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Etiology and epidemiology of digital dermatitis in Australian dairy herds

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ABSTRACT

Bovine digital dermatitis (BDD) is an important cause of lameness in dairy cows worldwide. However, very little is known about this disease in Australian herds, which are predominantly managed on pasture. The primary objectives of this cross-sectional study were to describe the presence and prevalence of BDD in Australian dairy herds and to characterize the microbiota of healthy skin and M4 lesions of BDD-affected, pasture-managed cows. Cows from 71 dairy herds were examined at milking time to identify the presence of BDD lesions. True prevalence was estimated using Bayesian methods with informative priors for sensitivity and specificity. Biopsy samples (n = 60) were collected from cows with and without BDD lesions in 7 pasture-based herds. The microbiota in the superficial and deep strata of each tissue biopsy were characterized via sequencing of the V3–V4 region of the bacterial ribosomal RNA gene. Lesions were detected in 1,817 (11.5%) of 15,813 cows and in 68 of 71 (95.8%) herds. The median herd-level apparent and true prevalences of BDD were 8.5% and 18.1%, respectively, but prevalences varied considerably between farms. On farms with BDD, M4 lesions accounted for 70% to 100% of all lesions (interquartile range = 95.1%–100%, median = 100%); M2 lesions (i.e., large ulcerative lesions) were observed at low prevalence (<2.2%) in the few herds (7/71, 9.9%) where they were found. There was a significant difference in the composition of the microbiota between healthy skin and M4 lesions but not between superficial and deep tissue layers. Several gut- and effluent-associated bacterial taxa, including *Lentimicrobium* and *Porphyromonas*, which have previously been associated with BDD, were abundant in BDD lesions but not in control biopsies. Our study supports the idea that such taxa are involved in, although possibly not essential to, lesion development and persistence in pasture-managed cows in Australia. Our results also suggest that *Dichelobacter* may contribute to the disease process. We conclude that BDD is likely to occur in most Australian dairy farms, but that further studies are needed to identify its effect on cow welfare and productivity. Further investigation of the etiology of BDD in Australian dairy herds is also necessary to inform prevention and control strategies.

Key words: digital dermatitis, microbiome, true prevalence, lameness

INTRODUCTION

Lameness is recognized as one of the most important diseases of dairy cows worldwide, as it impairs cow welfare and production, threatens economic returns for producers and ultimately, the sustainability of food production systems (Garbarino et al., 2004; Amory et al., 2008; Cha et al., 2010; Bruijnis et al., 2012). Bovine digital dermatitis (**BDD**; also referred to as papillomatous digital dermatitis; Hanna et al., 1994) is the most common infectious cause of lameness in dairy cattle herds worldwide (Laven and Logue, 2006). The disease manifests as an ulcerative or proliferative lesion of the skin at the interdigital cleft. The clinical manifestations of BDD range from a small, active focus of <2 cm in diameter to raised or papilliform-like projections (Döpfer et al., 1997).

First described in Italy in 1974 (Cheli and Mortellaro, 1974), BDD has since been reported worldwide, with surveys estimating the percentage of herds with lesions to be 74.4% and 93.6% in North America (Cramer et al., 2008; Solano et al., 2016) and 91% to 98.8% in Europe (Holzhauer et al., 2012; Oliveira et al., 2017; Pirkkalainen et al., 2021). Although BDD has been reported in Australia, its prevalence and effects in Australian dairy herds are not well described. In the largest study conducted in Australia to date, BDD lesions were found in 13 out of 13

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Victorian farms, with an average herd-level prevalence of 19.1% (range: 6.2%–32.1%; Coombe et al., 2018). Surveys involving small numbers of herds (<5) have also identified herds with BDD lesions in Queensland and Victoria (Coombe et al., 2013; Ardila et al., 2015; Hesseling et al., 2019). Recent studies of pasture-based cows in New Zealand, which has similar farming practices to large parts of Australia, identified BDD in 63.8% (Yang et al., 2017) and 59.2% (Yang et al., 2019b) of herds, with herd-level prevalences of up to 12.7% (Yang et al., 2017) and between 3.1% and 6.3% (Yang et al., 2019a).

The etiopathogenesis of BDD is not fully understood, but the disease is thought to have a mixed bacterial etiology, based on the abundance and diversity of bacteria identified in lesions in histological and molecular studies (Wyss et al., 2005; Santos et al., 2012; Krull et al., 2014; Nielsen et al., 2016), the efficacy of antimicrobial therapy (Demirkan et al., 1999; Nishikawa and Taguchi, 2008; Berry et al., 2010), and a near-absence of fungal or viral DNA in tissue biopsies (Krull et al., 2014). Several bacterial genera have been implicated in the disease process (Krull et al., 2014; Nielsen et al., 2016; Klitgaard et al., 2017; Espiritu et al., 2020; Caddey and De Buck, 2021), but the essential causative agent or agents have not been definitively identified. The spirochaete *Treponema* is often described as the primary causative agent of BDD (Collighan and Woodward, 1997; Pringle et al., 2008; Evans et al., 2009; Nielsen et al., 2016; Mamuad et al., 2020). Spirochaetes are frequently reported in BDD lesions and are often the dominant bacterial genera (Klitgaard et al., 2013; Krull et al., 2014; Nielsen et al., 2016; Klitgaard et al., 2017). They are also regularly associated with regions of tissue necrosis (Choi et al., 1997; Yano et al., 2010; Santos et al., 2012; Klitgaard et al., 2013). Furthermore, spirochaetes have been implicated in the etiopathogenesis of other infectious diseases of the ruminant hoof, such as contagious ovine digital dermatitis (**CODD**; Collighan et al., 2000; Demirkan et al., 2001). The *Treponema* spp. most often associated with BDD are *Treponema medium*, *Treponema denticola*-like, and *Treponema phagedenis*-like (Evans et al., 2008, 2009, 2012; Clegg et al., 2016; Nielsen et al., 2016). Some uncertainty surrounds the role of *Treponema* in the development of BDD lesions, however, as some *Treponema* challenge studies failed to reproduce BDD lesions (Döpfer et al., 1997; Gomez et al., 2012), and the presence and abundance of spirochaetes in BDD lesions are not consistent across studies (Milinovich et al., 2004; Hesseling et al., 2019). Other bacterial genera, including *Porphyromonas* (Grund et al., 1995; Collighan and Woodward, 1997; Santos et al., 2012; Nielsen et al., 2016), *Fusobacterium* (Nielsen et al., 2016), *Bacteroides* (Koniarová et al., 1993; Collighan and Woodward, 1997; Yano et al., 2010; Santos et al., 2012), and *Dichelobacter* (Capion et al., 2012; Rasmussen et al., 2012; Gilhuus et al., 2013; Knappe-Poindecker et al., 2013; Knappe-Poindecker et al., 2014; Krull et al., 2014; Nielsen et al., 2016) have also been implicated in the development of BDD, although they are generally regarded as secondary invaders.

Next-generation sequencing has been employed in the study of several polymicrobial diseases of the ruminant foot, including ovine footrot (Maboni et al., 2017; McPherson et al., 2019; Clifton et al., 2022), CODD (Duncan et al., 2021), and BDD. The microbiota of the BDD lesion has been described in studies conducted in the United States (Santos et al., 2012; Krull et al., 2014; Zinicola et al., 2015), Denmark (Nielsen et al., 2016), Japan (Gotoh et al., 2020), South Korea (Espiritu et al., 2020; Mamuad et al., 2020), Brazil (Moreira et al., 2018), Australia (Hesseling et al., 2019), and Canada (Caddey et al., 2021). A meta-analysis of previous microbiota studies identified similarities in the composition of the microbiota of BDD lesions across studies irrespective of region and methodological variations, with *Treponema*, *Mycoplasma*, *Porphyromonas*, and *Fusobacterium* the genera that best differentiated BDD lesions from healthy skin (Caddey and De Buck, 2021).

Current knowledge of the epidemiology and etiology of BDD in Australian dairy cow herds is limited. A greater understanding of the epidemiology of BDD in Australian herds is needed to determine the effects of this disease on animal health, productivity, and welfare. Characterizing the etiology of BDD lesions will help to inform strategies for controlling, preventing, and treating the disease. The primary objectives of this study were to describe the presence and prevalence of BDD in Australian dairy herds and to characterize the microbiota of healthy skin and BDD lesions of cattle in BDD-affected, pasture-managed cows. A secondary objective was to explore potential herd-level risk factors for cow-level BDD prevalence.

MATERIALS AND METHODS

All protocols were approved by the University of Sydney's Animal Ethics Committee (project no. 2021/1860).

Herd Recruitment

Herds (n = 71) were recruited from New South Wales (**NSW**, n = 33), northern Queensland (**QLD**, n = 18), and northern Victoria (**VIC**, n = 20) between June 2021 and July 2022. We aimed to enroll 70 herds, as each field investigator (n = 3) considered that enrolling 20 to 30 herds was feasible. Consequently, we estimated that we would be able to estimate the prevalence of BDD-affected herds within ±12 percentage units of the "true" prevalence, as-

suming a prevalence of 60%, as recently observed in New Zealand (Yang et al., 2017, 2019b), which has similar farming systems to Australia. A convenience sampling method was used. Herds were eligible for inclusion if they were known to the study investigators (most were clients of Livestock Veterinary Services, Tableland Veterinary Service, Malanda, Australia, and Rochester Veterinary Practice, Rochester, Australia), milking more than 25 cows, and had water hoses in the milking parlor to enable cleaning and examination of hind feet during milking time. Biopsy samples were collected from grazing herds in NSW (n = 3) and QLD (n = 3). We restricted our sampling to grazing cows for biopsy collection and microbiome analysis because most cows in Australia are managed in pasture-based systems, and because few studies have investigated BDD etiology in grazing cows. Biopsy herds were also selected according to criteria of convenience (proximity to investigators clinic, willingness to be involved). For prevalence estimation, nongrazing herds were also considered.

Sampling Strategy

For the prevalence survey, the first 7 herds had all lactating cows examined for BDD lesions, as it was not known whether cows with BDD were more likely to present at particular stages of the milking order, which occurs with lame cows (Sauter-Louis et al., 2004). After analyzing these findings, we concluded that selecting cows from any stage of the milking order was unlikely to bias prevalence estimates (data shown at https://rpubs.com/samrowe/bdd_epi_sampling). This enabled field investigators to examine a sample of cows from the remaining herds that were recruited, which facilitated the recruitment of more herds and larger herds. The sample size required to detect at least one BDD-affected cow was calculated using the 'rsu.sssep.rs' function in epiR (Martin et al., 1992; Stevenson, 2015) using the following assumptions: cow-level sensitivity (**Se**) = 0.63 (Yang and Laven, 2019), target herd sensitivity = 0.95, within-herd prevalence = 0.03 (i.e., a very low prevalence). This approach was used to ensure that enough cows were scored to detect the presence of BDD in herds with low prevalences, and thus give a valid estimation on the proportion of BDD-endemic herds in Australia. In situations where 2 or more cows with lesions were detected in a herd, the minimum sample size was recalculated using the 'epi.sssimpleestb' function in the epiR package using the following assumptions: Se = 0.63, specificity (**Sp**) = 1.0, within-herd prevalence = 35% (the prevalence observed in the first 7 herds visited), confidence level = 0.95, and desired level of precision = ± 0.1. Therefore, this 2-stage sampling approach aimed to maximize herd sensitivity and prevalence precision. Sample size calculations can be seen at https://rpubs.com/samrowe/bdd_epi_sampling. When herds were split into management groups (e.g., early- and late-lactation cows), field investigators used the sampling approach described above for each management group.

Examination at Milking Time

Cows were examined for BDD lesions at milking time. Hind feet were hosed clean with water and then visually examined using a headlamp. Feet were scored for BDD status using the system devised by Döpfer et al. (1997) and modified by Berry et al. (2012): M1 = a small (<2 cm), circumscribed lesion, with small red foci; M2 = as for M1 but larger (>2 cm) with extensive red mottling; M3 = healing stage with a dry rubbery scab; M4 = chronic stage, raised surface with brown or black papilliform or mass-like projections; M4.1 = chronic stage but with small active focus. Healthy feet were assigned the score M0. Field investigators recorded scores using audio recordings with smartphones and corded microphones. Field investigators were trained in this lesion scoring system with a set of images used by Vanhoudt et al. (2019) to describe interobserver agreement for BDD scoring (http://bit.ly/M-score_survey). These images were divided into training (n = 40) and testing (n = 41) images. After training, field investigators independently scored the previously unseen test images. Scores were used to measure agreement between field investigators (Fleiss kappa) and with lameness experts (Cohen's kappa) in the Vanhoudt et al. (2019) study. A questionnaire was completed before examination of cows, to collect demographic information about the farms, their husbandry practices, and their experiences with BDD. The questionnaire was refined from a previous version used in a survey of BDD in New Zealand dairy herds (Yang et al., 2018).

Biopsy Collection and Storage

In biopsy herds, field investigators recorded the tag number for the first 10 cows with lesions (cases) as seen during milking, along with an adjacent cow without lesions (control; 1 per case). Therefore, ratio of enrollment for cases and controls was 1. The farm was visited within 2 wk of the milking visit for biopsy collection. Subjects had their limb restrained with ropes in a standard cattle chute (i.e., not a specialized hoof trimming chute), and the foot was rinsed gently with clean water to remove gross debris, without dislodging epithelial tissue. The lesion type was re-scored after closer examination, and a punch biopsy (6-mm diameter, 10-mm depth) was collected from the skin at the plantar aspect of the interdigital cleft after applying local anesthesia (injection of 10–20 mL of lignocaine hydrochloride proximal to the intended biopsy

site). This was collected from the center of the lesion in BDD-affected cows and from an equivalent region in nonaffected cows. Only one biopsy was collected from each cow. Pain relief was provided to cows (ketoprofen 3 mg/kg intramuscular, Ilium Ketoprofen Injection, Troy Animal Health Care, Glendenning, NSW, Australia). Biopsies were immediately placed into a sterile 2-mL microcentrifuge tube (DNA LoBind, Eppendorf, Hamburg, Germany) containing 1 mL of RNAlater (Thermo Fisher Scientific, Waltham, MA) and transported to the laboratory in a cooler box with ice bricks. Biopsies were stored at −20°C for up to 30 d before processing.

Statistical Analysis: Estimating Prevalence of BDD

An analysis log can be found at https://rpubs.com/samrowe/bdd_epi. Audio recordings of herd visits were transcribed into a spreadsheet and then imported into R for analysis (R Core Team, 2018). The apparent prevalence of BDD for each herd was calculated by dividing the number of cows with at least one lesion by the number scored. A weighted average was calculated for herds that were split into milking groups. The true prevalence for each herd was estimated using Bayesian methods, using the "truePrev" function in the prevalence package (Devleesschauwer et al., 2013). This method uses Markov chain Monte Carlo simulation to produce posterior estimates for true prevalence, based on prior knowledge of test Se and Sp, and prevalence, as well as the following function describing the relationship between all parameters in the model:

$$\text{Apparent prevalence} = \text{Se} \times \text{True Prevalence} + (1 - \text{Sp}) \times (1 - \text{True Prevalence}).$$

The prior distributions for Se [β(17.996, 10.152)] and Sp [β(100, 2)] were based on a study by Yang and Laven (2019), who found that the Se for our method of detection was 0.63 (95% CI: 0.46–0.78) and the Sp was 0.99 (0.97–1.0). A uniform prior distribution between 0 and 1 was used for true prevalence. The model settings included 2 chains, burn-in = 10,000 iterations, and total values included in final output per chain = 10,000.

Extraction of Bacterial DNA from Tissue Biopsies

Each tissue biopsy was removed from the RNAlater, placed on a plastic Petri dish over ice, and dissected using a sterile scalpel blade. The biopsy was cut in half lengthways and separated into 2 subsections: the superficial stratum (0–2 mm below the skin surface) and the deep stratum (2–4 mm beneath the skin surface). The outer edges of each subsection were trimmed until the weight of the subsection was 200 mg (±10 mg). A DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) was used to extract DNA from each subsection. Each subsection was placed into a 1.5-mL microcentrifuge tube containing 180 mL of Buffer ATL, 40 mL of Proteinase K (Qiagen, Hilden, Germany), and one 5-mm stainless-steel bead (Sigma-Aldrich, St Louis, MO). Tubes were incubated overnight at 56°C on at oscillating heat block at 400 rpm. The contents of the tube were then transferred to a new 2-mL tube containing 30 mg of 0.1-mm silica-zirconia beads, and bead-beating was performed using a Bead Ruptor 4 (Omni International, Tulsa, OK) at maximum speed for 3 min. Thereafter, DNA was extracted as per the manufacturer's protocol. A negative extraction control (RNAlater only) was also included with each batch of DNA extractions. The DNA was eluted into 50 mL of Buffer AE (Qiagen) and stored at −20°C before analysis. Concentration and purity of DNA were evaluated with a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Rockland, DE).

PCR Amplification, Library Preparation, and Next-Generation Sequencing

The PCR amplification, library preparation, and next-generation sequencing were performed by the Australian Genome Research Facility (AGRF; Melbourne, Australia). The PCR amplification of the V3–V4 region of the bacterial 16S rRNA gene was performed using the primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′). These primers have been used previously to characterize the microbiota of BDD lesions in Australia (Hesseling et al., 2019), and the V3–V4 region is reported to be appropriate for the identification of *Treponema* (Klitgaard et al., 2013). Library preparation was performed with the Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA), and sequencing was performed on an Illumina MiSeq system using 300 base-pair paired-end chemistry.

Analysis of Next-Generation Sequencing Data

Bioinformatics were performed with QIIME2 v2020.6 (Bolyen et al., 2019). Primer and adapter trimming was performed using the QIIME2 cutadapt plugin with the flags --p-match-adapter-wildcards, --p-match-read-wildcards, and --p-discard-untrimmed. Quality trimming, merging of paired-end reads, chimera filtering, dereplication, and feature table construction were performed using the QIIME2 DADA2 plugin (Callahan et al., 2016). Twenty base-pairs were trimmed from the 5′ end of each read, and the forward and reverse reads were truncated at 240 and 200 bp, respectively. The default settings were used for all other parameters. Unique sequences were

grouped into amplicon sequence variants (**ASV**, also referred to as features) using a threshold of 99% similarity. An alignment of the representative sequences was created and a phylogenetic tree was constructed using the align-to-the-tree-mafft-fasttree command in the QIIME2 phylogeny plugin. Alpha rarefaction plots were generated and reads inspected to determine the minimum number of reads required to ensure that the true diversity of the bacterial communities in each sample was captured. Taxonomy was assigned to ASV using a naïve Bayes classifier trained on the SILVA v13.8 database, with 16S sequences trimmed to retain the V3–V4 hypervariable region only, using the 341F and 806R primer sequences. This strategy is reported to enable greater classification depth than the use of a classifier trained on full-length 16S rRNA sequences (Werner et al., 2012). Chloroplast, mitochondrial, archaea, and eukaryotic sequences were filtered from the feature table and the representative sequences before analysis.

Statistical analysis of the count data was performed with RStudio v2023.06.0 (R Core Team, 2018). Analysis was restricted to biopsies from M4 lesions, as an insufficient number of biopsies were collected from lesions of other scores to enable statistical analysis. The feature table was rarefied to 10,000 reads per sample, and α diversity was calculated for each sample using 4 metrics: the number of observed features, Shannon's diversity index, Simpson's diversity index, and inverse Simpson's index with the R package phyloseq v1.44.0 (McMurdie and Holmes, 2013). Differences in α diversity between samples representing the superficial and deep tissue stratum of healthy (score M0) and BDD-affected feet (score M4) were analyzed with a Wilcoxon rank sum test with the R package phyloseq. Beta diversity analyses were performed to evaluate the level of dissimilarity of the microbial communities in samples from BDD-affected and unaffected feet. Count data were centered log ratio transformed using the R package microbiome v1.22.0, and a distance matrix was generated using Euclidian distances with the R package phyloseq. Distances were analyzed using the "ADONIS" function, and a dispersion test was performed using the "betadisper" function to confirm homogeneity of variances, using the R package vegan v2.6.4 (Oksanen et al., 2023). A significant *P*-value of 0.05 was used for all α and β diversity analyses.

Differential Abundance Analysis

Linear discriminant analysis effect size (**LEfSe**; Segata et al., 2011) was used to identify differentially abundant features in metadata categories of interest. Before analysis, feature tables generated by QIIME2 were collapsed to the genus level, and data were converted into relative frequencies. The table was then exported as a text file and formatted in Excel per the requirements for analysis with LEfSe. The LEfSe analyses were performed using the default parameters.

Risk Factors for Cow-Level BDD

A secondary objective was to identify associations between herd-level risk factors and BDD prevalence. The factors evaluated for potential association with true herd-level prevalence of BDD are shown in Table 1. Unconditional prevalence odds ratios (**POR**; Pearce, 2004) were calculated using generalized linear mixed models on a cow-level data set using the lme4 package (Bates and Maechler, 2010). No multivariable models (i.e., a methodology used to derive causal estimates) were reported, as it was decided that the prevalence observed in our study was likely to be a poor proxy for incidence rates and POR a poor proxy for causal incidence rate ratios (Rothman and Lash, 2021).

RESULTS

Description of Farms in Study

Farms (n = 71) were recruited from 5 regions in NSW and from a single region in both VIC and QLD (Table 2). Median herd size was 243 (range 59–7,990), median daily milk yield was 23 kg (15–42), and calving patterns were seasonal (5.6%), split (21.1%), and year-round (73.2%). Most herds (87.3%) used grazing as part of their management system and had purchased cows or bulls from other farms in the past 20 years (98.6%). When farmers were asked whether they thought BDD was causing lameness in their cows, 53.5% answered that they had not seen lesions, 8.5% indicated that they had seen lesions but did not think they were causing lameness, and 38.0% indicated that they had seen lesions causing lameness. Lameness management strategies used by farmers are summarized in Table 1.

Prevalence of BDD

A total of 15,813 cows were examined for BDD lesions, which accounted for 50.4% of the 31,284 lactating cows in the 71 herds recruited into the study. Of the cows scored, 1,817 (11.5%) had lesions. Although lesions were seen in almost all herds (68/71, 95.8%), the within-herd apparent and true prevalences were low to moderate for most herds (Figure 1). Table 2 shows herd-level prevalences of BDD, stratified by region. The median herd-level apparent and true prevalences of BDD were 8.5% and 18.1%, respectively, but this

Table 1. Unconditional associations between herd-level management strategies and cow-level prevalence of bovine digital dermatitis lesions in Australian cows

Risk factor	Number of herds (%)	POR[1]	95% CI
Predominant management system			
Gazing only	19 (26.7)	Ref	
Grazing and feed pad	43 (60.6)	3.2	1.6–6.4
Barn	9 (12.7)	10.6	3.9–28.5
Use of foot mats			
Never	55 (22.5)	Ref	
Intermittent (<3 mo/yr)	13 (18.3)	1.7	0.7–4.0
Regular	3 (4.2)	2.6	0.5–13.4
Use of foot bathing			
Never	57 (80.3)	Ref	
Intermittent (<3 mo/yr)	8 (11.3)	1.4	0.4–4.8
Regular	6 (8.4)	1.3	0.5–3.7
Farm currently conducts preventative trimming			
No	57 (80.3)	Ref	
Yes	14 (19.7)	2.8	1.3–6.3
Has the farmer ever used a veterinarian or professional hoof trimmer for preventative or therapeutic trimming?			
No	0 (0.0)		
Veterinarian only	47 (66.1)	Ref	
Trimmer only	8 (11.3)	6.3	2.5–15.9
Veterinarian and trimmer	16 (22.5)	3.1	1.5–6.4
Who currently does most hoof trimming?			
Farmer	32 (45.1)	Ref	
Veterinarian	32 (45.0)	0.8	0.4–1.5
Professional hoof trimmer	7 (9.9)	5.3	1.8–15.3
Have lactating cows been purchased in the last 20 yr?			
No	10 (14.1)	Ref	
Yes	61 (85.9)	2.0	0.8–5.1
Have adult bulls been purchased in the last 20 yr?			
No	13 (13.3)	Ref	
Yes	58 (81.7)	0.8	0.3–1.8

[1]Prevalence odds ratio (POR) was estimated using univariable generalized linear mixed models, adjusting for the clustering of cows within herds.

Table 2. Apparent and adjusted estimates for herd-level prevalence of bovine digital dermatitis (BDD) in farming regions in Australia

Item	Number of herds (% of total)	Apparent prevalence of BDD		True prevalence of BDD	
		Median (%)	Range (%)	Median (%)	Range (%)
All herds	71 (100.0)	8.5	0–53	18.1	0.0–80.6
New South Wales					
Bega Valley	5 (7.0)	8.7	2.3–27.6	17.8	2.8–42.3
Central West	6 (8.5)	25.1	10.3–53.0	42.0	14.6–80.6
Greater Western Sydney	5 (7.0)	33.0	11.5–35.6	38.8	16.4–55.6
North Coast	5 (7.0)	12.7	4.8–32.8	23.0	5.4–51.4
South Coast	12 (16.9)	14.6	2–35.7	22.8	2.7–55.9
Queensland					
Atherton Tablelands	18 (25.4)	3.5	0–17.4	8.0	0.0–26
Victoria					
Northern Victoria	20 (28.2)	5.2	0–26.9	10.8	0.0–41

varied considerably between farms and by region. The apparent prevalences of lesions other than M4 were low in most herds. The median (range) within-herd apparent prevalences were 0.0% (0.0%–0.7%) for M1, 0.0% (0.0%–2.2%) for M2, 0.0% (0.0%–1.6%) for M3, 8.5% (0.0%–5.7%) for M4, and 0.0 (0.0%–8.6) for M4.1. On farms with BDD, M4 lesions accounted for 70 to 100% of all lesions (interquartile range = 95.1%–100%, median = 100%). M2 lesions were identified at low prevalences (<2.2%) in the few herds (7/71, 9.9%) where they were found.

Interobserver Agreement Among Field Investigators

Interobserver agreement among the 3 field investigators for classification of 41 test images for the presence or absence of BDD lesions (i.e., a 2-point score) was high, as evidenced by a Fleiss kappa value of 0.92 (95% CI: 0.73–1.0). The agreement when using the 6-point scoring system was 0.50 (95% CI: 0.40–0.60). When each field investigator was individually compared against expert evaluation of the same images, weighted Cohen's kappa ranges were 0.88 to 1.0 and 0.60 to 0.76 for 2- and 6-point scoring systems, respectively.

Next-Generation Sequencing Data

A total of 6,976,696 sequencing reads were obtained from 127 samples (Supplemental Table S1, see Notes) collected from 7 herds (NSW = 3, QLD = 4), including 64 tissue samples from healthy feet, 60 tissue samples from M4 BDD lesions, and 3 negative extraction controls (i.e., one for each batch of DNA extractions). The raw sequencing data were uploaded to the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra; BioProject ID: PRJNA1021941). A total of 4,063,836 reads remained after quality filtering and removal of chimeric reads (Supplemental Table S1). Data from 80 tissue samples were analyzed, including 41 samples from healthy feet (19 samples from the superficial stratum, 22 samples from the deep stratum) and 39 samples from BDD lesions (21 samples from the superficial stratum, 18 samples from the deep stratum). A minimum of 10,000 reads per sample was deemed the minimum number of reads required to capture full diversity in each sample based on the generation and inspection of α rarefaction curves. Data from 47 tissue samples were excluded from all analyses as they contained an insufficient number of reads (Supplemental Table S1). After denoising (Supplemental Table S1), no reads remained for negative extraction control AM76, whereas 1,121 and 167 reads remained in the other negative extraction controls. The taxonomic composition of these were inspected before analysis of the full data set. Three bacterial genera were identified in the negative control samples, 2 of which (*Cutibacterium*, formerly a member of the genus *Propionibacterium*, and *Hyphomonas*) represented the majority of reads in these samples. The genus *Cutibacterium* was also detected in tissue biopsies but represented a small proportion (0.002%–0.1%) of the data from tissue biopsies. The genus *Hyphomonas* was not detected in any tissue biopsies. The genus *Cutibacterium* has previously been identified as a contaminant of DNA extraction kits and PCR reagents (Glassing et al., 2016).

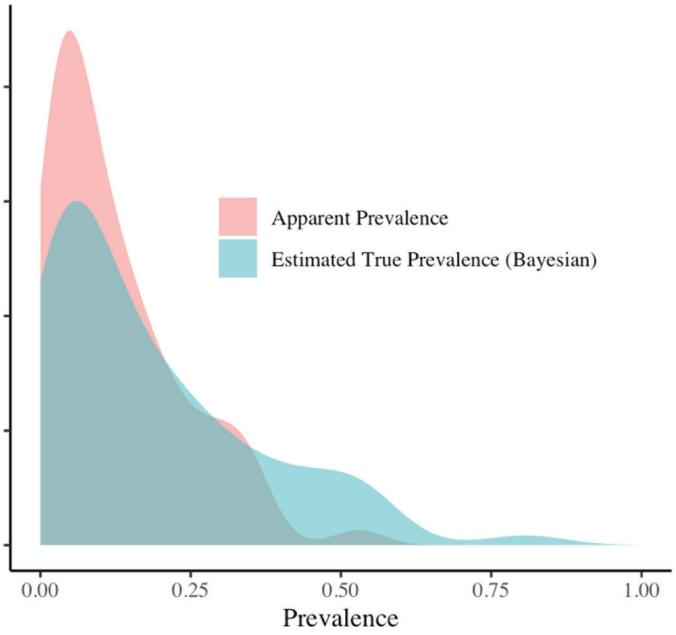

Figure 1. Density plot showing the distribution of the apparent and estimated true prevalence of bovine digital dermatitis in 71 dairy herds in Australia. The estimated true prevalence values were higher than the apparent prevalence values, as evidenced by a greater density of herds toward the right side of the plot. The median apparent and true prevalences were 8.5% (range 0–53.0) and 18.1% (range 0–80.6) as shown in Table 2. Y-axis indicates density of herds at a given prevalence value.

Microbiota Diversity

There was no difference in α diversity between samples from healthy skin and BDD lesions at either depth according to any of the diversity metrics evaluated ($P > 0.05$). By contrast, β diversity was different ($P = 0.001$) between samples from healthy skin and M4 lesions at both depths (Supplemental Figure S1). There was no difference in homogeneity of variances between samples from BDD-affected or unaffected feet ($P > 0.05$). Both α and β diversity were also compared between the superficial and deep strata of BDD-affected feet; however, there was no difference in α or β diversity between the superficial and deep strata of M4 lesions according to any of the diversity metrics evaluated ($P > 0.05$).

Microbiota Taxonomy

Taxonomic analyses were performed at the phylum and genus levels. At the phylum level, healthy feet were dominated by *Actinobacteriota* and *Firmicutes* regardless of tissue depth (Figure 2). At the genus level, similar genera were dominant in both the superficial and deep tissue strata, namely *Kocuria* (46.9% and 48.4%, respectively), *Corynebacterium* (16.8% and 12.6%, respectively), and *Propioniclava* (2.9% and 2.4%, respectively). The deep stratum also commonly contained *Dermatophilus* (5.2%) and an unclassified bacteroida (2.4%; Figure 3).

In M4 lesions, the phyla *Bacteroidota*, *Firmicutes*, and *Actinobacteriota* were dominant at both depths (Figure 2). At the genus level, similar genera were dominant in both the superficial and deep tissue strata, namely *Lentimicrobium* (31.4% and 53%, respectively), *Corynebacterium* (10.8% and 7.1%, respectively), and *Porphyromonas* (5.5% and 3.1%, respectively). Additionally, *Peptostreptococcales-Tissierellales* W5053 (3.4%) and an unclassified *Peptostreptococcaceae* (3.5%) were also abundant in the superficial stratum (Figure 3), and *Clostridium* (3.6%) was also abundant in the deep stratum (Figure 3).

Given the dominance of *Lentimicrobium* in tissue samples from M4 lesions, an NCBI-BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed for each feature classified as *Lentimicrobium* by the QIIME2 classifier to obtain further information (Supplemental Table S2). The top hit for each query was recorded, and hits with an E-value of 0 and a percentage identity >99% were considered. Of the 40 sequences queried, 12 met these criteria. The most frequently occurring result, which was the top hit for 6/12 queries, was a 16S rRNA sequence from an uncultured *Bacteroidetes*/*Bacteroidota* previously identified in BDD lesions (Yano et al., 2010), with an additional sequence showing >98% identity. The top hit for 5 of these queries was an uncultured *Peptostreptococcus* identified in the microbiota of human squamous cell carcinomas (Wang et al., 2017), with an additional 4 sequences showing 96% to 99% identity to that sequence. The top hit (E-value = 0, percentage iden-

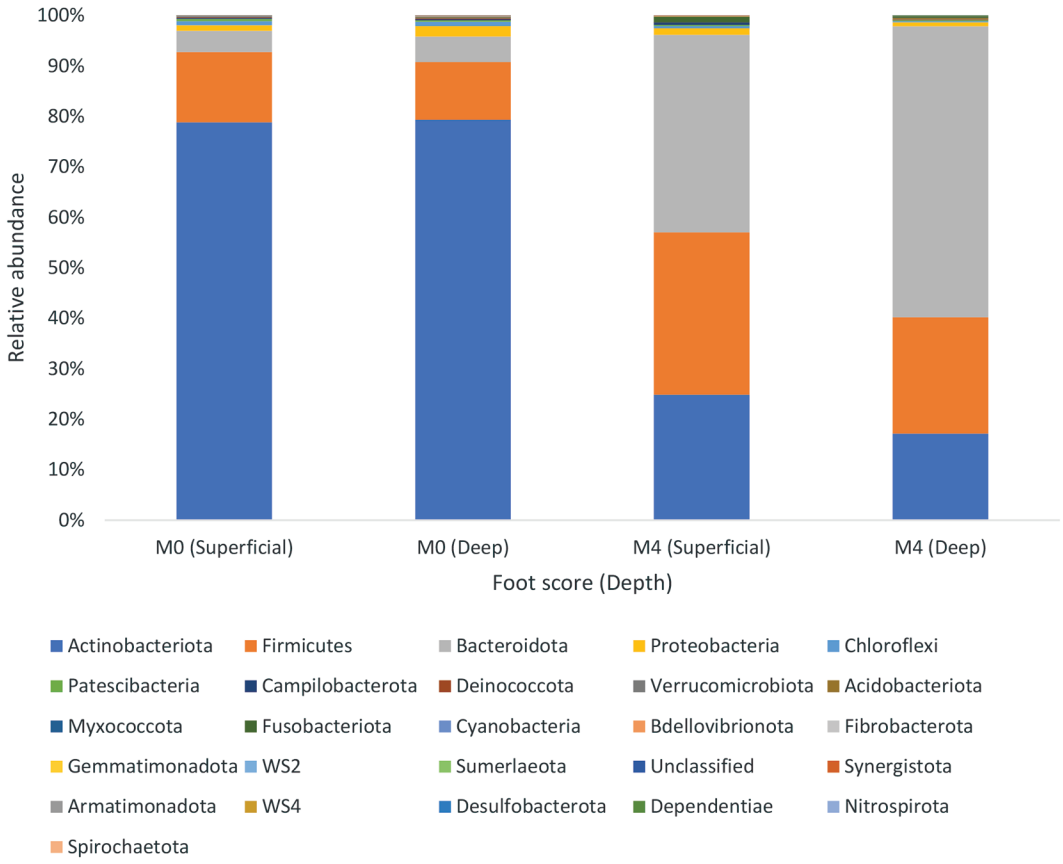

Figure 2. Bacterial phyla identified in tissue samples from healthy skin (score M0; 19 from the superficial stratum, 22 from the deep stratum) and bovine digital dermatitis lesions (BDD; score M4; 21 samples from the superficial stratum, 18 samples from the deep stratum). Samples were collected from 7 dairy cow herds (3 in New South Wales, 4 in northern Queensland) with a history of BDD.

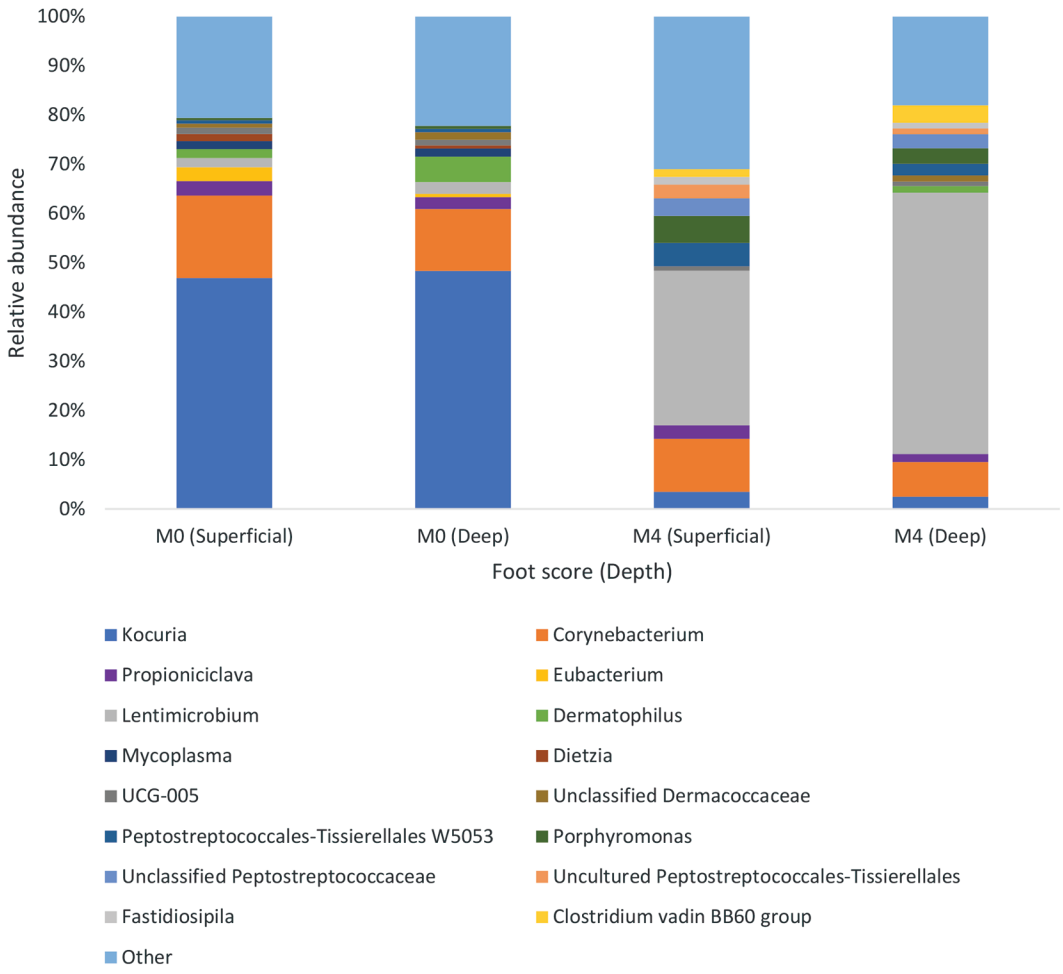

Figure 3. Ten most abundant bacterial genera identified in tissue samples from healthy skin (score M0; 19 from the superficial stratum, 22 from the deep stratum) and bovine digital dermatitis lesions (BDD; score M4; 21 samples from the superficial stratum, 18 samples from the deep stratum). Samples were collected from 7 dairy cow herds (3 in New South Wales, 4 in northern Queensland) with a history of BDD.

tity = 100) for one of these queries was a sequence from an uncultured *Lentimicrobiaceae* identified in organic wastewater (Gagliano et al., 2020).

Treponema was identified in the deep stratum of 2 biopsies collected from healthy feet, 6/21 biopsies from the superficial stratum of BDD-affected feet, and 6/19 biopsies from the deep stratum of affected feet. Relative abundance of *Treponema* spp. was low (0.01%–0.12% and 0.006%–0.03% for superficial and deep strata of affected feet, respectively).

Dichelobacter was identified in the superficial and deep stratum of biopsies from healthy feet (6/19 biopsies each, 0.01%–0.13% and 0.03%–0.73% of the total population, respectively). In contrast, *Dichelobacter* was one of the most abundant genera in samples from lesions, representing up to 4.9% of the community in the superficial stratum and up to 1.36% of the community in the deep stratum.

LEfSe

A LEfSe analysis was used to identify differentially abundant features in the superficial and deep stratum of biopsies collected from healthy and BDD-affected feet. In lesions, the bacterial taxa with the highest linear discriminant analysis (**LDA**) scores were the phylum *Bacteroiodetes*/*Bacteroidota*, the family *Lentimicrobiaceae*, the genus *Lentimicrobium*, and the order *Sphingobacteriales*, with slightly higher scores for the superficial stratum (LDA score = 4.1) than for the deep stratum (LDA score = 3.6), with the exception of *Bacteroidetes*/*Bacteroidota*, which had an LDA score of 3.6.

In the superficial stratum of healthy feet, the bacterial genera with the highest LDA scores were *Corynebacterium* (LDA score = 4.1) and *Kocuria* (LDA score = 3.5; Figure 4). In the deep stratum of healthy feet, the highest LDA scores were for the phylum *Actinobacteriota* (LDA

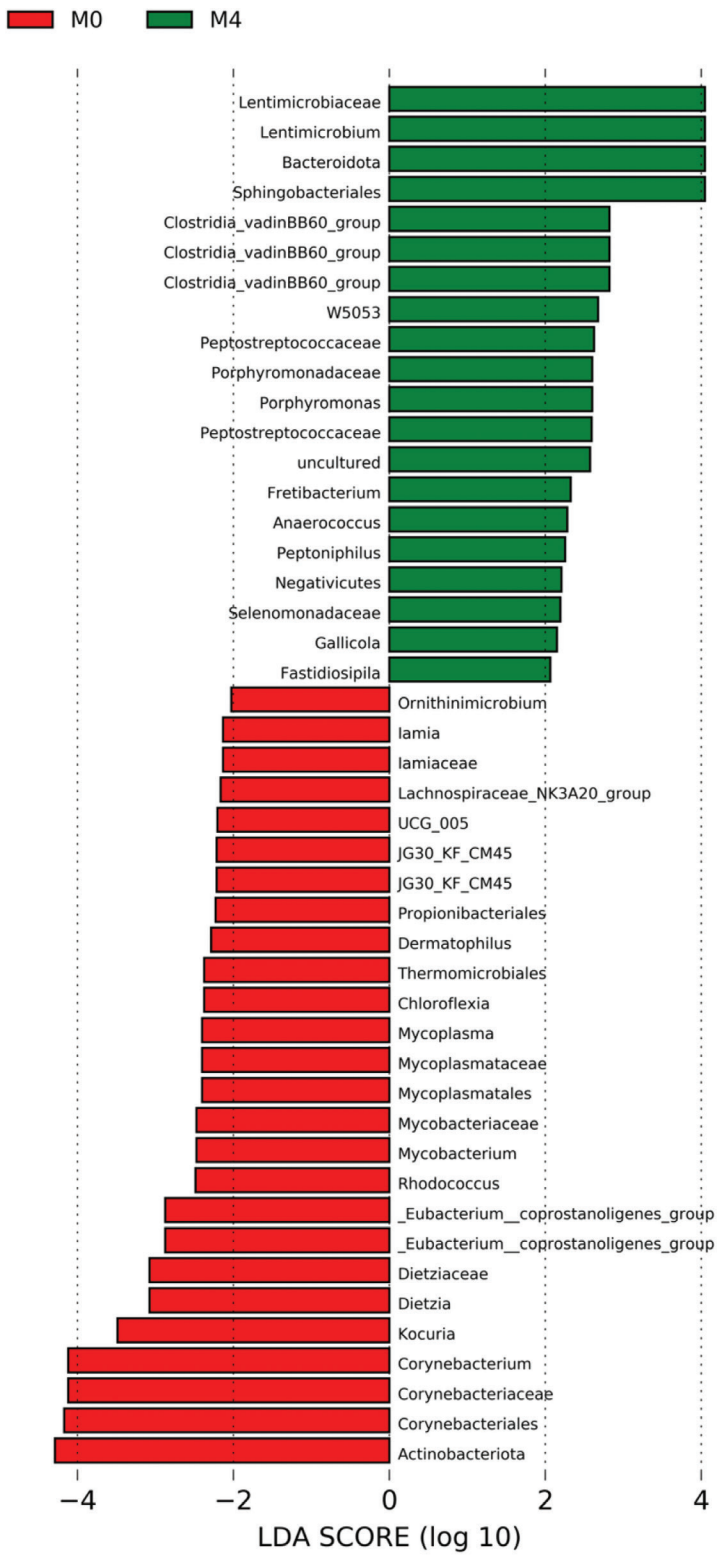

Figure 4. Linear discriminant analysis effect size (LEfSe) was used to identify bacterial genera that were preferentially abundant in tissue samples from healthy skin (score M0; 19 from the superficial stratum, 22 from the deep stratum) and bovine digital dermatitis lesions (BDD; score M4; 21 samples from the superficial stratum, 18 samples from the deep stratum). Samples were collected from 7 dairy cow herds (3 in New South Wales, 4 in northern Queensland) with a history of BDD. Taxa with linear discriminant analysis (LDA) score ≥2 were classified as preferentially abundant in the category of interest. Taxa associated with M0 lesions were assigned negative scores, whereas those associated with M4 lesions were assigned positive scores.

score = 3.9), the genus *Kocuria* (LDA score = 3.5), the family *Corynebacteriaceae* (LDA score = 3.3), and the genus *Corynebacterium* (LDA score = 3.3; Figure 5). A LEfSe analysis was also used to compare the bacterial populations in the superficial and deep strata of tissue samples from M4 lesions only. No differentially abundant taxa were identified in either stratum.

Risk Factors for Cow-Level BDD

Unconditional associations between herd-level management strategies and cow-level prevalence of BDD lesions are shown in Table 1. Odds of BDD at the cow level were higher when cows were managed in barns (POR = 10.6, 95% CI: 3.9–28.5) and feed pads (POR = 3.2, 95% CI: 1.6–6.4) when compared with grazing-only systems. Prevalences were higher for cows on farms where preventative trimming was used (POR = 2.8, 95% CI: 1.3–6.3), and where professional hoof trimmers conducted the majority of the trimming (POR = 5.3 vs. farmer trimming, 95% CI: 1.8–15.3). Prevalence odds ratio estimates for biosecurity practices, use of foot mats and foot baths, were imprecise as evidenced by wide 95% confidence intervals.

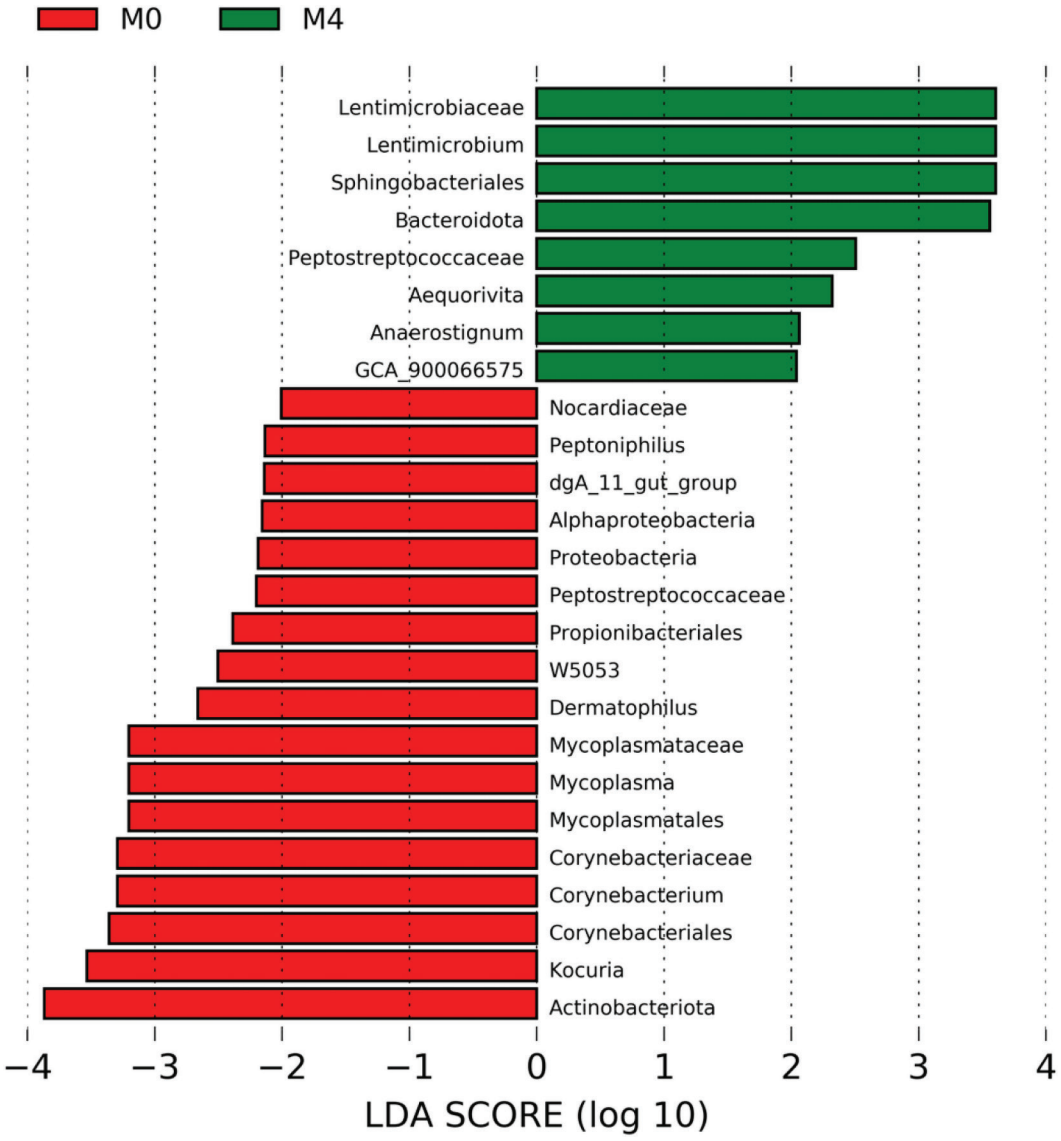

Figure 5. Linear discriminant analysis effect size (LEfSe) was used to identify bacterial genera that were preferentially abundant in tissue samples from healthy skin (score M0; 19 from the superficial stratum, 22 from the deep stratum) and bovine digital dermatitis lesions (BDD; score M4; 21 samples from the superficial stratum, 18 samples from the deep stratum). Samples were collected from 7 dairy cow herds (3 in New South Wales, 4 in northern Queensland) with a history of BDD. Taxa with linear discriminant analysis (LDA) score ≥2 were classified as preferentially abundant in the category of interest. Taxa associated with M0 lesions were assigned negative scores, whereas those associated with M4 lesions were assigned positive scores.

DISCUSSION

BDD Occurs on Most Australian Farms

The findings from the current study (95.8% of herds having lesions) and previous surveys (Coombe et al., 2018; Hesseling et al., 2019) indicate that BDD is likely to be present in most Australian herds. The widespread presence of BDD is consistent with findings from herds in Europe and North America. For example, the proportion of herds with BDD lesions was 74.4% (Cramer et al., 2008) and 93.6% (Solano et al., 2016) in Canada, 97% in Denmark (Oliveira et al., 2017), 98.8% in Finland (Pirkkalainen et al., 2021), and 91.0% in the Netherlands (Holzhauer et al., 2006). Furthermore, farmer-reported presence of BDD in Britain and the United States, which may be underestimated, was 79.0% (Barker et al., 2010) and 70.2% (USDA, 2009), respectively. By contrast, a recent survey in New Zealand found that less than half (49.6%) of pasture-based herds had cattle with BDD lesions (Yang et al., 2019b). It is unclear when the disease was introduced into Australia and how it is has spread since it was first documented in 1994 (McLennan and McKenzie, 1996). The spread was likely facilitated by the movement of cows and bulls between herds (Rodriguez-Lainz et al., 1999; Wells et al., 1999; Yang et al., 2019a), which was practiced in 70 out of 71 herds in the current study. Previous studies have documented suboptimal biosecurity practices in Australian dairy farms (Beggs et al., 2015). Furthermore, the use of outside labor in the form of veterinarians (45.0% of herds in the current study) or hoof trimmers (9.9%) for preventative or therapeutic hoof trimming may have also contributed to spread between herds (Wells et al., 1999; Yang et al., 2019b).

Effects of BDD on Australian Farms Are Currently Unclear

Although BDD appears to be present on most Australian farms, its effects on cow welfare and production are not clearly understood. This knowledge gap exists because no study in Australia, including the current study, has been designed to evaluate this. We hypothesize that the BDD-associated lameness and production losses in the average Australian herd are likely to be less than in European and North American herds, where effects are well known (Cramer et al., 2009; Bruijnis et al., 2012; Gomez et al., 2015). This is for several reasons. First, the overall prevalence was relatively low in many herds in the current study, with the median observed and true prevalences being 8.5% and 18.5%, respectively. Surveys of herds in other countries have found average or median herd apparent prevalences to range from 12% to 30.9% (Holzhauer et al., 2006; Cramer et al., 2008; Solano et al., 2016; Pirkkalainen et al., 2021). Furthermore, we found that M2 lesions, which are considered to be the most active and painful form of BDD (Holzhauer et al., 2008), were not detected in 90.1% of herds in our study and, when present, were only found in up to 2.2% of animals. In contrast, cross-sectional studies found M2 prevalences of 6% in Canada (Solano et al., 2017a), 16% in Denmark (Capion et al., 2008), and 5.7% in Finland (Pirkkalainen et al., 2021), with a 3-wk cumulative incidence of M2 lesions in herds in the Netherlands from 20.5% to 25% (Barkema et al., 1994; Holzhauer et al., 2008).

Given that the effect of BDD on Australian farms is poorly understood, we do not recommend wholesale uptake of foot bathing until further research is conducted. Although foot bathing with copper sulfate or formaldehyde has been shown to effectively reduce BDD prevalence when used appropriately (Laven and Hunt, 2002; Holzhauer et al., 2012; Solano et al., 2017b), we are also aware that the practice can be costly to the producer and may be harmful to humans and the environment (Mal et al., 2002). However, we do recommend that herds and their hoof-health advisors monitor for BDD lesions and provide medical treatment to individual cows with painful lesions. Furthermore, care should be taken to make sure that hoof trimming equipment is regularly disinfected to prevent the transmission of BDD-associated pathogens (Gillespie et al., 2020).

Bacterial Populations of Feet with BDD Lesions Are Dysbiotic

The role of the skin microbiota in the development and persistence of foot lesions has been investigated for several diseases of the ruminant foot, including BDD (Krull et al., 2014; Zinicola et al., 2015; Caddey et al., 2021; Duncan et al., 2021; Clifton et al., 2022; Bay et al., 2023). Broadly, the development of BDD lesions corresponds with a marked shift in the composition of the skin microbiota at all taxonomic levels and a decrease in diversity (Santos et al., 2012; Zinicola et al., 2015; Nielsen et al., 2016; Moreira et al., 2018; Caddey and De Buck, 2021). In the present study, there was no difference in α diversity between healthy and BDD-affected feet in either the superficial or deep stratum ($P > 0.05$). However, there was a difference ($P < 0.001$) in β diversity according to all 3 metrics evaluated. In particular, there was a marked increase in the relative abundance of the phylum *Bacteroidota*/*Bacteroidetes* in lesions, and a corresponding decrease in the relative abundance of the phylum *Actinobacteriota* (Figure 2). It is unclear whether this dysbiosis drives lesion development or is simply a manifestation of the disease process.

Gut- and Effluent-Associated Bacteria Were Abundant in Lesion Biopsies

Consistent features of the microbiota of BDD lesions have been identified across different studies. A recent meta-analysis of data from 8 studies of the microbiota of BDD lesions identified 4 genera that best differentiated the microbial populations of BDD lesions from those of healthy skin: *Treponema*, *Porphyromonas*, *Mycoplasma*, and *Fusobacterium* (Caddey and De Buck, 2021). Of these, *Porphyromonas* was the only genus identified as differentially abundant in BDD lesions in the present study. This genus has been implicated in the etiopathogenesis of several infectious diseases of the ruminant hoof (Nattermann et al., 1993; Sweeney et al., 2009; Maboni et al., 2017; Kontturi et al., 2019), including BDD (Moe et al., 2010; Krull et al., 2014; Nielsen et al., 2016). However, few studies have focused on the specific role of this genus in lesion development, and its significance is largely unknown. In the present study, *Treponema* was detected in the superficial (6/20) and deep (6/19) strata of tissue samples collected from BDD lesions but represented only a small proportion of the total community (0.01%–0.12%). This finding is in agreement with previous Australian studies, which have identified treponemes in BDD lesions (McLennan and McKenzie, 1996) but found that their presence and abundance is inconsistent (Rasmussen et al., 2012; Duncan et al., 2021). This differs from studies conducted in the United Kingdom and the United States, where treponemes are often one of the most abundant genera in BDD lesions (Klitgaard et al., 2013, 2017; Krull et al., 2014; Nielsen et al., 2016).

The genus *Lentimicrobium*, which is a member of the phylum *Bacteroidota*, was the dominant bacterium in tissue biopsies collected from BDD lesions, representing 53% and 31.4% of the community in the superficial and deep strata, respectively (Figure 3). To our knowledge, this genus has not been described in BDD lesions previously. The genus *Lentimicrobium*, which was first described in 2016 (Sun et al., 2016), has been reported most often in effluent (Sun et al., 2016; Guo et al., 2022; Liu et al., 2022) and soil (Lin et al., 2021; Deng et al., 2022; Li et al., 2022), but has also been identified in the gastrointestinal contents of sheep (Li et al., 2020), cattle (Islam et al., 2021; Ryu et al., 2022), and goats (Tian et al., 2022). The virulence characteristics of the genus have not been described, but the genus is strictly anaerobic and motile, with optimal growth occurring at 30 to 37°C (Sun et al., 2016). As such, the hyperkeratotic or necrotic tissue of foot lesions may provide a favorable, anaerobic environment for the genus to proliferate. Given the association of this genus with feces and effluent, exposure to this genus on a dairy farm is highly likely. Of the 40 representative sequences classified as *Lentimicrobium* in the present study, 5 shared >99% sequence similarity with a 16S rRNA sequence from an uncultured *Bacteroidota* (accession no. GQ424184.1) identified in clone libraries prepared using DNA extracted from BDD lesion biopsies in Japan (Yano et al., 2010). This sequence was one of the most prevalent sequences identified in the library, representing up to 32% of the clones sequenced in each library (Yano et al., 2010). The investigators classified this sequence as a member of the phylum *Bacteroidota* due to its similarity to that of a sequence from another uncultured *Bacteroidota* (accession no. AY548787) identified in waste water (Kaksonen et al., 2004). These findings provide further evidence for the environment as a reservoir for key BDD pathogens, some of which originate from the gut of livestock, and effluent as a possible vehicle for transmission of these organisms on dairy farms.

Dichelobacter May Contribute to the Persistence of Lesions

The genus *Dichelobacter* is best known as the primary causative agent of ovine footrot (Beveridge, 1941); however, *Dichelobacter* has been implicated in other infectious diseases of the ruminant hoof, including BDD (Rasmussen et al., 2012; Moreira et al., 2018; Caddey and De Buck, 2021). *Dichelobacter* is not regarded as a primary causative agent of BDD, and it is unclear whether the bacterium contributes to the development of foot lesions. In the present study, *Dichelobacter* was one of the most abundant genera in the superficial stratum of lesions, representing up to 4.9% of the community (Figure 3). The genus was also present in the deep stratum of lesions but represented a smaller proportion of the community (up to 1.6%). Given the presence and abundance of *Dichelobacter* in the superficial stratum of lesions, coupled with the capacity of this organism to cause foot lesions in ruminants, we propose that the role of *Dichelobacter* in the development and persistence of BDD lesions warrants further study.

Strengths and Limitations of the Study

One strength of this study was the enrollment of a large number of herds from the major dairy farming states in Australia. However, herds were recruited by convenience sampling, with most herds being a client of Livestock Veterinary Services, Tableland Veterinary Service, or Rochester Veterinary Practice. It is therefore possible that the findings from our study may not be representative of all herds in Australia. Another important limitation of our study is the use of a relatively insensitive screening test, which involved visual examination at milking time after hosing with water (Solano et al., 2017a; Cramer et

al., 2018; Yang and Laven, 2019). We decided to use the method previously described by Yang and Laven (2019) so that results could be compared between studies, and because their method was considered to be the most user-friendly approach for study investigators. It should be noted that other scoring methods exist, including the use of hoof trimming chutes (Cramer et al., 2018) and mirrors (Relun et al., 2011).

To address the limitations in diagnostic sensitivity of our screening approach, we adjusted the necessary sample size to achieve an acceptable herd sensitivity and estimated true prevalence using Bayesian methods. Furthermore, the rigor of our sampling approach is supported by a high level of agreement between observers (Fleiss kappa value of 0.92; 95% CI: 0.73–1.0). However, it should be noted that the Fleiss kappa value for the 6-point scoring system was lower (0.50, 95% CI: 0.40–0.60), indicating that some misclassification within BDD-affected limbs may have occurred.

We discourage readers from making causal conclusions from the unconditional associations for BDD risk factors evaluated in this study. This is because we did not control for confounding and because our measure of disease frequency (prevalence) is unlikely to be a robust proxy for disease incidence. Given that the objective of most disease control programs is to prevent new cases (i.e., incidence), risk factor studies should be designed to estimate incidence directly, or use designs where the measure of association is an appropriate proxy for incidence. Although POR have been shown to be an effective proxy for causal incidence rate ratios (Rothman and Lash, 2021), this requires several assumptions, including that the factor of interest is not associated with the removal of animals with the disease from the population (e.g., culling), which we cannot be sure of in this case.

Previous studies indicate that the composition of the microbiota of BDD lesions is similar between active lesions regardless of the stage of disease, but differs from that of inactive lesions (Zinicola et al., 2015). In the present study, only samples from chronic M4 lesions were analyzed. As such, the microbial populations identified in these samples may only be representative of the inactive, chronic stage of disease. Further investigation is necessary to determine the composition of the microbiota in the active stages of disease, and how it may differ to that of inactive lesions. Sequencing data from approximately one-third of the samples collected had to be excluded from the microbiota analysis as there were an insufficient number of reads for these samples following quality filtering, which suggests that an insufficient amount of microbial DNA was obtained from these biopsies. Future studies may benefit from the inclusion of an additional step in the DNA extraction protocol to concentrate the microbial DNA before analysis.

CONCLUSIONS

We conclude that BDD is likely to occur in most Australian dairy farms, but the effect on cow welfare, health, and production has not yet been determined. Further investigations into the risk factors for incident cases of BDD are also needed to identify control strategies. Due to these considerable knowledge gaps, we do not yet recommend wholesale adoption of control practices such as foot bathing in Australian herds. Our study identified an association between several bacterial genera and M4 lesions; however, further investigation is required to determine what role, if any, these genera play in this stage of disease. Moreover, we identified *Lentimicrobium*, an organism previously detected in effluent and the ruminant gut, as the most common genus in superficial and deep tissue layers of chronic M4 lesions. Further investigations into the etiology of BDD in Australian dairy herds, particularly during the early, active stage of lesion development, is necessary to inform prevention and control strategies.

NOTES

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Nonstandard abbreviations used: ASV = amplicon sequence variant; BDD = bovine digital dermatitis; CODD = contagious ovine digital dermatitis; LDA = linear discriminant analysis; LEfSe = linear discriminant analysis effect size; NSW = New South Wales; POR = prevalence odds ratios; QLD = Queensland; Se = sensitivity; Sp = specificity; VIC = Victoria.

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