Mat	Yes	6
T	Jutland	Conventional	Holstein	Firm	Mat	Yes	6
U	Jutland	Conventional	Holstein	Firm	Sand	NN	6
V	Jutland	Conventional	Holstein	Slated	Mat	Yes	6

X	Jutland	Conventional	Holstein	Slated	Mat	NN	6
Y	Funen	Conventional	Jersey	Slated	Mat	NN	4
Z	Funen	Conventional	Jersey	Slated	Mat	NN	5

352 NN: Not known

353

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

356

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

359

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

364