Do footbath disinfectants modify the dynamics of the skin microbiota in dairy cattle affected by digital dermatitis?

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Abstract

Bovine digital dermatitis (DD), is a highly prevalent disease among dairy cattle characterized by ulcerative and painful lesions. While multiple risk factors are involved in the disease, its precise etiology remains unclear and the effectiveness of the current control strategies still highly variable. However, and even if the main role of *Treponema* spp. in the development of the disease is consistently recognized, the importance of other bacterial communities and the factors affecting the skin environment remains uncertain. The objective of this study was to investigate the dynamics of microbiotas from feet affected by DD, before and after the implementation of disinfectant footbaths under field conditions. During this investigation, the diversity, structure, and composition of microbiotas from DD lesions over time were explored according to different clinical factors. The findings of this investigation confirmed a particular

microbiota related to DD lesions dominated by *Treponema* spp. and highly different from those microbiotas of healthy skin. However, the diversity and structure of microbiotas from DD lesions did not vary according to the usage of footbaths or individual topical antibiotic treatments. Otherwise, microbiotas from proliferative lesions evidenced a different structure and diversity in comparison to non-proliferative lesions. The composition of microbiotas changed over time revealing the potential main role of *Treponema* spp., *Fusobacterium* spp., *Mycoplasma* spp. and *Porphyromonas* spp. in the dynamics of DD lesion progression. Further studies are necessaries to confirm if the clinical evolution of DD lesions is driven by a particular microbiota and how this microbiota induces disease.

Key words: Bovine digital dermatitis, Microbiota, Polymicrobial, Footbaths, Multifactorial.

Highlights

Multiple bacteria have been identified in DD lesions. However, a large part of these microorganisms are inhabitants of the foot skin and the farm environment. For the first time, the microbiotas of DD lesions were followed over 45 days under field conditions to explore their evolution over time and how footbaths practices may affect their dynamics. The results of this investigation confirmed a particular microbiota related to DD lesions dominated by *Treponema* spp. and highly different from those microbiotas of healthy skin. The microbiotas from DD lesions evolved over the study period and the differential bacteria were identified. Further studies are guaranteed to determine the role of the bacteria composing these microbiotas on the lesion occurrence and its outcome.

Introduction

The increased prevalence of lameness and its impact on the welfare and productivity of animals reflects an important concern facing modern dairy farming. One of the main causes of infectious lameness is bovine digital dermatitis (DD)[1], a multifactorial and polymicrobial disease spread across the world and characterized by ulcerative and painful lesions which might persist as a chronic condition. DD lesions progress in dynamic ways conditioned by multiple environmental factors that influence the spread of associated-pathogens and the integrity of the foot skin. Currently, the control strategies implemented in dairy farms such as footbaths or individual treatments, have demonstrated variable effectiveness in the healing and prevention of DD lesions[2,3].

Although the precise etiology of DD remains unclear, the maceration of the foot skin and the presence of *Treponema* spp. have been consistently identified as the major etiological components of DD[4–7]. However, multiple other bacteria have also been associated with DD infection in different studies, such as *Mycoplasma* spp.[8,9]; *Fusobacterium necrophorum*[10]; some Bacteroides, specifically *Porphyromonas levii* [11,12]; different Proteobacteria, such as *Campylobacter* spp. and *Dichelobacter nodosus* [13–15], and finally a broad range of other Firmicutes[16]. Nevertheless, the large part of these DD-associated pathogens (including the *Treponema* spp.) are inhabitants of the foot skin, the rumen, and the gastrointestinal tract of ruminants, and thereby, of the farm environment [17–19]. Together, all these findings have reinforced the concept of DD as a polymicrobial disorder suggesting that a particular microbiota may drive the lesion environment and affect its outcome.

The development of collective and individual treatment strategies has been focused on the incriminate DD associated-pathogens, mainly against *Treponema* spp. Nevertheless, these treatments influence the structure of the microbial communities

that constitute the foot skin barrier. The disruption of the skin microbiota can lead to dysbiosis and consequently induce colonization of DD-associated pathogens and the consequent apparition of DD lesions. Hypothetically, a successful control strategy should regulate microbiota for preventing or decreasing the proliferation of the DD-associated pathogens.

Next-generation sequencing had enlarged the perspectives to explore deeply the dynamics of the entire skin microbiota, beyond the restrictions imposed by the culture of pathogens or the pre-selection of targeted species. Through the sequencing analysis of the 16S ribosomal RNA (rRNA), this investigation explored the dynamics of DD microbiota over time and the short-term effects of a new disinfectant footbath biocide on the DD microbiotas gathered from the foot skin of 10 diseased cows from 5 different dairy farms followed over 45 days.

Results

The clinical evolution of the lesions according to the lesion type and the M5 scoring are presented in Table 1. Regarding the lesion type recorded, by the end of the study period, the number of proliferative lesions remained unchanged in the footbath group and only one additional proliferative lesion was recorded in the control group. Independent of the allocation group, none of the DD lesions evidenced a transition to a healing or healthy status (M3-M0). Before the study start, 2 of the feet allocated in the control group received a topic antibiotic treatment (12 and 21 days, respectively). During the study period, 3 feet from the control group, were treated with topic antibiotic treatment at days 17, 35 and 39, respectively, and one foot from the footbath group was treated on day 17.

According to the histologic evaluation of epidermal damage, the 10 samples of healthy skin were considered at score 1 and most of the DD lesions were considered at score 3 (n=32), as expected (Table 1). The lesions considered at EDS 3 were systematically associated with bacterial invasion of spirochaetal origin (Figure 1), whereas lesions at EDS 2 seemed less frequently associated with bacterial invasion (BIS=1 (n=3), BIS=2 (n=3), and BIS=3 (n=1)) (Table 1). Otherwise, samples considered at EDS 1 were never associated with bacterial invasion. While during the study, all 10 initial DD lesions studied remained among the clinical stages M2, M4 or M4.1, 4 of those lesions presented the lowest BIS (2 allocated at the nT group). Likewise, only two DD lesions (M4 and M3) were considered at EDS 1.

From the raw reads processing, 1571 non-chimeric unique amplicon sequence variants (ASV) were identified. After removing low prevalence ASV occurring in less than two samples, 1398 ASV were conserved. Finally, the phylogenetic tree-based agglomeration conducted to the identification of 139 unique ASV including 8 uniquely named taxa on the phylum level and 24 on the genus level (Figure 1S). The set of microbial proxies studied according to every sampling time are presented in Table 1. For the within-group comparisons, no noticeable differences were found in any of the microbial proxies studied before footbath implementation (Healthy skin at day 0 Vs. DD lesion at day 0), or during the study (day 0 Vs. day 15; day 0 Vs. day 30; day 0 Vs. day 45). Likewise, no between-group differences (nT Vs. T) were found in any of the microbial proxies studied between healthy skins, or between DD lesions at day 0, day 15, day 30 or day 45. However, when comparing all the microbiotas of healthy skin to those of DD lesions from day 0 (independent of the allocation group), noticeable differences were detected between the microbiotas evenness ($p = 0.001$), diversity (p

= 0.001), and dissimilarity (p = 0.027). Furthermore, when comparing together the microbiotas from DD lesion collected at day 45 to all those from day 0, noticeable differences were detected in all the microbial proxies studied. When comparing DD lesions over time, noticeable differences were only detected for the evenness at every sample time comparison studied (day 0 Vs. day 15; day 0 Vs. day 30; day 0 Vs. day 45).

According to the clinical variables explored and the histological evaluation, no differences in the richness were detected for any of the comparisons (Table 2). Likewise, the usage of footbaths or IndTx did not affect the microbiota's evenness, diversity or dissimilarity. Otherwise, proliferative lesions presented decreased dissimilarity and increased evenness and diversity in comparison to non-proliferative lesions. The DD lesions considered at M2, M4 or M4.1 stages did not differ between them in any of the microbial proxies studied. However, healthy skin microbiotas had noticeably higher evenness, diversity, and dissimilarity in comparison to any other lesion. Similarly, microbiotas from samples considered at the lowest scores according to the BIS and EDS, had noticeably higher evenness, diversity, and dissimilarity (Table 2).

Regarding the composition of the microbiota, the most frequent phylum found among the healthy skin samples were Firmicutes (41.98%), Actinobacteria (17.80%), Spirochaetes (15.88%), Bacteroidetes (15.88%), and Proteobacteria (7.57). Otherwise, before the start of the study, the most frequent phylum found in DD lesions were Spirochaetes (44.43%), Bacteroidetes (29.95%), Firmicutes (14.16%), and Tenericutes (8.27%) (Figure S1).

The evolution over time of the DD lesion microbiotas is presented in figure S2. The microbiotas from healthy skin at day 0 and DD lesions at day 45 have a more disperse configuration compared to the other time points (Figure S2 A). Nevertheless, when comparing all-time points together, no visual differences or cluster formations were evidenced (Figure S2 B).

The figure 2A displays the microbiotas according to the usage or not of footbath disinfectants during the study period (DD samples from Day 15 until the end of the study). Both microbiotas seem to share a similar cluster, however *Fusobacterium* spp. are placed outside the cluster in DD lesions that did not receive the footbath disinfectant. Comparing the samples from feet that received an IndTx during the interval before the next biopsy (from day 0) with those that did not receive any IndTx, the samples seem to share the same cluster (figure 2B). However, among the treated samples, *Fusobacterium* spp. is completely separated from the cluster formed by the remaining bacteria. The scatter plot of the lesion type suggests distinctive clusters for proliferative and non-proliferative lesions, though both microbiotas have a similar structure (Figure 2C). Finally, according to the clinical score, a large part of the healthy skin microbiotas seems to separate from the cluster formed by DD lesions. The M4.1 lesions are placed in the middle of M2 and M4 lesions. (Figure 2D). Otherwise, according to the histological evaluation, scores 0 and 1 seems to group separately from scores 2 and 3 when comparing bacterial invasion scores (Figure S3 A). Likewise, score 1 and 2 seems to group separately from score 3 when comparing epidermal damage scores (Figure S3 B).

Finally, the dynamics of DD microbiota composition were explored through a differential abundance analysis (Figures 3 and 4). Regarding the baseline comparison

between healthy skin and DD lesions at day 0, 55 ASV noticeably changed in the control group and 70 ASV in the footbath group, and between both groups, they share 27 common ASV. In both groups, DD lesions at day 0 have i) increased abundances of *Treponema* spp., *Mycoplasma* spp., *Fretibacterium* spp. and *Campylobacter* spp. and ii) decreased abundances of *Porphyromonas* spp. In the control group, while *Fusobacterium* spp. abundances increased, those of *Corynebacterium* spp. and other unidentified Actinobacteria decreased. In the footbath group, while *Prevotella* spp. abundances increased, those of *Fusobacterium* spp. change in both directions. Otherwise, when comparing the abundances of DD samples from day 15 to those of day 0 in the control group, it was observed the increase of *Fusobacterium* spp. and the decrease of *Acholeplasma* spp., *Porphyromonas* spp. and other unidentified Bacteriodetes and Firmicutes. Otherwise, for the same same time comparison, in the footbath group the abundances of *Campylobacter* spp., *Treponema* spp., other Spirochaetes, and other Actinobacteria decreased, while those of *Fusobacterium* spp. increased. For the same time comparison, the abundances of *Porphyromonas* spp. and the other unidentified Bacteriodetes and Firmicutes varied inconsistently. For the comparison between day 30 and day 15, in the control group abundances of *Fusobacterium* spp., *Porphyromonas* spp., *Corynebacterium* spp. and other unidentified Actinobacteria noticeably increased, while those of *Mycoplasma* spp. *and Acholeplasma* spp. decreased and those of *Treponema* spp. varied inconsistently. Otherwise, for the same time comparison in the footbath group, abundances of *Acholeplasma* spp., *Porphyromonas* spp., *Treponema* spp., and other Spirochaetes and Actinobacteria increased, while those of *Mycoplasma* decreased. For the comparison between day 45 and day 30, in the control group the abundances of *Acholeplasma* spp., *Fretibacterium* spp., and *Prevotella* spp. increased, while those of

Treponema spp. *and Fusobacterium* decreased, and those of the unidentified Bacteriodetes and Firmicutes varied inconsistently. Finally, for the same time comparison in the footbath group, abundances of *Mycoplasma* spp. *and Prevotella* spp. increased, while *Acholeplasma* spp. decreased. Additionally, independent of the allocation group, from day 30 until the end of the study, the abundances of unidentified Bacteriodetes and Firmicutes varied inconstantly. Although the abundances of the aforementioned bacteria presented noticeable changes among the time points studied, however, some bacteria presented noticeable changes at specific time points (Figure 3).

Discussion

The present investigation describes the differences between microbiotas from healthy skin and those affected by DD lesions under field conditions. Additionally, for the first time, the microbiota dynamics within affected feet were investigated during a period of 45 days measuring the impact of recognized factors, such as the usage of footbath disinfectants. As expected, the microbiotas from DD lesions were dominated by *Treponema* spp. and their configuration was largely different from those from healthy skin microbiotas in terms of diversity, structure, and composition. Among the microbiotas of DD lesions, noticeable changes were evidenced between the samples at day 0 and those obtained at day 45. However, neither the usage of footbath disinfectants nor IndTx seemed noticeably impact the general diversity and structure of microbiotas. Otherwise, the microbiotas from non-proliferative lesions were noticeably different from those of proliferative lesions. From the findings of this investigation, a dynamic evolution was evidenced on the abundances of *Treponema* spp., *Fusobacterium* spp., *Mycoplasma* spp. and *Porphyromonas* spp. during the 45

days of follow-up. Changes in the microbiota composition over time varied according to the usage or not of footbaths. This investigation demonstrates for the first time how microbiotas from DD lesions change over time under field conditions.

Our findings support the polymicrobial nature of DD highlighted in previous investigations based on metagenomics approaches and are in line with previous findings signaling *Treponema* spp. as the microorganism most related to DD lesions in comparison to healthy skin[5,8,9]. Moreover, the skin microbiotas gathered in this study partially share the same structure of microbiotas previously investigated on healthy skin and DD lesions from dairy cattle [5,20]. In detail, the main bacteria linked to DD lesions in previous investigations that were again identified by our findings include: *Treponema* spp., *Acholeplasma* spp., *Fusobacterium* spp.[5,8,9], *Porphyromonas* spp., *Prevotella* spp.[12], *Corynebacterium* spp.[9], *Tissierella* spp., *Mycoplasma* spp.[8], unclassified Bacteriodetes, Firmicutes and Proteobacteria[11,12,20]. Contrarily, some bacteria previously associated with DD, such as *Dichelobacter nodosus* [14,21] was not related in this study to the lesions. Furthermore, the present study identified *Fretibacterium* spp. an anaerobic bacteria not previously involved with DD but related to human periodontitis, a disease that as well is associated with *Treponemas* spp.[22]. Although notably changes in the abundances of *Fretibacterium* spp. were identified in microbiotas from DD lesions in comparison to those from healthy skin, the identification of this specific genus might be linked to regional particularities of the farms sampled. However, being the first skin microbiotas assembled from dairy cattle in France, no regional comparison can be done. Additionally, the fact that previous studies have not linked this bacteria genus to DD lesions might be explained by the original statistical procedures implemented in the analyses of our present study. Indeed, the relative abundances of every sample were calculated after the standardization of the observed

taxonomic groups according to their variance and not through procedures implemented previously such as the rough normalization which may entail the loss of valuable information[23]. In detail, when using rough normalization procedures for data analyses, information from samples with a scarce number of sequences is excluded from the dataset. In specific cases, such as healthy skin samples, the exclusion of this information may conduct to neglect the healthy nature of the sample which should have low numbers of sequences in comparison to diseased samples. The within cow design of this study offered the possibility of exploring the dynamics of microbiotas according to the clinical evolution of each animal. Indeed, the simple comparison between microbiotas from DD lesions obtained from different animals at diverse states may underestimate or neglect the impact of individual and environmental factors (i.e. foot conformation or barn hygiene) on the clinical outcome of lesions and their microbial configuration.

One of the main limitations of this study was related to the difficulties encountered in interpreting microbiotas obtained from an anatomical region which inevitably is in close contact with the ground and thereby with dirtiness. Indeed, the distinction of pathogens among environmental microorganisms is a challenge. Furthermore, colonization by opportunistic pathogens could be favored by the successive incisional biopsies performed during the follow-up of the lesions. Another limitation of the present investigation was related to the small sample size studied. Larger samples may highlight with more precision the benefit of control strategies for DD in increasing the microbiota diversity of the skin. As several risk factors at the farm level are involved with the disease, such as hygienic conditions, differences in the microbial profile according to the farms were expected. However, the small number of farms (n=5) and

the small number of individuals sampled within farm restrain the exploration of factors related to farm hygiene. Otherwise, while in this study the changes evidenced in the microbiota allowed the clear distinction between diseased (M2-4-4.1) and healthy states (M0), differences between allocation groups were not detected. Therefore, the potential usefulness of this tool for measuring any effectiveness of control strategies seemed inferior or at least different to standard observational methodologies such as DD stages or lameness scoring. In terms of ethics, feasibility, and more importantly precision, invasive methodologies (biopsies) are limited in large samples and, as pointed above, entails a challenge for the interpretation. Another point concerns the fact that the effect of footbath and IndTx could be highly affected by the exacerbated anti-inflammatory response of the animals after each biopsy. Therefore, measuring local immune responses may enhance the precision of the true effect of control interventions. Regarding the histological evaluation of the samples, this additional assessment clearly allowed the distinction of the bacterial invasion advance between the samples. Although BSI was in concordance with the clinical evaluation of the samples and the dynamic evolution of the microbiota abundances, the EDS results were more difficult to interpret due to the potential effect of the recurrent biopsy procedures. Lastly, the washing and scrubbing procedures performed during the biopsy procedures might have modified the skin microbiota.

Among the different clinical factors studied, the structure and diversity of microbiotas from DD lesions were not affected by the usage of IndTx. This could be explained by the short and not persistent effect of this strategy over time[24]. Otherwise, the findings of this study indicate noticeable differences between the microbiotas of non-proliferative and proliferative DD lesions. The presence of proliferative tissues is

associated with chronical processes and thereby to lesions prone to relapse [25]. The hyper-proliferation of tissues surrounding the lesion may alter the environment of the skin and the topical activity of footbath and IndTx[26–28], thus influencing a distinctive microbiota. Recently, *Treponemas spp.* have been related to some non-infectious diseases inducing lameness[29]. Further studies are needed to evaluate the role of microbiota in the dynamics of those non-infectious lesions that are related to the same risk factors recognized for DD. Other recognized factors influencing the occurrence of DD lesions at individual level, such as parity or days in milk, were not studied due to the small number of cows evaluated at every time point and the large variability among them.

Beyond the recognition of the pathogenic role of some specific bacteria, exploring the microbiotas from DD lesions at the community level may highlight how the dynamics of skin microbiota determine the persistence and the occurrence of DD lesions. Indeed, the 16S rRNA gene analyses may capture broad shifts in community diversity over time, but with limited resolution and lower sensitivity compared to metagenomic data. Nevertheless, the results of this investigation reveal the dynamic nature of microbiotas from DD lesions and highlight the potential importance of specific bacteria in the progress of the lesions over time. Future studies may benefit from shotgun sequencing tools, enhancing the sensibility in the recognition of pathogens and inhabitants and their specific role in the disease. Therefore, further investigations linking the skin microbiota to different herd management practices might enhance the current understanding of the disease and promote the development of successful control strategies.

In conclusion, we have described the dynamics of the skin microbiota in feet affected with DD lesions. The diversity and structure of microbiotas from DD lesions were different from those of healthy skin but did not vary according to the usage of footbaths or IndTx. Otherwise, microbiotas from proliferative lesions evidenced a different structure and diversity in comparison to non-proliferative lesions. The composition of microbiotas changed over time revealing the potential main role of *Treponema* spp., *Fusobacterium* spp., *Mycoplasma* spp. and *Porphyromonas* spp. in the dynamics of DD lesion progression. Further studies are needed to understand how risk factors associated with DD at farm and individual levels may affect the interactions between the bacteria composing the skin microbiota, and how these interactions may determine the clinical evolution of DD lesions.

Methods

Ethics statement

The research protocol was reviewed and approved by the Ethics Veterinary Committee in Clinical Research and Epidemiology from the Veterinary School of Nantes, ONIRIS, France (CERVO, France) (registered number: CERVO-2016-12-V).

Study population

Five dairy farms were included in the study. The herds were composed exclusively of Holstein cows to limit the putative breed effect, and on average 90 cows were milked twice a day. The herds were housed in cubicles and the prevalence of active DD lesions at the start of the study ranged from 20% to 40%. Cows were milked in a rotatory or conventional milking-parlor.

These recruited farms participated simultaneously in a controlled clinical trial which evaluates the effectiveness of a footbath solution on the control of DD lesions [30]. The solution administered in footbaths was a biocide composed by lactic acid (30%; w/w) and glycolic acid (10%; w/w) as active substances (Pink-Step™, Qalian, France). During the trial, each farm implemented a split-footbath which allowed to concurrently administer the Pink-step™ solution through one side of the footbath whilst the other side of the footbath remained empty and was used as a control. The frequency of administration was of 2 days (4 consecutive milkings) every week. Additionally, farmers were allowed to detect and then treat individually the severe cases of DD by using 2 applications of oxytetracycline (30 mg/ml) (Oxytetrin™, MSD Animal Health/Intervet, France)) 2 days apart. For the current investigation, from each farm, the owners have chosen two animals suffering from a DD lesion. Therefore, the study population consisted of 10 different affected feet from 10 different cows, 5 of those feet belonged to the control side (no disinfectant) and the remaining 5 to the footbath solution side, allowing to explore the putative short-term effects of the footbath solution on the skin microbiota composition. The sampled cows were between their first and fifth parity, and their days in milk ranged from 3 to 378 at the start of the study.

Follow-Up and Data Collection

Farms were visited by 2 investigators trained by practical lessons to practice the biopsies. To perform the biopsies every cow was carefully restrained in a trimming chute. The skin of the concerned foot was washed with water and then local anesthesia was provided using Procaine 2% (Procamidor™, Axience, France). Thereafter, the foot skin was rinsed and brushed with a PBS solution before to perform the sample with a sterile biopsy punch (6 mm). The incisional samples were again washed with sterile

PBS solution, cut tangentially into two approximately equal parts. One half was stored in 10% neutral buffered formalin solution for histological evaluation, and the other half was stored in sterile 2.0 ml microcentrifuge tubes at -20°C for DNA extraction and sequencing analysis. Finally, an aerosol bandage of aluminum (Aluspray™, *Vetoquinol*, France) was sprayed directly over the incisional biopsy wound of each cow. Additionally, in the case of pain detected by farmers, NSAIDs were provided after the biopsy sampling.

The biopsy sampling began immediately before the start of the footbath trial. During the first sampling and at this unique occasion each cow was sampled twice at the same foot, one skin sample on the healthy area free of any DD lesion, and the second skin sample directly on the DD lesion. Thereafter, during the following 3 visits, single DD lesion biopsies were sampled at intervals of 15 days to allow the partial healing of the skin. The location of each biopsy intended to be sampled approximately closer or over the site where the precedent sample was taken. In total, each animal was sampled 4 times during the study period of 45 days. Thus, at the end of the study, a total of 50 biopsies were recovered, 25 from affected feet allocated to the control untreated group (nT) and 25 from affected feet allocated to the footbath treatment group (T). All lesions sampled were photographed and scored using the M5 score system[28]. Records of every lesion include the information relative to the farm and cow characteristics and specifically if any concomitant treatment was administered between the samplings.

Histopathology

The paraffin sections of every tissue sample were stained with *Hematoxylin-Eosin-Safran* (HES) for the general histopathological evaluation and with *Warthin Starry*

Silver (WSS) to evaluate the presence and degree of spirochaetal invasion. The degree of epidermal damage (EDS) was assessed according to a modified score system described in a previous study[14]. A score of 1 was defined as normal skin or presenting mild acanthosis and hyperkeratosis without any other significant microscopic lesions associated and without bacteria. Score 2 was defined as skin with marked acanthosis and hyperkeratosis, without erosion or ulceration, with or without pustules, with or without hemorrhages and erythrocytic crusts with or without dermal fibrosis. Finally, score 3 was defined as skin presenting a segmental epidermis, localized to extensive, necrotizing to necrosuppurative epidermis with balloon degeneration of epithelial cells, necrotic vasculitis and intralesional bacteria (spirochaetes, bacilli, and coccobacillus). Otherwise, the total bacterial invasion score (BIS) encompassed also the spirochaetes identified by the WWS staining. The score 0 represented the absence of invasive bacteria; score 1, a low number of invasive bacteria; score 2, a moderate number of invasive bacteria; score 3, a high number of invasive bacteria.

DNA Extraction, 16S rRNA amplification, illumina MiSeq sequencing.

Bacterial DNA extraction was enhanced adding to every sample 20 µl of proteinase K, 180 µl of tissue lysis buffer, and 40 µl of lysozyme (Qiagen, Valencia, CA, USA). Thereafter, all the samples were incubated for 12 h at 56°C and then processed directly for DNA extraction using the Powerlyzer Powersoil Kit (Quiagen, Valencia, CA) according to the manufacturer's instructions. Extractions were performed in rounds of 10 samples and in every round, an additional empty tube was processed in parallel to serve as a negative extraction control. Finally, the resulting supernatant of each sample was transferred to a labeled microcentrifuge tube and stored at -20°C

The Quant-iT dsDNA Assay Kit (Thermo Fisher Scientific, MA, USA) was used to measure DNA concentrations prior to PCR amplification. The V4 region of the bacterial 16S rRNA gene was amplified by PCR, using Illumina Nextera XTIndex Primer 1 (N7xx) and Primer 2 (S5xx), where barcodes were unique to each sample. The PCR protocol consisted of an initial denaturation at 95℃ for 10 minutes followed by 40 cycles of 95℃ for 30 seconds and 60℃ for 1 minute and the real-time fluorescence data acquisition occurred at the end of each annealing/extension phase. All PCR assays use the same PCR cycling conditions allowing parallel testing of all PCR assays. The amplicons were extracted and purified from the gel using the Zymo gel extraction kit according to the manufacturer's instructions and quantified using the Quant-iT dsDNA Assay Kit (Thermo Fisher Scientific, MA, USA). Finally, the purified amplicons were sequenced using the MiSeq reagent kit v2 (13 cycles) on the MiSeq platform (Illumina, Inc., CA, USA).

Sequencing data processing

The generated 16S rRNA raw gene sequences were preprocessed to remove any remaining, barcodes or adapters using Trimmomatic v0.38[31]. Raw reads were then processed using R[32], implementing the established DADA2 workflow[33]. The sequence variants in each sample were inferred using the “divisive amplicon denoising algorithm”, a parameterized model of substitution errors to distinguish sequencing errors from real biological variation [34]. The inferred forward and reverse sequences were then merged, removing sequences that did not perfectly overlap and subsequently chimeras were removed. Thereafter, to these ASV taxonomic labels were assigned through a Bayesian classifier using the Ribosomal Database Project (RDP) v16 training set[35,36]. The phylogenetic relatedness of the ASV identified was

calculated using the R package *decipher*[37] and, spurious ASV not occurring in two or fewer samples were removed. Finally, the ASV were agglomerated according to a cophenetic distance inferior to 0.03.

Statistical analyses

The statistical analyses were performed using R[32]. The analysis strategy consisted of the following 4 steps: i) Investigate the dynamics of the skin microbiota on feet suffering lesions of DD using different microbial proxies of community diversity and structure. ii) Investigate the dynamics of the skin microbiota on feet suffering lesions of DD using different microbial proxies of community diversity and structure. iii) Explore whether the microbiotas studied were affected by different factors that have been previously identified to impact the clinical evolution of DD lesions (Clinical variables). iv) Identify differential ASV that may affect the evolution of DD lesions over time.

The clinical variables explored were the allocation group (nT Vs. T), the usage or not of individual topical antibiotic treatments (IndTx) during the study, the M5 stages recorded, the lesion type (proliferative or not), and lastly the bacterial invasion and lesion scoring performed during the histopathologic evaluation. The diversity and structure of microbiotas were explored using the R packages *microbiome* and *phyloseq*[38]. In detail, four different proxies including richness (count of observed ASV), diversity (Shannon index), evenness (Pielou index), and dissimilarity were determined in every sample. General changes in the microbiota abundances were investigated through the Pielou and the Shannon indexes. While the Pielou index measures the evenness of ASV abundances and how abundances are distributed [39], the Shannon index determines the microbial diversity by encompassing the richness and evenness metrics. Otherwise, the Bray-Curtis distances were calculated to explore the

dissimilarity, the degree of difference between the microbiotas studied according to the different clinical variables studied. All microbial proxies were compared between the allocation groups (nT Vs. T) over time by using the Mann-Whitney U test, and within the group over time by using the Wilcoxon signed-rank test. Likewise, all microbial proxies were compared between the clinical variables studied using Kruskal-Wallis and pairwise Mann–Whitney U tests. The impact of the clinical variables on the structure of microbiotas over time was visualized using the Bray-Curtis distances and nonmetric multidimensional scaling ordination (NMDS). In the scatter plots created, high dissimilarity of microbiotas was represented by long distances between dots, whereas microbiotas with a similar composition were clustered together.

Finally, the dynamic on DD microbiota composition was explored by identifying specific ASV changing over time and according to the allocation group through a differential abundance analysis using the R package *DEseq2*[23]. *DEseq2* uses a negative binomial distribution and implement the variance stabilization transformations of the ASV read counts identified in the raw data set to account for overdispersion. A single model was fitted including the main effects of the allocation group and the sample time. Within the allocation groups, a code for animal identification was created and included as a fixed effect in the model to account for dependencies between the multiple samples within the same cow. For each allocation group, the noticeable effects (p-value ≤ 0.05) on the counts of ASV were investigated between the microbiotas of: i) healthy skin Vs. of DD lesions at day 0, ii) DD lesions at day 0 Vs. DD lesions after 15 days, iii) DD lesions at day 0 Vs. DD lesions after 30 days and, iv) DD lesions at day 0 Vs. DD lesions after 45 days. No comparisons between the allocation groups were performed during the

differential abundance analysis. All figures were created with the R packages *ggplot2* and *plotly* [40,41].

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Author contributions

J.M.A., D.D., K.O., A.R., N.B., and R.G. designed the study. J.M.A., S.L., D.D., and K.A. analyzed the data. J.M.A. and R.G. interpreted results. J.M.A. and R.G. drafted the manuscript. All authors revised critically the manuscript for important intellectual content and approved the final version to be published.he authors acknowledge the farmers and veterinarians who have contributed to this study.

Figure 1. Skin biopsy from a dairy cow with an active bovine digital dermatitis lesion (M2). Colonizing bacteria within surface lesions and defined intra-lesional spirochetes within inflammatory loci are easily evidenced with the Warthin Starry silver staining.

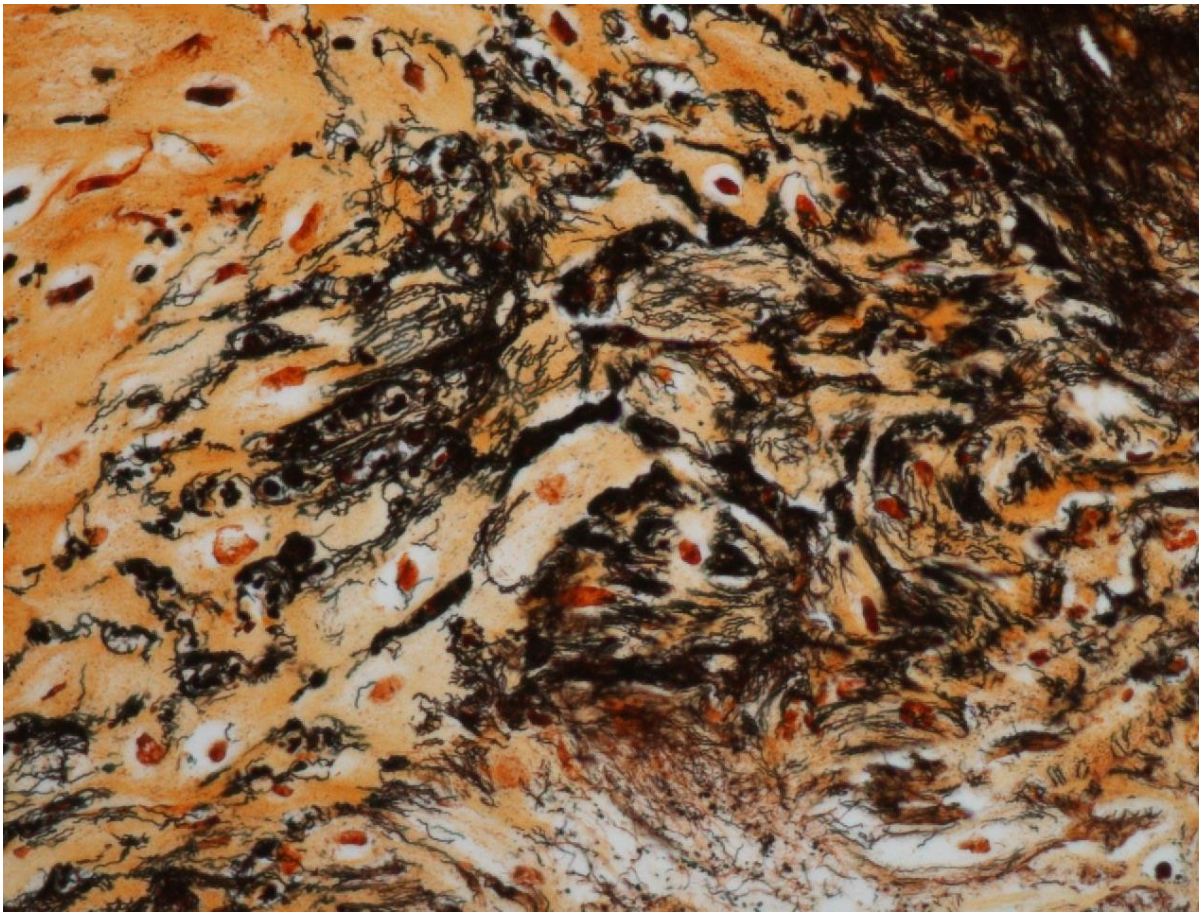

Figure S1. Relative abundances of the ASV present in the samples according to the allocation group over time. Taxonomic classification at the phylum level (A) and at the genus level (B).

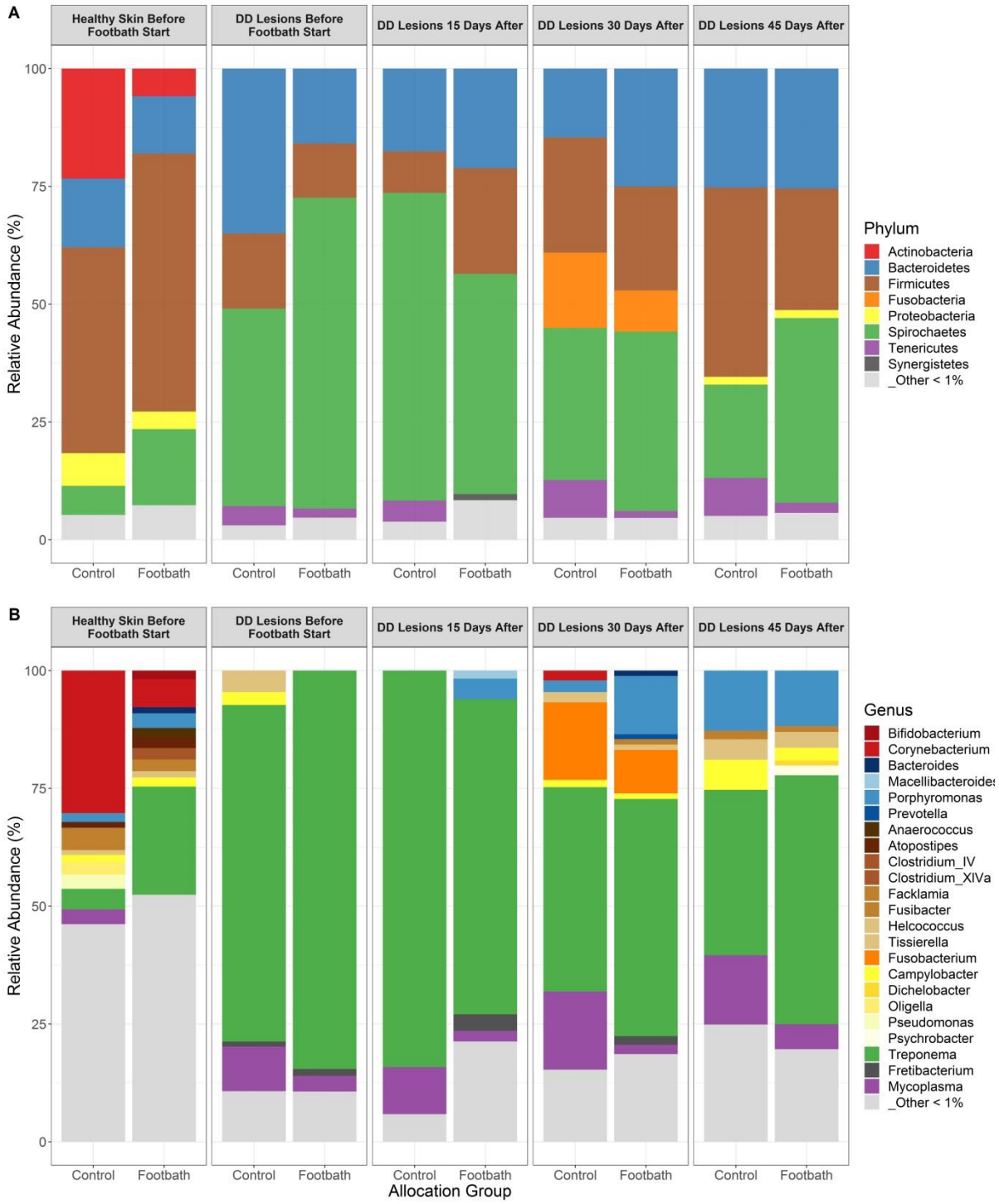

Figure S2. The composition of skin microbiotas from feet suffering bovine digital dermatitis (DD) before and across the 45 days of study follow-up. Microbiotas are visualized by dots representing the ASV present in the samples. The size of the dots represent the mean relative ASV abundance and are colored according to their taxonomic classification at the Phylum level. The ordination was constructed using a Bray-Curtis matrix and Nonmetric multidimensional scaling (NMDS).

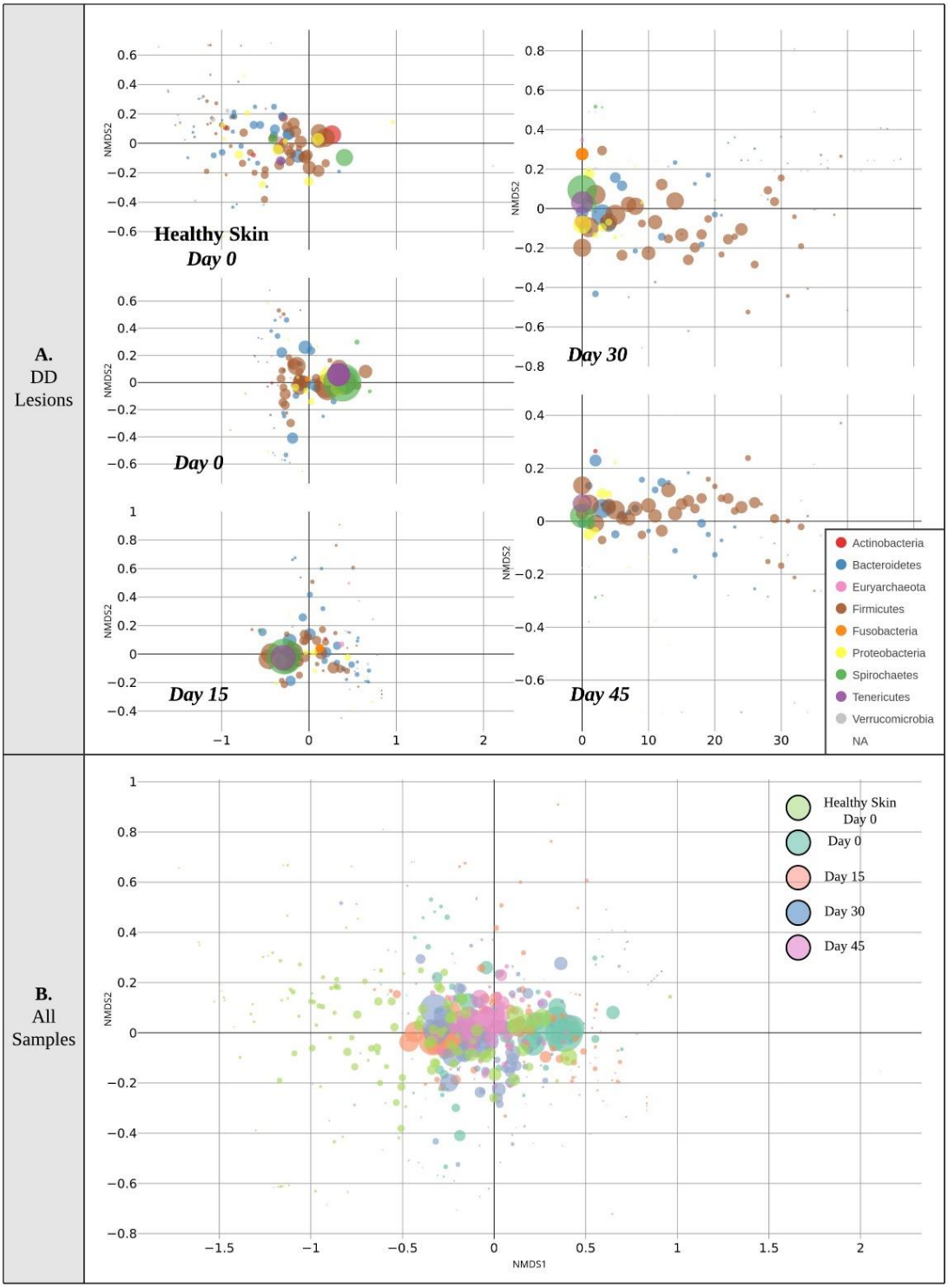

Figure S3. Nonmetric multidimensional scaling (NMDS) scatterplot of Bray-Curtis dissimilarities of the skin microbiotas from feet suffering bovine digital dermatitis (DD) lesions. Microbiotas are visualized by dots representing the amplicon sequence variants (ASV) present in the samples according to the histological evaluation for bacterial invasion (A) and epidermal damage (B). The size of the dots represent the mean relative ASV abundance and are colored according to their taxonomic classification at the Phylum level.

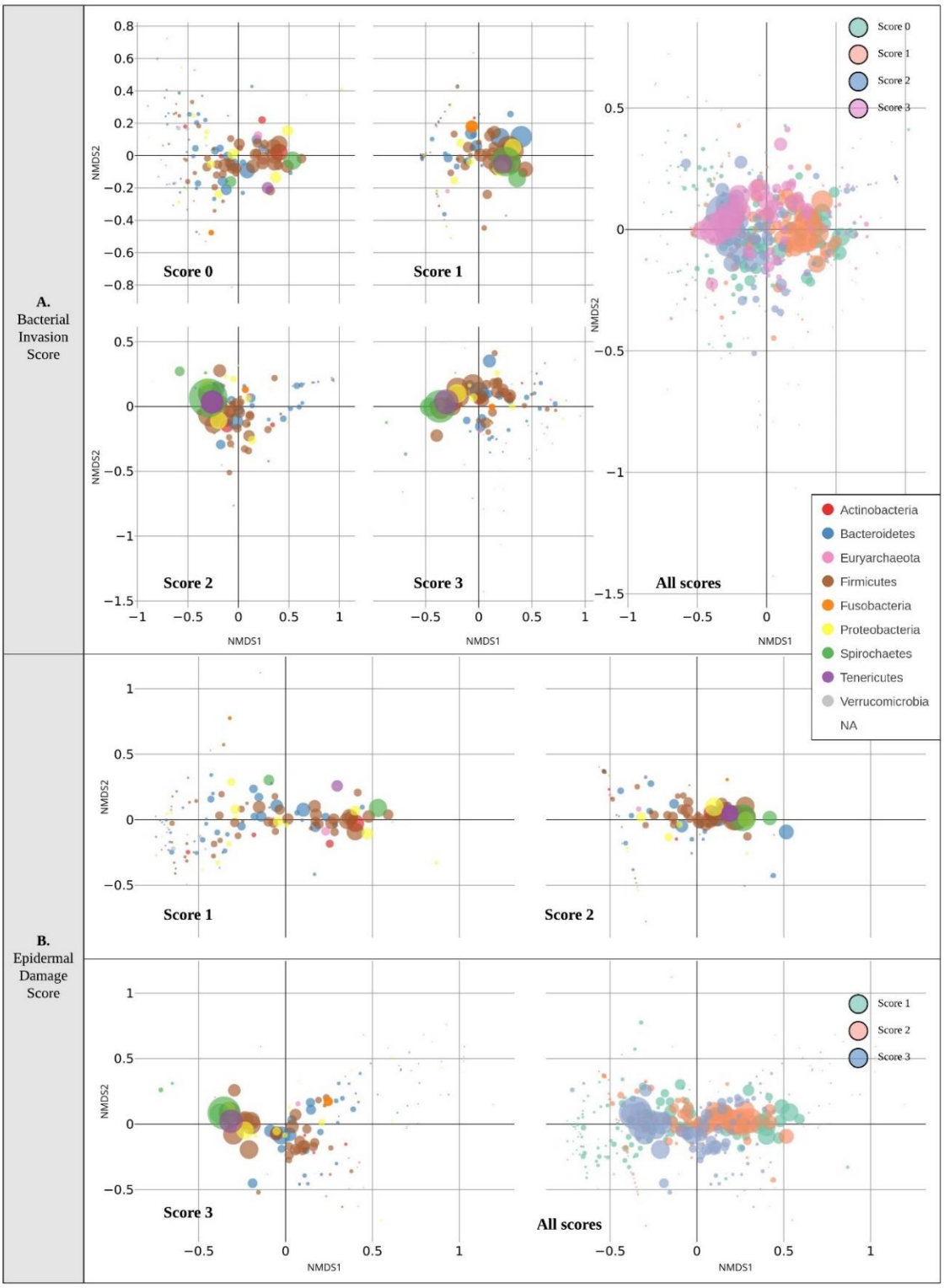

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Figure 2. Nonmetric multidimensional scaling (NMDS) scatterplot of Bray-Curtis dissimilarities of the skin microbiotas from feet suffering bovine digital dermatitis (DD) lesions. Microbiotas are visualized by dots representing the amplicon sequence variants (ASV) present in the samples according to the usage of footbath disinfectant (A), the usage of individual topical antibiotic treatments (B), the lesion type (C) and the M5 score lesion score (D). The size of the dots represent the mean relative ASV abundance and are colored according to their taxonomic classification at the Phylum level.

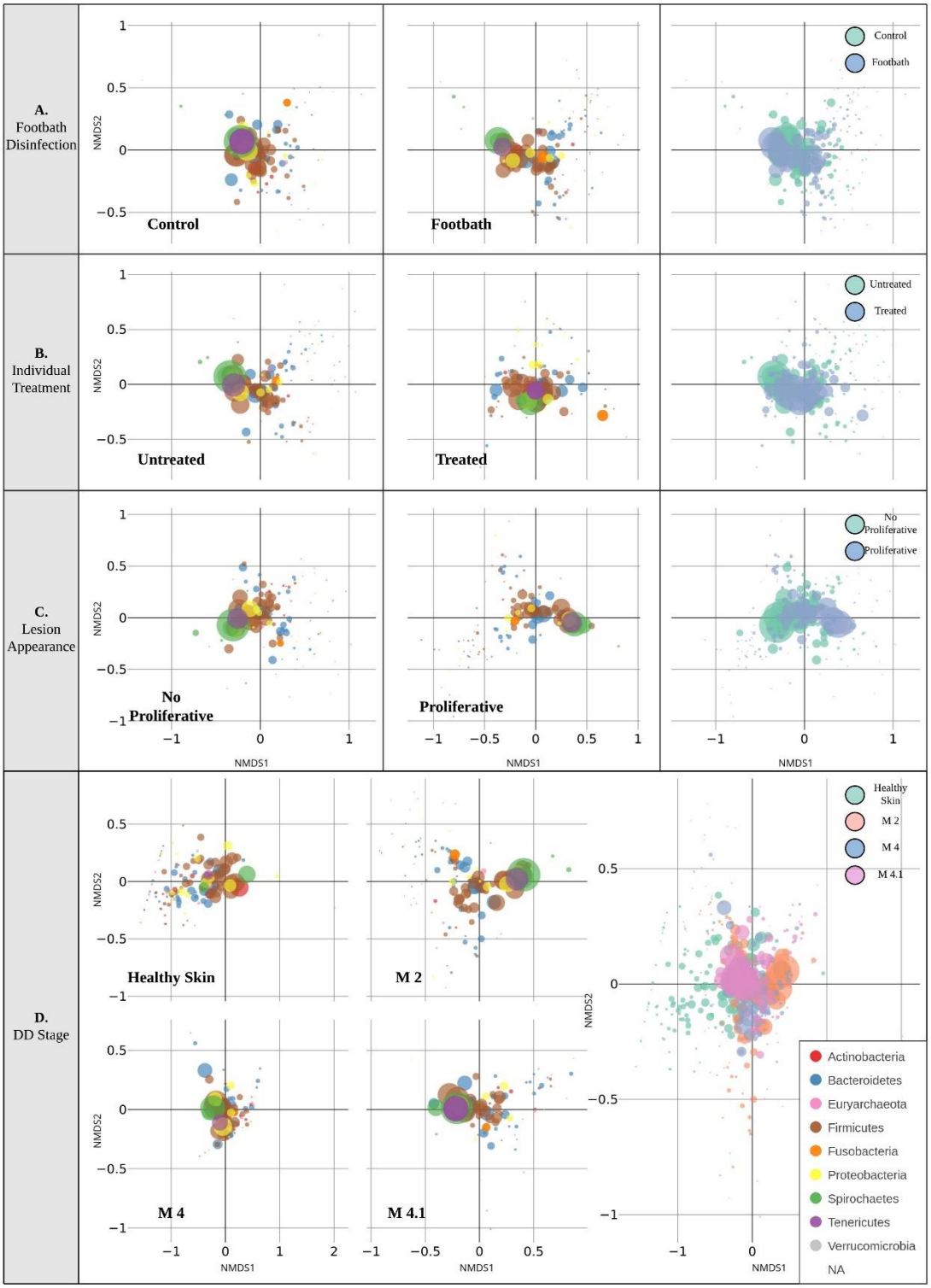

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Figure 3. Differential abundance of amplicon sequence variants (ASV) present in skin microbiotas from feet suffering bovine digital dermatitis (DD) lesions. (**A**) DD lesions versus healthy skins at day 0 before footbath implementation (BF), (**B**) DD lesions at day 15 versus DD lesions at day 0 BF, (**C)** DD lesions at day 30 versus DD lesions at day 15, (**D**) DD lesions at day 45 versus DD lesions at day 30. Bubbles represent the ASV classified at the genus level (x-axis) that showed statistically noticeably changes (y-axis) on a log2-scale. The sizes of the bubbles represent the mean relative ASV abundance and are colored according to their phylum-level classification. Unclassified ASV at the genus level are groped at the Phylum level (*).

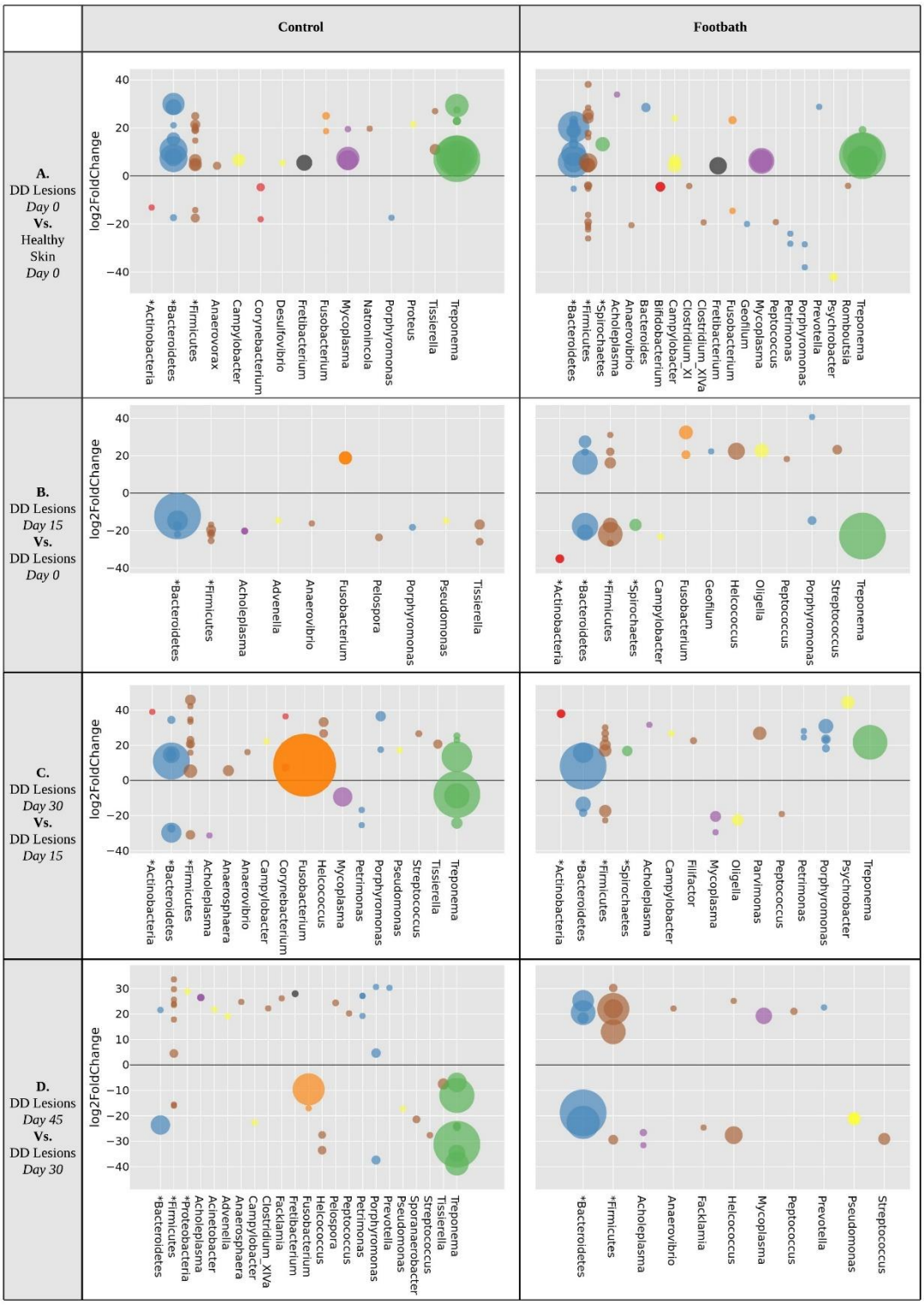

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Table 1. Clinical variables and microbial proxies studied in 10 dairy cows with bovine digital dermatitis lesions followed over 45 days according to the usage or not of disinfectant footbaths.

Variables Studied	Untreated Feet (n=5)					Footbath Treated Feet (n=5)					All samples (n=10)				
	Before Footbath Start		After Footbath Start			Before Footbath Start		After Footbath Start			Before Footbath Start		After Footbath Start		
	Healthy Skin	DD Lesions	DD Lesions			Healthy Skin	DD Lesions	DD Lesions			Healthy Skin	DD Lesions	DD Lesions		
	Day 0		Day 15	Day 30	Day 45	Day 0		Day 15	Day 30	Day 45	Day 0		Day 15	Day 30	Day 45
Clinical evolution															
Lesion stage															
M0	5	-	-	-	-	5	-	-	-	-	10	-	-	-	-
M1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M2	-	3	3	3	1	-	4	4	4	2	-	7	7	7	3
M3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M4	-	2	-	-	2	-	1	-	-	1	-	3	-	-	3
M4.1	-	-	2	2	2	-	-	1	1	2	-	-	3	3	4
Lesion Type															
Proliferative	-	1	1	2	2	-	3	4	4	3	-	4	5	6	5
Histopathology															
Bacterial Invasion Score															
0	5	-	1	-	1	5	-	-	2	-	10	-	1	2	1
1	-	3	-	1	-	-	-	2	-	1	-	3	2	1	1
2	-	2	1	1	-	-	2	2	-	-	-	4	3	1	-
3	-	-	3	3	4	-	3	1	3	4	-	3	4	6	8
Epidermal Damage Score															
1	5	-	1	-	-	4	-	-	-	1	9	-	1	-	1
2	-	3	-	-	1	1	-	1	-	1	1	7	1	-	2
3	-	2	4	5	4	-	5	4	5	3	-	3	8	10	7
Microbial proxies															
Richness	469.60 (402.00)	286.20 (94.41)	198.00 (64.18)	258.00 (90.87)	419.60 (113.80)	483.40 (379.38)	238.40 (142.85)	384.20 (137.81)	383.00 (218.43)	390.20 (150.38)	476.5 (368.57)	262.30 (116.90)	291.10 (141.08)	320.50 (170.92)	404.90§ (126.68)

Evenness	0.76 (0.09)	0.39 (0.14)	0.48 (0.11)	0.49 (0.17)	0.63 (0.07)	0.83 (0.04)	0.46 (0.10)	0.58 (0.20)	0.53 (0.13)	0.57 (0.13)	0.80 (0.08)	0.43* (0.13)	0.53§ (0.16)	0.51§ (0.14)	0.60§ (0.10)
Diversity	4.34 (0.58)	2.25 (0.89)	2.50 (0.47)	2.70 (1.02)	3.77 (0.58)	4.96 (0.75)	2.53 (0.85)	3.38 (1.20)	3.13 (1.00)	3.39 (0.94)	4.66 (0.72)	2.39* (0.84)	2.94 (0.98)	2.92 (0.98)	3.58§ (0.76)
Dissimilarity	0.40 (0.17)	0.23 (0.03)	0.22 (0.04)	0.22 (0.03)	0.15 (0.04)	0.33 (0.09)	0.27 (0.07)	0.24 (0.06)	0.26 (0.10)	0.21 (0.04)	0.37 (0.14)	0.26* (0.06)	0.24 (0.05)	0.25 (0.08)	0.19§ (0.05)

[a]All variables are presented as mean (standard deviation).
*Noticeable differences between the DD lesions and healthy skin from day 0.
§ Noticeable differences between the DD lesions from days 15, 30 or 45 and DD lesions on day 0.

Table 2. Effect of different clinical variables on the microbial proxies studied in 10 dairy cows with bovine digital dermatitis lesions followed over 45 days.

Microbial proxies[1]	Clinical variables													Histological Evaluation						
	Footbath Treatment			Topic Antibiotic Treatment			Lesion Type			Lesion stage				Bacterial Invasion Score				Epidermal Damage Score		
	Control (n=15)	Footbath (n=15)	p	Untreated (n=34)	Treated (n=6)	p	Non-proliferative (n=20)	Proliferative (n=20)	p	Healthy Skin (n=10)	M2 (n=23)	M4 (n=6)	M4.1 (n=11)	0 (n=14)	1 (n=7)	2 (n=8)	3 (n=21)	1 (n=11)	2 (n=7)	3 (n=32)
Richness	291.87 (128.92)	385.80 (159.78)	0.08	309.00 (148.33)	380.33 (119.31)	0.16	300.50 (148.30)	338.9 (143.37)	0.31	476.50 (368.57) [a]	315.22 (150.61) [a]	413.17(174.67) [a]	278.09 (99.85) [a]	481.93 (320.75) [a]	322.86 (167.15) [a]	312.88 (152.41) [a]	287.76 (108.09) [a]	480.91 (358.41) [a]	346.29 (150.05) [a]	307.47 (136.36) [a]
Evenness	0.53 (0.13)	0.56 (0.15)	0.71	0.52 (0.14)	0.49 (0.16)	0.51	0.46 (0.16)	0.57 (0.10)	0.02	0.80 (0.08) [a]	0.49 (0.16) [b]	0.56 (0.17) [b]	0.55 (0.09) [b]	0.73 (0.17) [a]	0.46 (0.25) [ab]	0.48 (0.10) [b]	0.54 (0.10) [b]	0.75 (0.16) [a]	0.56 (0.18) [ab]	0.52 (0.14) [b]
Diversity	2.99 (0.89)	3.30 (0.98)	0.36	2.97 (0.96)	2.89 (1.02)	0.95	2.61 (1.06)	3.30 (0.72)	0.01	4.66 (0.72) [a]	2.80 (1.01) [b]	3.37 (1.22) [b]	3.06 (0.65) [b]	4.29 (1.07) [a]	2.67 (1.59) [ab]	2.72 (0.63) [b]	3.06 (0.73) [b]	4.38 (1.11) [a]	3.26 (1.17) [ab]	2.93 (0.91) [b]
Dissimilarity	0.25 (0.06)	0.25 (0.06)	0.90	0.27 (0.06)	0.22 (0.05)	0.07	0.28 (0.06)	0.24 (0.06)	0.02	0.38 (0.13) [a]	0.27 (0.06) [b]	0.23 (0.06) [b]	0.23 (0.06) [b]	0.35 (0.13) [a]	0.25 (0.04) [ab]	0.26 (0.05) [ab]	0.25 (0.06) [b]	0.38 (0.13) [a]	0.24 (0.04) [b]	0.26 (0.06) [b]

[1]All measures are presented as mean (standard deviation).

Means within the same comparison with different subscripts are noticeably differences from one another ($p < 0.05$).