



## Bovine digital dermatitis in Victoria, Australia

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**Aims** The objectives of this study were to estimate the prevalence of digital dermatitis (DD) in Victoria, Australia, and to investigate which organisms are consistent with typical DD lesions. The prevalence and causative pathogens of DD are not clear yet in Australia and this paper is one of the first to explore these questions in this country.

**Methods** Examination and sampling of limbs was undertaken at three knackeries in Victoria, Australia. Limbs were classified as normal (N), active DD-lesion (A), dried or chronic DD-lesion (D) or suspected case of DD (S). A total of 823 cows were examined. Six skin biopsies were taken at each knackery, from which DNA was extracted for diversity profiling. Histochemical staining of samples was performed on eight of the skin biopsies.

**Results** DD was detected in 29.8% of all cows. The prevalence of DD was significantly higher in dairy cows (32.2%) than in beef cows (10.8%). The differential abundance of *Treponema*-species was significantly increased in dried lesions, compared with the normal skin biopsies. *Actinobacteria*, *Proteobacteria*, *Firmicutes* and *Tenericutes* were found to be significantly different in abundance in the DD lesions compared with normal skin biopsies. Silver staining of samples showed only mild inflammation and in two samples organisms with morphology consistent with *Spirochaetes* were detected.

**Conclusions** The calculated prevalence indicates that DD is present in Victoria, Australia. The results of diversity profiling showed that the presence of *Treponema*-species was significantly different between the samples of DD lesions and normal skin.

**Keywords** Digital dermatitis; *Treponema*; south-east Australia; silver staining; diversity profiling

**Abbreviations** AGFR, Australian Genome Research Facility; DD, Digital dermatitis; LFC, Logarithmic Fold Change; OTU, Operational taxonomic unit; PCR, Polymerase chain reaction

Aust Vet J 2019;97:404–413

doi: 10.1111/avj.12859

### Introduction

Bovine digital dermatitis (DD), also known as papillomatous digital dermatitis or hairy heel warts, is a contagious disease and an important cause of lameness in cattle worldwide<sup>1</sup>.

The disease was first described in 1974 in Italy<sup>2</sup>. DD is mostly found on the hind feet and causes lameness, reduced milk production, reproductive performance and animal welfare<sup>3–7</sup>. A single case of DD has an estimated cost of US\$133, the result of increased labour for treatment and prevention, production losses and an increased risk of culling of affected cows<sup>4,5,8,9</sup>. Development of the characteristic DD lesions is associated with *Treponema*-species, and these spirochaetes are likely to be the primary pathogens of DD<sup>10–12</sup>. Risk factors for the disease are wet and dirty walking surfaces, low parity, the existence of other hoof disorders and housing system<sup>13,14</sup>. Close contact between cows is necessary for the transmission of DD<sup>15</sup>.

Although *Treponema*-species have been suggested to be the primary pathogen of DD in multiple studies, it must be considered that DD is most likely a polymicrobial disease for which the causative pathogens are not fully understood. Suggested secondary pathogens include *Bacteroidetes*, *Fusobacteria*, *Tenericutes*, *Firmicutes*, *Proteobacteria*, and *Actinobacteria*<sup>16–21</sup>. Transmission experiments using pure cultures of *Treponema*-species have generally failed to produce significant DD; to cause infection it seems necessary to abrade the skin before DD may develop<sup>22</sup>. Furthermore, a killed vaccine against *Treponema* provided poor protection against DD development<sup>23</sup>, which also supports the suggestion of DD as a polymicrobial disease.

Visual examination of the plantar aspect of the feet is a standard method to diagnose DD, which is characterised by ulcerative and/or proliferative lesions. The classification of lesions is based on a scoring system developed by Dopfer et al., (1997) and describes five different stages of DD<sup>10</sup>. Briefly, M1 is a circumscribed, active and ulcerative lesion of 0–2 cm diameter, not painful on palpation. The ulcerative red-grey stage is referred to as M2, with a diameter more than 2 cm and often painful on palpation. M3 defines a healing lesion covered by a scab and is typically seen after a treatment with antibiotics. This stage is in most cases not painful on palpation. M4, the chronic stage, is characterised by hyperkeratosis and generally not painful on palpation. M4.1 is also a chronic stage, but with a small area of ulceration<sup>10,24</sup>.

*Treponema*-species are difficult to culture, so a more successful way to identify these species is by PCR<sup>25–28</sup>. Another commonly used technique for analysis is haematoxylin-eosin (HE) staining or silver staining<sup>10,16,18,29</sup>. However, there is no gold standard test to confirm a macroscopic diagnosis of DD<sup>10</sup>. At the time of writing, a 16S rRNA diversity analysis, using amplification and sequencing on samples of DD-like lesions has never been performed in Australia.

DD is widespread around the World. It is reported in various countries in Europe<sup>25,30–33</sup> and North and South America<sup>15,36–38</sup>. Affected herds are also reported in countries such as Israel, Japan and

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Iran<sup>16,34,35</sup>. In Australia, until recently limited published reports or research about DD have been available, except for one single suspected case in 1996, near Brisbane<sup>39</sup>, and cases of DD in one herd in Victoria, Australia<sup>40</sup>. However, since 2015, there has been an ongoing study in Queensland investigating the prevalence of DD<sup>41</sup>. Prior to our study, a pilot study among 13 herds in Victoria, Australia was conducted. The herds were examined for DD and all herds were positive, with a mean within-herd prevalence of 19.11%<sup>42</sup>.

Lameness is an important issue for the Australian dairy industry, because of the potential financial and welfare-related consequences. The deregulation of the Australian dairy industry in 2000 led to reduced milk prices, increased herd sizes and more intensive feeding systems, including the use of feedpads. These circumstances may contribute to an increasing DD prevalence and DD may be an emerging disease in Australia. In 2004, Milinovich et al. developed a questionnaire, sent to Australian veterinarians, to investigate whether DD occurs in Australia<sup>29</sup>. One of the 88 respondents had seen cases or suspected cases, but many assumed that they had diagnosed these lesions as other claw diseases. More surprising, 38.1% of respondents had not heard of DD, so these findings underline the importance of education for veterinarians and farmers, on how to diagnose, treat and prevent DD. The prevalence and causative subspecies of *Treponema* of DD are not clear in Australia and this information is important to aid in the development of education, treatment methods and preventive measures. The objectives of this study were to estimate the prevalence of DD in Victoria, Australia, and to investigate which *Treponema*-species or other bacteria are associated with typical DD-lesions.

## Materials and Methods

### Data collection and sample size

Five researchers from the University of Melbourne investigated the prevalence of DD and collected samples from DD-like lesions. The investigation was conducted at three knackeries in Victoria, Australia: Rochester (36°21'38.4"S 144°41'58.0"E), Maffra (37°58'08.5"S 146°58'59.8"E) and Camperdown (38°14'22.3"S 143°08'59.5"E). A knackery is a processing facility for deceased cattle that are not fit for human consumption or for transportation, and the reason for culling these cows was unknown. Rochester and Maffra were visited twice, Camperdown once. Each visit was undertaken by one researcher, who observed and examined the limbs of cattle processed during one week.

A veterinarian (J.E. Coombe) who was experienced in cattle lameness trained all researchers. This training included an explanation of differences between DD and other hoof disorders, and on the first day of examination the observations of researchers were confirmed by the veterinarian. All limbs of all mature cattle (cattle with a parity of 1 or more) processed were examined during that week. This was to keep the survey population as homogenous as possible. Both fore and hind limbs of knackery cattle were examined, after washing with tap water with a low-pressure hose, for existence and stage of DD. The limbs of one cow were attached/grouped so limbs could be linked to each other.

After examination, limbs were classified as: Normal (N), defined as no visible lesion or palpable thickening of the epithelium (Figure 1A); an active DD lesion (A), defined as a red, erosive, acute or ulcerative lesion (Figure 1B) and related to M1 and M2 of the scoring system of Dopfer et al., (1997); a dried or chronic DD lesion (D), a hyperkeratotic, wart-like proliferative lesion with thickening of the epithelium (Figure 1C) and comparable with M4 of Dopfer's system<sup>10</sup>; or a suspected case of DD (S) classified as a palpable thickening of the epithelium but no visible lesions (Figure 1D). The sample size on cow-level was calculated. Because the prevalence of DD is unknown, it was assumed to be 50%. Based on this estimate, sample size of 385 cows was needed to reach a maximum error of 5% at 95% confidence. In total, 3292 limbs of 823 cows were examined.

### Collection of skin biopsies

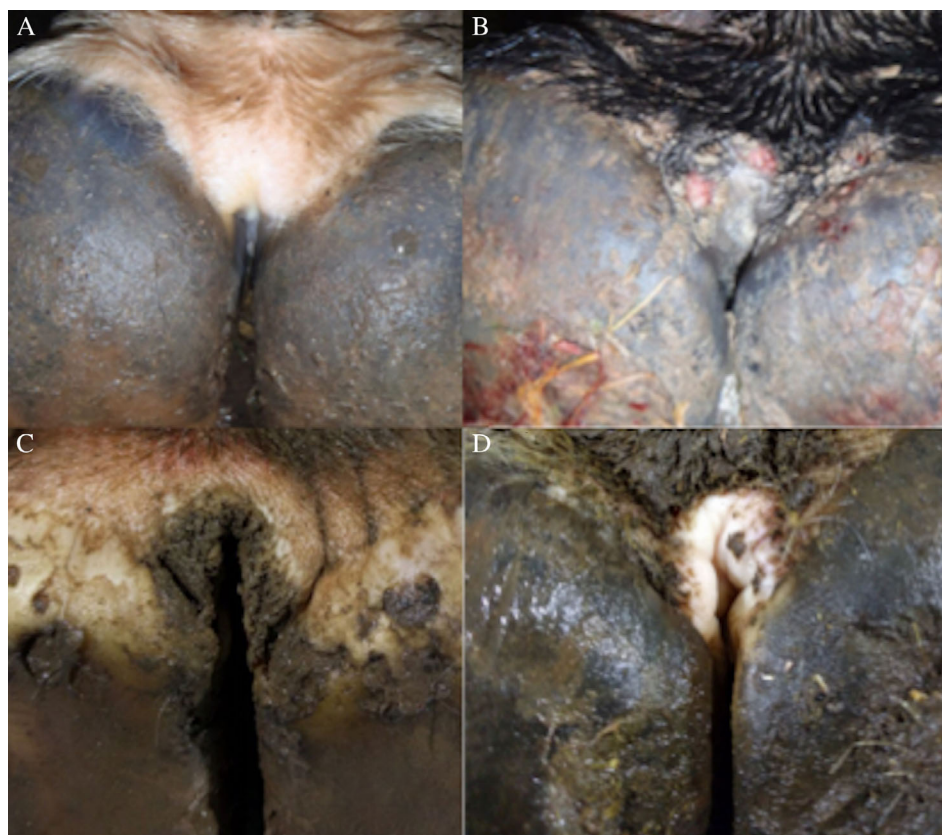
Besides examination, six biopsy samples were collected at each knackery location for 16S rRNA diversity profiling. This included six samples from each location: two samples from normal skin (at the heel cleft, corresponding to the typical site of a DD lesion), two samples from active lesions (on the border of the lesion and normal skin) and two samples from dried lesions (a biopsy of the hyperkeratotic skin), taken from each of six feet. These samples were collected using a sterile scalpel blade, the biopsy was placed in RNA-stabilisation solution at room-temperature and then transported to the Australian Genome Research Facility (AGRF) for DNA-extraction, PCR amplification and next generation sequencing. The primers used for amplification were 314F (5' CCTAYGGGRBGCASCAG 3') and 806R (5' GGACTACNNGGTATCTAAT 3'), designed to target the V3 and V4 region of the 16S rRNA gene. The sequencing was undertaken on the Illumina MiSeq platform to produce paired end reads, each 300 bp in length.

In addition, eight of the skin biopsies were, after 16S rRNA diversity profiling, also submitted for processing and histopathological examination. Six of the submitted samples were taken in August and were classified as two normal skin biopsies (sample number 1 and 2), two active lesions (sample number 3 and 4) and two dried lesions (sample number 5 and 6). Two samples of active lesions (number 9 and 10) were taken in September and also submitted. These 8 samples were randomly chosen for histopathology by casting lots.

### Data analysis

**Observational data.** Data were analysed using the statistical software package SPSS (version 24.0 IBM corporation). A case of DD was defined as a cow with score of A or D on at least one foot. Cows that had unclear data in regard to their classification of DD on one or more limbs were excluded from the analysis. Data were analyzed through the use of  $\chi^2$  test. The significance threshold for the  $\chi^2$  test was set at  $\alpha < 0.05$ .

**Diversity profiling.** Quality filtering and operational taxonomic unit (OTU) assignment was performed using the software USEARCH<sup>43</sup> and QIIME 1.9.1<sup>44</sup>. An OTU refers to a cluster of closely related individuals, and in the context of uncultured bacteria serves as a rough equivalent to a microbial species. In 16S rRNA diversity sequencing each unique sequence cluster can be thought of



**Figure 1.** Examples of classifications of lesions of culled cows at knackeries. A) normal, healthy skin, B) active DD-lesion, C) dried, chronic DD-lesion, D) suspected case of DD.

as individual species, however as bacteria are not wholly sequenced, nor cultured, it is typical to conservatively call these individuals OTUs. The individual components of the data processing mirrored those previously described in detail by Legione et al., (2018)<sup>45</sup>. Briefly, paired sequence reads were merged to obtain full length (~492 bp) sequences, trimmed to remove primer regions, and filtered for quality. For quality filtering, merged sequences with a combined probability of 1 or more erroneous bases were excluded to ensure that only highly accurate reads were included in the following clustering process. Additionally, merged sequences shorter than 400 bp were removed. These filtered reads were then clustered into OTUs (i.e. novel sequence groups) using a threshold of 97% nucleotide identity. This threshold is commonly used as the cut off between species when assessing the 16S rRNA region. Chimeras, a common term for artefact sequences introduced during PCR cycling, were removed *de novo*. This process compares each new OTU to the previously defined, and more abundant, OTUs. The software then removes any newly defined OTU that has sequence similarity to parts of more than one defined OTU, based on the probability that chimeric sequences will be at a lower abundance. Taxonomy was assigned using the Greengenes taxonomy database through sequence similarity between the OTU sequence and the database sequences.

The relative abundance (proportion in each sample) of OTUs was utilised to compare the phylum presence in each sample. For alpha

diversity, rarefaction was undertaken to perform species richness through both OTU abundance and Chao1<sup>46</sup>, and non-parametric comparisons were performed between the three sample groups (active lesions, dried lesions or normal skin biopsies). Briefly, rarefaction is the process of subsampling reads from each sample to the same level to allow more accurate comparison of diversity. In this instance, merged reads were extracted from each sample to a depth of 50,000 reads. The Chao1-indices between the groups were statistically tested by using a two-sample t-test, using nonparametric methods with 10,000 Monte Carlo permutations. Beta diversity was assessed by using both weighted (quantitative) and unweighted (qualitative) UniFrac distances<sup>47</sup> in conjunction with a phylogenetic tree built using the unweighted pair group method with arithmetic mean (UPGMA) from an alignment of the representative OTU sequences. To compare the beta diversity of the microbiomes between the three different sample groups, distance and dissimilarity were used by utilising a permutational ANOVA (PERMANOVA) with 10,000 permutations. A test for multivariate homogeneity of group dispersions (PERMDISP) with 999 permutations, was also utilised to identify which of the tested groups had significantly different beta diversity. Statistical comparisons of the differential abundance of OTUs between the sample groups were also obtained, to determine if particular OTUs were over-presented in each group. This was undertaken by transforming the raw OTU abundance data using DESeq2<sup>48</sup> and undertaking a negative binomial Wald test to



compare transformed OTU abundance between groups. The analysis method normalises reads across samples to allow comparison, rather than losing information by rarefying samples, in order to compare species abundance between groups<sup>49</sup>. Normalising reads through the use of DESeq2 has been found to be a method that offers a reliable sensitivity on smaller datasets (less than 20 samples)<sup>48,50</sup>. Results were considered to be significant when  $P < 0.05$ , after adjustment for false discovery using the method described by Benjamini & Hochberg (1995). The relative abundance of OTUs of importance were considered based on OTUs that were suggested as secondary causative pathogens in previous studies of DD<sup>16–21</sup>.

The read data discussed in this publication have been deposited in the NCBI sequence read archive database and are accessible through accession number SRP131302.

**Histopathology.** Eight skin biopsies were submitted for histological examination. The tissues were fixed with a 10% neutral buffered formalin solution and processed routinely. 3 micron sections were stained with haematoxylin and eosin (H&E) for routine examination, and Warthin Starry (WS) silver stain. H&E stained sections were examined for acanthosis of the epidermis, loss of structure in the epidermis and dermis, uniformity of the stratum corneum and spinosum, bacterial colonisation, presence of spirochaetes, epidermal damage and inflammatory infiltrate in the dermis, according to published principles of bovine digital dermatitis diagnosis, with the examiner blinded to the stage of lesion represented by each section. WS stained sections were examined for presence of spirochaetes.

## Results

### Prevalence

The study population, after excluding cases with missing data, was 823 cows. Within the study population, 88.6% (730/823) were dairy cattle and 11.4% (93/823) were beef cattle. The total prevalence of DD was 29.8% (245/823) (Table 1). DD-lesions were diagnosed mainly on the hind legs (97.1%, 238/245). The prevalence of DD was

significantly higher in dairy cows than in beef cows;  $n = 823$ ,  $\chi^2 = 18.14$ , degrees of freedom (df) = 1,  $P < 0.001$ . Of the cows with DD ( $n = 245$ ), 19.6% (48/245) of the cows had active lesions and 84.9% (208/245) of the cows had dried lesions, because 66.9% (164/245) of the cows with DD had more than one foot affected (Table 1). There was a significant difference, ( $n = 823$ ,  $\chi^2 = 51.86$ , df = 4,  $P < 0.001$ ), between the prevalence of DD and the geographic location of the knackery and time of examination (Table 2).

### Diversity profiling

Across 18 samples, a total of 4,202,787 paired reads were obtained, with a median of 231,144.5 (range: 89,552 - 373,989). After merging paired reads, trimming 5' and 3' ends, quality filtering to reduce errors and excluding merged sequences shorter than 400 bp, a total of 2,714,524 reads were suitable for OTU clustering. After dereplication, 989,648 unique reads were obtained, 817,074 of which were singletons. This resulted in 172,574 reads suitable for clustering into OTUs. Of these unique reads, 59,655 were determined to be chimeric and discarded. The remaining reads were clustering into 5036 OTUs, after excluding 11 which were either chloroplasts or mitochondria. In total 2,698,890 reads, from the 3,076,122 merged reads (87.7%) were matched to the clustered OTUs. These clustered OTUs ranged from 50,449 to 234,324 within samples. A total of 1684 of the OTUs detected across the three sample groups were shared between them.

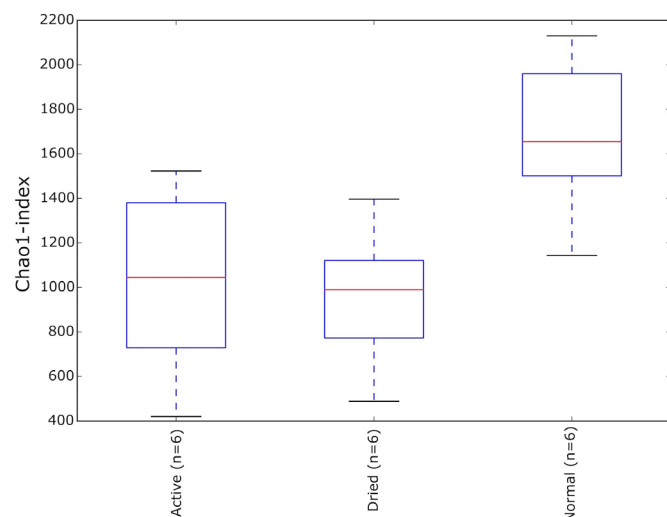
Analysis of the Chao1 index (Figure 2), after rarefaction of samples to 50,000 reads, highlights that the mean richness of OTUs in normal skin biopsies was significantly higher ( $t = -3.64$ ,  $P = 0.018$ ) than in dried lesions. There was no significant difference between the Chao1 from the active lesion samples and the dried lesion samples ( $t = 0.31$ ,  $P = 1.000$ ) or between the active lesion samples and the normal skin biopsies ( $t = -2.78$ ,  $P = 0.092$ ). There were no significant differences between the mean Chao1 of active, dried or normal skin biopsies on location-level.

**Table 1.** The percentage of cases of different stages of DD among dairy cows, beef cows and total examined cows

Sample group	Dairy cows	% (95% CI)	Beef cows	% (95% CI)	Total cases	% (95% CI)
Cases of DD	235/730	32.2 (29, 36)	10/93	10.8 (4, 17)	245/823	29.8 (27, 33)
Active lesions	46/235	19.6 (14, 25)	2/10	20.0 (19, 21)	48/245	19.6 (15, 25)
Dried lesions	200/235	85.1 (81, 90)	8/10	80.0 (79, 81)	208/245	84.9 (80, 89)
Lesions on > 1 foot	159/235	67.9 (62, 74)	5/10	50.0 (12, 88)	164/245	66.9 (61, 73)

**Table 2.** Prevalence of DD per location, date and season

Location	Rochester	Maffra	Rochester	Maffra	Camperdown	Total
Sampling date	May 2017	May 2017	July 2017	August /Sept 2017	Sept 2017	
Season	Autumn	Autumn	Winter	Winter/Spring	Spring	
DD cases/sample	21/179	7/35	56/137	23/106	138/366	245/823
% (95% CI)	11.7 (7, 16)	20.0 (6, 34)	40.9 (33, 49)	21.7 (14, 30)	37.7 (33, 43)	29.8 (27, 33)



**Figure 2.** Chao1- index of the samples of active lesions, dried lesions and normal skin. The difference between the dried lesions and normal skin was significant ( $P = 0.018$ ).

When assessing beta diversity using PERMANOVA, there was a significant difference between at least two groups (Pseudo- $F = 1.44$ ,  $P = 0.037$ ) when comparing the unweighted UniFrac distances between the three groups. This suggests that there was a significant difference between sets of taxa in the three sample groups, based on the unweighted distance in the UPGMA tree<sup>47</sup>. The PERMDISP output of the unweighted UniFrac distances resulted in a significant difference in beta diversity between the dried lesions and the normal skin biopsies ( $F = 2.98$ ,  $df = 2$ ,  $P = 0.045$ ).

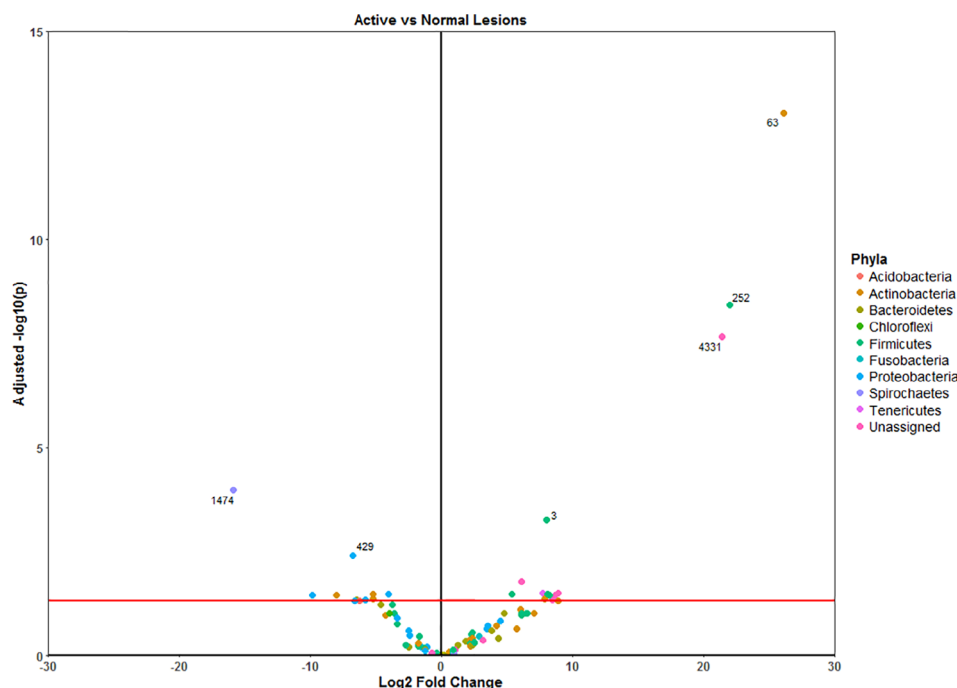
The DESeq2 analysis showed 82 OTUs with significantly different abundance between clinically normal and diseased cases; 26 of these

OTUs were found to be significantly different when the active lesions were compared with the normal skin biopsies (Figure 3), 71 were found to be significantly different when the dried lesions were compared with the normal skin biopsies (Figure 4). Fifteen of the 82 (18.3%) significantly differentially abundant OTUs were shared in both the active and dried group (Table 3).

In addition, Figures 5 and 6 were made based on OTUs that were suggested to be secondary causative pathogens in previous studies of DD<sup>16–21</sup>. This was undertaken in order to make a comparison with these previous studies more accessible. It is important to mention that these figures are based on the relative abundance of OTUs, and thus say something about the proportion of an OTU in a sample. This is different from the differential abundance in the results of the DESeq2 analysis, because the differential abundance is based on normalised data.

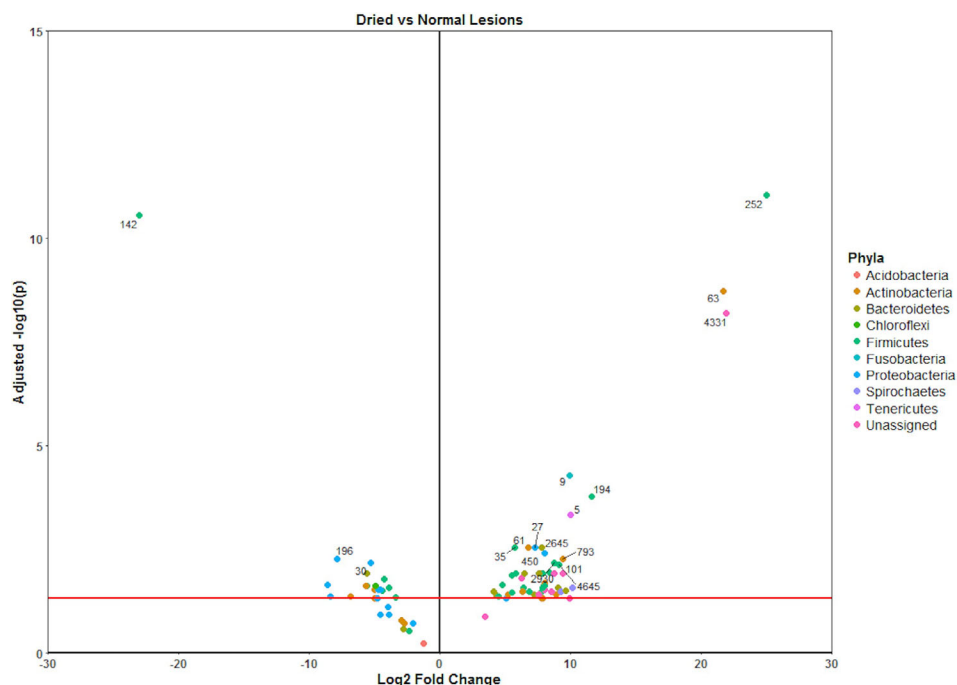
### Histopathology

Tissues were not identifiable as acute, chronic or not infected. Epidermal thickness was within normal limits for a bovine heel. Dermal inflammation was generally mild and limited to perivascular lymphohistiocytic infiltrates in the dermis. Sample 3 showed focal partial to full thickness keratin and epithelial loss adjacent to an extensive region showing loss of keratin structure, with superficial moderate infiltration with degenerate and fragmenting leukocytes and abundant colonies of small bacteria. Within the keratin, not directly associated with the eroded focus, was a cluster of bacteria including small numbers of long wavy organisms, presumed spirochaetes. Underlying epithelium showed superficial ballooning degeneration and hyaline, eosinophilic cytoplasm and in the underlying superficial dermis there was a very mild neutrophilic infiltrate.



**Figure 3.** Result of the DESeq2 analysis: a volcano-plot showing the significant OTUs when the active lesions were compared with the normal skin biopsies. The x-axis displays the LFC, the y-axis displays the inverse logarithmic P-value, adjusted for false discovery. The red line displays the equivalent of a P-value of 0.05, the points above this line are OTUs that had a significantly different abundance between Active and Normal samples. Positive LFC values reflect increases in OTUs in Active samples compared to Normal, and negative LFC values reflect a decrease. Labeled OTUs are those where the P-value < 0.01.

**Figure 4.** Result of the DESeq2 analysis: a volcano-plot showing the significant OTUs when the dried lesions were compared with the normal skin biopsies. The x-axis displays the LFC, the y-axis displays the inverse logarithmic P-value, adjusted for false discovery. The red line displays the equivalent of a P-value of 0.05, the points above this line are OTUs that had a significantly different abundance between Dried and Normal samples. Positive LFC values reflect increases in OTUs in Dried samples compared to Normal, and negative LFC values reflect a decrease. Labeled OTUs are those where the P-value < 0.01.



Sample 6 also showed focal ballooning degeneration of epithelium, associated with the os of a vibrissal follicle. The follicular lumen was plugged with disordered keratin and the luminal surface of the epithelium showed hypereosinophilia, infiltration by degenerate leukocytes, mainly neutrophils and sloughing of degenerate cells, probably epithelial, into the lumen. There was a mild lymphohistiocytic and neutrophilic infiltrate in the surrounding superficial dermis. No bacteria, including spirochaetal forms, were present. Sample 4 also had bacteria suggestive of spirochaetes in the keratin and superficial epidermis, in the absence of an associated lesion. Both animals in which spiral bacteria were seen were clinically acute cases.

## Discussion

The aims of this study were to estimate the prevalence of DD in Victoria, Australia, and to investigate which bacteria are consistent with typical DD lesions. Overall, the results showed a prevalence of 29.8% of DD, with a prevalence of 32.2% among dairy cattle and of 10.8% among beef cattle. These findings prove that DD is present in Victoria, Australia. These results are similar to a study in the south-eastern USA with a comparable study design<sup>36</sup>, which found a prevalence of 29% in dairy cattle and 4% in beef cattle. Another recent investigation into DD among dairy cattle has been performed in New Zealand<sup>52</sup>, a pasture-based dairy industry, with similar conditions to those in south-east Australia. This research concluded that 63.8% of 224 farms were affected with DD, although the prevalence at a cow-level was quite low (1.2%). Both the herd and cow-level prevalence were lower than countries in North America and Europe, which is thought to be related to the pasture-based system<sup>53</sup>. In our study, 97.1% of cows with DD had lesions on the hind feet. This supports the findings of previous studies, with fewer than 3% of affected cows found with lesions on the fore limbs only<sup>36,54</sup>.

The primers used in the PCR-based analysis were 314F and 806R designed to target the V3 and V4 region of the 16S rRNA gene; these regions amplify with the majority of treponemes identified from DD lesions<sup>26</sup>. The Chao1-index (Figure 2) shows that the richness of OTUs of normal skin biopsies is significantly higher than in dried lesions, which is in accordance with previous studies<sup>55,56</sup>. The DESeq2 results highlight 82 OTUs that had a significantly different abundance between diseased (Active/Dried) and Normal samples. The large number of potentially causative organisms may be reflective of previous assessments that DD is a polymicrobial disease. Two out of six biopsies of healthy skin contained OTUs that were taxonomically classified as *Treponema*-species (Figure 6; sample 13 and 14). Previous studies also found cases of healthy skin biopsies which were positive, and transmission experiments using pure cultures of *Treponema*-species have generally failed to introduce DD<sup>22</sup>. Our results and these previous findings confirm that DD is a polymicrobial and multifactorial disease; presence of *Treponema*-species only does not necessarily cause DD. Indeed, seven out of twelve biopsies of dried and active lesions were PCR negative for *Treponema*-species, and in only two of our silver stained biopsies were *Spirochaetes* detected. The negative results for treponemes in our samples may be due to the low sensitivity of the PCR rather than the absence of treponemes in the samples. The same conclusion was drawn from five biopsies of erosive dermatitis-lesions, which were obtained from a veterinary practice in Tasmania, Australia<sup>29</sup>. In that study, there were no *Spirochaete* organisms observed by silver staining, and these researchers suggest that their finding indicated that other hoof disorders with common conditions are easily mistaken for DD.

None of the silver stained samples showed severe signs of inflammation or were identifiable as acutely or chronically infected, and in only two out of eight samples *Spirochaetes* were detected. This is not

**Table 3.** Results of the DESeq2 analysis; LFC and *P*-values of the OTUs that were significantly different (*P* < 0.05) in regard to normalised abundance in both the groups of active lesions and dried lesions, compared with the normal skin biopsies. Positive LFCs are associated with an increase in OTU abundance compared to the normal skin sample group, negative LFCs are associated with a decrease in OTU abundance compared to the normal skin sample group

OTU	Absolute read counts			Active vs. normal		Dried vs. normal		Phylum	Class	Order	Family	Genus
	Normal	Active	Dried	LFC <sup>a</sup>	<i>P</i> -value <sup>b</sup>	LFC <sup>a</sup>	<i>P</i> -value <sup>b</sup>					
63	0	4622	56	26.09	< 0.001	21.67	< 0.001	Actinobacteria	Actinobacteria	Actinomycetales	Intrasporangiaceae	
2143	10	560	228	7.83	0.044	8.03	0.021	Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	Trueperella
4234	603	1	0	-8.04	0.036	-6.89	0.043	Actinobacteria	Actinobacteria	Actinomycetales	Williamsiaceae	Williamsia
31	446	9296	3206	5.36	0.034	4.51	0.043	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	
194	5	512	495	8.14	0.036	11.63	< 0.001	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	
252	0	66	325	22.02	< 0.001	24.99	< 0.001	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	Peptococcus
2148	1	267	88	8.25	0.036	7.97	0.024	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	
2930	2	349	219	8.07	0.033	8.76	0.007	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	
91	2247	1	4	-9.89	0.036	-8.40	0.044	Proteobacteria	Deltaproteobacteria	Myxococcales		
1474	4	0	336	-15.89	< 0.001	9.26	0.033	Spirochaetes	Spirochaetes	Spirochaetales	Spirochaetaceae	Treponema
5	545	62385	25867	7.70	0.031	10.00	< 0.001	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	Mycoplasma
657	10	1398	644	8.49	0.048	9.38	0.012	Unassigned				
1420	3	767	203	8.68	0.036	8.05	0.030	Unassigned				
3501	1	196	27	8.86	0.031	8.75	0.012	Unassigned				
4331	0	124	36	21.38	< 0.001	21.91	< 0.001	Unassigned				

<sup>a</sup>Logarithmic Fold Change in differential abundance <sup>b</sup>The *P*-value is adjusted for false discovery, according to Benjamini & Hochberg (1995).

in accordance with previous studies conducted in the UK, the Netherlands and USA<sup>10,57,58</sup>, and the reason for the discrepancy is unclear, but may relate to sampling issues and the fact that dead material was sampled. These prior studies described DD lesions histologically as having *Spirochaetes* clearly present, microabscesses in the epidermis and dermis, a proliferative epidermis and showing progressive inflammatory patterns. One of the examined tissues, sample 4, was PCR negative for *Treponema*-species based on sequencing, but *Spirochaete*-like organisms were seen in the histological examination. Even though, this concerned only one sample, this finding could suggest that *Spirochaete*-like organisms, for example *Leptospira* or *Brachyspira*, are easily mistaken for *Treponema*. Therefore, looking for *Spirochaetes* in a histological sample, with the purpose of detecting *Treponema*, may not be a reliable method for the confirmation of a diagnosis of DD. However, sample 4 was also negative for *Leptospira* and *Brachyspira*, according to the results of the 16S rRNA diversity profiling. The *Spirochaete*-like organisms seen in the histological sample might be other organisms, or the 16S rRNA diversity profiling was not sensitive enough to detect these organisms.

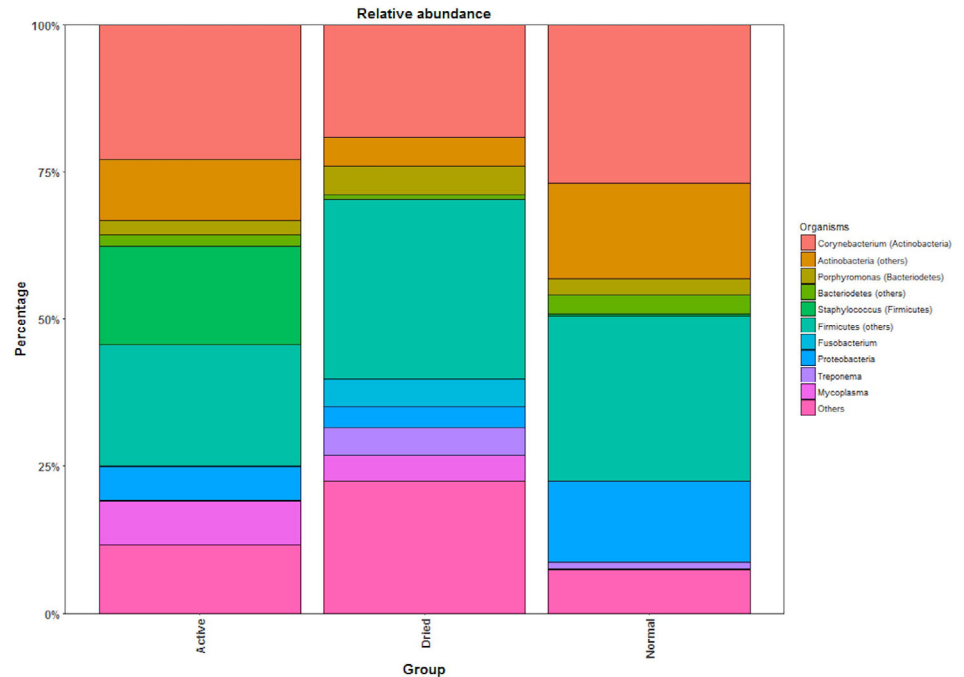
We identified 5 OTUs from the phylum *Firmicutes* and 2 OTUs from the phylum *Actinobacteria* that were significantly increased in differential abundance in the DD lesions (Table 3). This is not in accordance with a previous study that concluded the opposite; *Firmicutes* and *Actinobacteria* are predominant in samples of healthy cows<sup>28</sup>. One OTU classified as *Proteobacteria* was found to be decreased in differential abundance in DD lesions, which is in accordance with a previous research among Danish cattle<sup>31</sup> (Table 3).

*Staphylococcus* was increased in relative abundance in the samples of active lesions (Figure 5), this is due to sample 15 and 16 (Figure 6), which contained relatively high amounts of this bacterium. *Staphylococcus* is not considered to play a role in the aetiology of DD, however, it is an opportunistic environmental pathogen and an important causative agent of mastitis<sup>59</sup>. Thus, *Staphylococcus* in our samples might be due to environmental contamination.

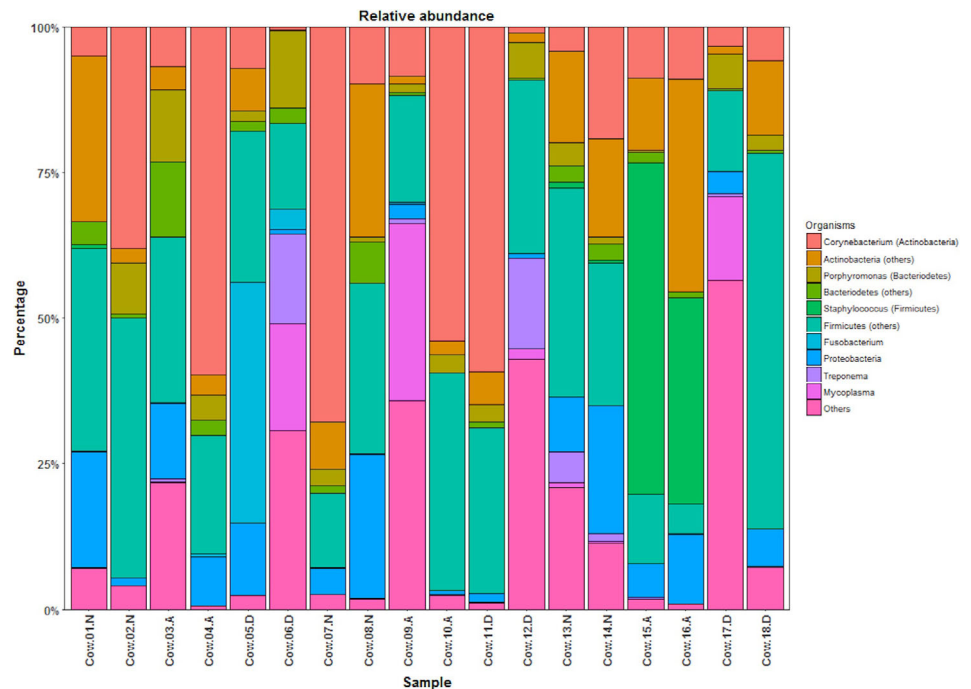
The literature surrounding the causative pathogens of DD is inconsistent, which supports the assumption that DD is a polymicrobial and multifactorial disease. Considering the significant results in differential abundance for pathogens associated with DD lesions, when compared with the results from other studies, the findings of this study may indicate that the aetiology of DD in Australia could differ from the aetiology of DD in other countries. Analysis of the microbial components of DD from more samples would be needed to determine whether this is the case.

The survey population for this research was cattle from knackeries. These cows are not fit for human consumption or for transportation, and the reason for culling was unknown. It is possible that lameness was the reason these cows were sent to a knacker, which may have increased the calculated DD prevalence for this survey population<sup>36</sup>, because DD is an important cause of lameness<sup>1</sup>. Conversely, the time to inspect the feet was limited, so it is possible that small DD lesions have been missed and therefore the calculated prevalence is underestimated. Furthermore, clinical signs of lameness were not taken into account in this study, because cows were already deceased before their limbs were examined. So, no conclusions can be drawn

**Figure 5.** Relative abundance of bacteria of interest in active, dried and normal skin biopsies. Each group (active, dried and normal) contained 6 samples. The relative abundance of 'others' are organisms that were not deemed notable in previous studies into DD.



**Figure 6.** Relative abundance of bacteria of interest per sample. D; dried lesion, A; active lesion, N; normal skin biopsy. The relative abundance of 'others' are organisms that were not deemed notable in previous studies into DD.



about the signs affected cows might show when they are infected with DD.

It is strongly recommended for future studies to collect farm and cow information. Ideally, cows from different farms would be examined, across the seasons, because these data can add valuable insight into potential risk factors such as farm type, geographical distribution, clinical signs, and the prevalence at a herd-level of DD in

Australia. A possible seasonal pattern can be investigated if the case history of affected cows is known and if examination is done in different seasons, this could give an insight into a potential role of rainfall on the occurrence of DD as well.

The present study is the first to investigate the prevalence and causative pathogens of DD in Victoria, Australia. The prevalence was found to be higher in dairy cattle than in beef cattle, and the overall



prevalence was 29.8%, proving that DD is present in Victoria, Australia. Species richness (Chao1-index) and phylogenetic diversity (UniFrac) were significantly decreased in dried lesions, and 11 OTUs classified to the phyla *Spirochaetes*, *Actinobacteria*, *Firmicutes*, *Proteobacteria* and *Tenericutes*, were found to be significantly differentially abundant in both the active and dried lesions. Additional research is necessary to investigate the true prevalence, risk factors and causative pathogens and to develop better treatments and preventive strategies for this emerging disease in Australia. It is important to make farmers and veterinarians aware of DD, in order to develop adequate preventive measures

# Conflict of interest statement

None of the authors has a personal or financial relationship with other organisations or people that could influence or bias the content of the paper.

# Acknowledgments

The authors would like to thank the staff at Maffra, Rochester and Camperdown knackeries for their assistance with the project. This research was funded by Dairy Australia.

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(Accepted for publication 17 June 2019)