UNIVERSITE BRETAGNE LOIRE

Oniris
ÉCOLE NATIONALE VÉTÉRINAIRE, AGROALIMENTAIRE ET DE L'ALIMENTATION
SANTÉ ET ALIMENTATION AU CŒUR DE LA VIE

Thèse de Doctorat

Juan Manuel ARIZA

Mémoire présenté en vue de l'obtention du
grade de Docteur d'Oniris - École Nationale Vétérinaire Agroalimentaire et de l'Alimentation Nantes-Atlantique
sous le sceau de l'Université Bretagne Loire

École doctorale : *Ecologie, Géosciences, Agronomie, ALimentation*

Spécialité : *Epidémiologie, évaluation des risques*
Unité de recherche : *Biologie, Epidémiologie et Analyse de Risque en santé animale (BIOEPAR), INRA, Oniris*

Soutenue le *27 février 2018*

Évaluation de l'efficacité et conditions optimales d'utilisation d'une solution de désinfection collective pour la maîtrise de la dermatite digitée en troupeaux bovins laitiers.

JURY

Rapporteurs :	**Karin ORSEL,** Professeur, University of Calgary, Canada **Jonathan HUXLEY,** Professeur, University of Nottingham, United Kingdom
Examinateurs :	**Monique ZAGOREC,** Directrice de recherche, INRA, France **Adrian STEINER,** Professeur, University of Bern, Switzerland
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Assessment of the effectiveness of a new footbath biocide and the best management practices for the prevention and treatment of the bovine digital dermatitis in dairy cows.

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Table of Contents

List of Tables

Chapter I.

Chapter II.

A.

Chapter III.

A.

B.

Chapter IV.

A.

B.

Chapter V.

List of Figures

Chapter I.

Chapter II.

A.

List of abbreviations

ANOVA: Analysis of variance

CFU: Colony-forming unit

CT: Collective treatments

bDD: Bovine digital dermatitis

DIM: Days in milk

DNA: Deoxyribonucleic acid

ELISA: Enzyme-linked immunosorbent assay

IR: Intensive regimen

GLM: Generalized linear model

HR: Hazard ratio

LC: Lactating cow

MB: Mesophilic bacteria

MR: Moderate regimen

NGS: Next-generation sequencing

NNT: Numbers needed to treat

NRCT: Non-randomised controlled trial.

NSAIDs: Nonsteroidal anti-inflammatory drugs

OM: Organic Matter

OR: Odds Ratio

OTU: Operational Taxonomic Units

PERMANOVA: Permutational multivariate analysis of variance

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

rRNA: Ribosomal ribonucleic acid

RCT: randomized controlled trials

TLA: Time since the last administration of an antibiotic

Chapter 1. INTRODUCTION

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A. Nature, History, and importance of Digital Dermatitis in Dairy Cattle

Lameness is one of the most important problems in dairy farming industries (Rutherford et al., 2009). Lameness is a clinical sign resulting from trauma, metabolic disorders or from foot disorders related or not to infectious causes. Foot disorders represent the cause of lameness in more than 90% of the cases. Despite numerous studies recognizing the economic and welfare consequences of lameness, its prevalence has increased in the last 20 years concerning even 40% of the farms, depending on their management and the country involved (Algers et al., 2009, Archer et al., 2010). A major concern carried by lameness is the indiscriminate usage of antibiotics for controlling a clinical sign rather than the pathological cause (Tisdall and Barrett, 2015). Bovine digital dermatitis (bDD) is currently one of the major causes of infectious lameness in dairy cattle. This disease is characterized by the chronic inflammation of the foot dermis leading to ulcerative lesions painful to the touch and prone to bleed. Over time, those ulcerative lesions could evolve to chronic stages characterized by the skin proliferation and as a result by their aspect papillomatous or hyperkeratotic often accompanied by hypertrophied hairs (Holzhauer et al., 2008). The lesions are commonly located at the plantar aspect of the inter-digital cleft (Read and Walker, 1998b). Consequently, due to the importance and the painful and persistent nature of the bDD lesions, the disease is considered as the main welfare issue facing intensive dairy industries (Bruijnis et al., 2012; Arnott et al., 2017).

Bovine digital dermatitis was first described clinically in the early seventies in Italy (Cheli and Mortellaro, 1974). In France, the first publication associating the disease with cows in late gestation dates from the eighties (Gourreau et al, 1992). Afterward, in 1992 for a first time, a potential pathogen was associated with bDD. Specifically, in these early investigations spirochetes from the genus *Treponema*, anaerobic bacteria complicated to culture, were isolated, (Read et al., 1992; Walker et al., 1995; Read and Walker, 1998b). However, it is likely that bDD was encompassed among the early descriptions of foot-rot where treponemes were the main pathogen associated. Therefore, according to those "foot-rot" records, the first veterinary evidence involving treponemes with lameness in livestock dates from 1936 from a case report concerning a sheep herd (Beveridge, 1936) The first outbreak of lameness associated with spirochetes in a dairy herd with hoof lesions was reported only in 1966 (Egerton and Parsonson, 1966). Therefore, since its official clinical description, the disease has been vastly spread among dairy herds, probably as a livestock trading consequence, and

thereby is considered as highly infectious based on the increasing incidences reported at within and between-herd levels.

Despite incremental progress understanding the disease, its precise etiology remains debated. Nevertheless, the presence of multiple specific *Treponema* species on feet suffering from cutaneous maceration is recognized as a major etiological component involved in the development of the disease (Gomez et al., 2012). The complexity of bDD lies in its poly-*Treponemal*, poly-microbial, and multi-factorial components. Indeed, the fact that multiple *Treponema*, multiple bacteria, and several risk factors have been implicated in the disease pathogenesis enlarges its already complex nature. Thus, it is hypothesized that risk factors which promote unhygienic and wet environments will determine the feet skin maceration, the proliferation of treponemes and the subsequent development of bDD lesions (Orsel et al., 2017).

bDD lesions lead to different degrees of lameness with consequences in the longevity and production of the diseased animals (Ettema et al., 2010). Thus, bDD was associated for example with an impaired reproductive performance evidenced by 20 more open days on average to conceive (Argaez-Rodriguez et al., 1997); and a decreased milk production of between 0.63 kg/day and 0.78 kg/day in average (Relun et al., 2013c). Across the years, the number of scientific publications in reference to bDD has been steadily increasing (Figure 1), the disease became popular in the industry and thereby several strategies of control focused in individual cases or in the entire herd are currently commercialized.

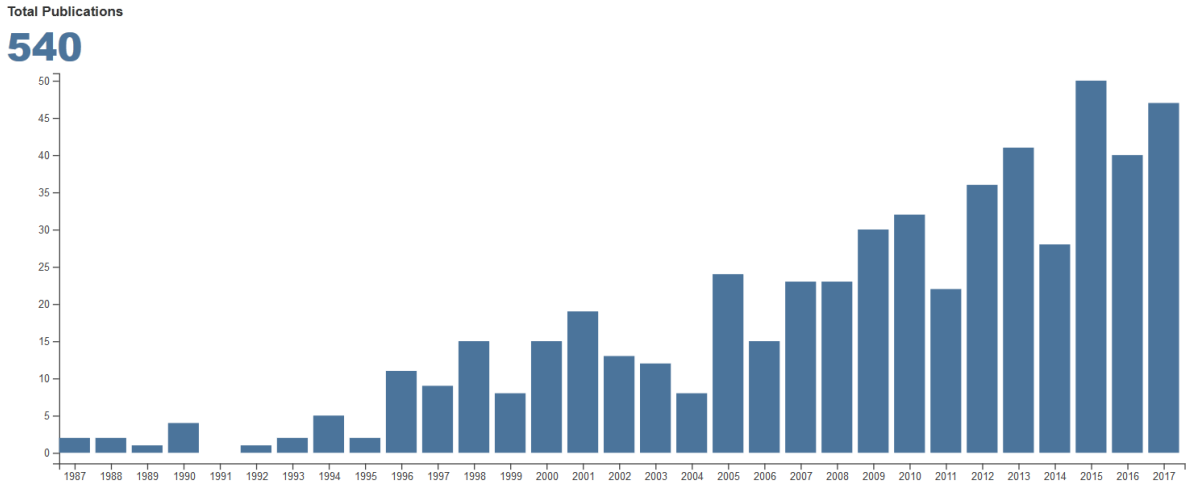

Figure 1. Number of publications retrieved in Web of Science database from the period between 1974 until the date. Search was performed including all known synonyms of bDD, and excluding any reference to other species or human medicine.

These strategies consist of basically the individual treatment of ulcerative lesions and the collective administration of topical disinfectants through footbaths to the entire herd. Besides the economic losses carried by the lameness consequences, the bDD-related costs are importantly increased by the implementation of these control strategies (Charfeddine and Pérez-Cabal, 2017). Furthermore, most of the bDD control strategies implemented currently are not supported by strong scientific evidence (Laven and Logue, 2006). Moreover, the implementation of these strategies is frequently perceived by farmers as time-consuming, laborious, expensive and often ineffective (Relun et al., 2013b).

Currently, an increased proportion of farming systems tend to keep their cows partially or permanently indoors. In North America in 2007, only 35.1% of the farms practiced grazing at least in the summer period (USDA, 2007). Likewise, in Europe since 2001 the number of zero-grazing farms has been growing from less than 20% to 30% in the Netherlands, 70% in Denmark (Reijs et al., 2013) and 69% in Great Britain (March et al., 2014). While bDD is highly present in zero-grazing farms, bDD is reported as well in farms with larger access to pasture (Pinedo et al., 2017). Interestingly, even if grazing practices advocate the reduction in all factors affecting the feet and environmental hygiene, herd prevalence over 60% have been reported in New Zealand where in pasture- based systems predominate across the territory (Yang et al., 2017a). Last reports from Europe and North America, reveals that the bDD prevalence between herds ranged from 21 to 96%, and from 2.9 to 30% at within herd level (Brown et al., 2000; Holzhauer et al., 2006b; Cramer et al., 2008; Solano et al., 2016; Yang et al., 2017). However, the bDD prevalence is highly dependent on several factors, such as the

season, the geographical location, the farm management practices and furthermore, the methodology implemented for the bDD diagnosis. Independent of the prevalence rates, once the disease attained a herd, this eradication seems to be impossible. Currently, bDD is a highly prevalent disease affecting dairy farms on a worldwide scale causing considerable economic losses.

B. State of the art and knowledge gaps about Bovine Digital Dermatitis

1. Clinical features of bDD: what we already know.

The main clinical issue associated with bDD is lameness. Moreover, lame cows could also present clinical signs such as loss of body condition, decreased milk production or impaired reproduction. Besides lameness, the characteristic signs of bDD include the ulcerative and proliferative aspect of the lesions and the bleeding and pain associated with ulcerative stages. Nevertheless, it is possible to find a large part of animals within an infected herd without clinical signs associated with bDD lesions (Frankena et al., 2009). As mentioned before, the lesions are commonly located on the plantar aspect of the inter-digital cleft (90%) (Relun et al., 2011). However, in the feet skin, it is common to evidence as well lesions on the dorsal aspect of the foot or close to the accessory dew-claws. Otherwise, bDD-like lesions in other anatomic locations or as a consequence of other disorders have been reported, such as hock lesions (Clegg et al., 2016a), or pressure sores (Clegg and Palfreyman, 2014). The characteristically bDD lesions evolve in a dynamic way, and different scoring systems have been proposed to represent and report the course of the disease (Döpfer et al., 1997; Manske et al., 2002; Berry et al., 2012; Krull et al., 2014). Among them, the more studied are the Iowa score system and the M stages system (Orsel et al., 2017).

Nevertheless, M stages system is currently the most broadly used in the scientific literature (Figure 2). This system consists of six different stages describing the morphological particularities of bDD lesions over their clinical evolution. However, the transitions between these lesion stages do not follow a strict sequential order. Among those six M categories, the M0 stage corresponds to the normal skin. Once maceration of the skin is attended by the wet and unhygienic environmental conditions, micro-wounds are generated allowing the proliferation of bDD pathogens-associated and thereby leading to the lesion occurrence. Thereafter, the M1 stage comprise early circumscribed lesions red to grey with a diameter inferior to 2 cm; the M2 stage corresponds to ulcerative and painful lesions larger than 2 cm

in diameter with a red-gray surface; the M3 is the healing stage with a scab covering the lesion surface; the M4 stage corresponds to chronic lesions not painful, characterized by the skin proliferation in a hyperkeratotic or papillomatous form; and finally, the M4.1 stage comprise as well chronic lesions but suffering a small area of ulceration. Whereas active lesions are related to the ulcerative stages (M1-M2-M4.1), the inactive lesions are related to the healing and chronic stages (M3-M4).

Histologically, active lesions are associated with ulceration, invasion of the stratum spinosum and/or papillary dermis by dense mats of spirochete-dominant bacteria and reactive inflammation. Active lesions are considered as the main contagious stage of the bDD. Likewise, chronic lesions are harder to differentiate and are characterized by the parakeratotic hyperkeratosis and spirochetal colonization of the stratum corneum (Berry et al., 2012). Although inactive-chronic lesions are tissues biologically diseased and infected, clinically these lesions stages are less related to lameness, pain or bleeding. Therefore, the transition of active lesions to inactive stages might be considered under the clinical regard as an improvement, however as pointed before the importance of these lesions as chronic reservoirs could carry important implications at long-term in the persistence of the disease into the herd and the risk of outbreaks. Figure 2 illustrates the multiple possible transitions between the M stages and the corresponding life cycle of treponemes according to the lesion stage.

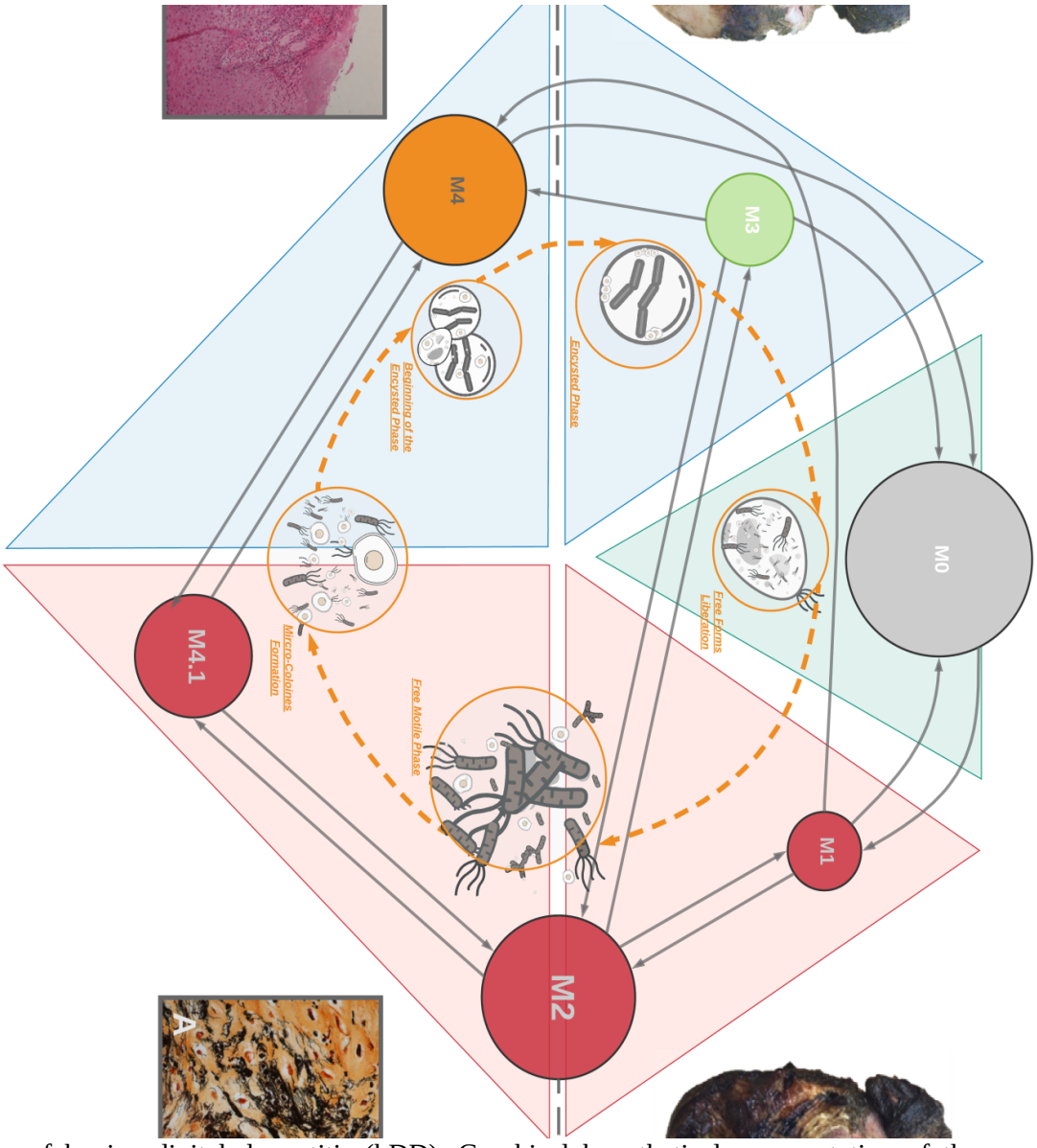

Figure 2. Dynamics of bovine digital dermatitis (bDD). Graphical hypothetical representation of the many possible transitions between the different M stages. Red triangles represent the active stages, blue triangles the inactive stages and triangle the healthy stage. The orange round circles represent every treponemal stage of their life cycle according to the ev of bDD lesions, going from a free and motile phase until an encysted phase. **A.** Histological section of an active bDD evidencing numerous intra-lesional spirochetes. **B.** Histological section of an inactive bDD lesion presenting market hyperke and acanthotic areas with exudation and bacteria

The average time elapsed between the occurrence of a lesion is estimated to at least 133 days in average, and are in accordance with the small incidence rates reported of 4 new cases per 100 cow foot-months (Relun et al., 2013b; Krull et al., 2016b). Otherwise, bDD lesions can clinically improve as soon as 8 days (Holzhauer et al., 2008) and late as 42 days on average (Nielsen et al., 2009). Together, these findings indicate a large range of transition time among the different stages (8 to 144 days). Although this large range could be attributed to the differences in the recording frequency between studies, these time differences could as well highlight the crucial impact of risk factors in the dynamics of the disease and the bacterial

communities involved. Thus, several studies reported that after the administrations of the appropriate treatment or once the environmental conditions are improved, the healing of the lesions might be attained after 28 days in average (Relun et al., 2012). However, in the literature, high recurrence rates are reported (Berry et al., 2012). The clinical aspects related to the treatment and prevention of bDD lesions will be deeply explored in a next and specific section of this chapter.

In general, bDD can be diagnosed by the simple observation or more precisely by other clinical methodologies. The direct observation of a bDD lesion in a trimming chute is considered as the gold standard diagnosis. Nevertheless, for practical concerns, alternative observational methods of detection have been investigated, such as the inspection of hind feet previously cleaned during the milking using a swiveling mirror and a powerful headlamp (Relun et al., 2011; Solano et al., 2017). Although, these methods are useful to distinguish in general the presence or absence of bDD lesions (Sp ≥ 80% and Se ≥ 90%), their accuracy in the distinction between active and inactive lesions is reduced (Sp ≥ 80% and 70% and Se ≥ 40% and 90 %, respectively). The principal differential diagnosis of bDD is interdigital dermatitis which primarily involves the interdigital skin and is characterized by fissuring, caseous necrosis of subcutis, and diffuse digital swelling (Read and Walker, 1998b). Nevertheless, due to the strong inter-relationship between both entities, the differentiation between them remains debated (Walker et al., 1995; Knappe-Poindecker et al., 2013). Other methodologies that could help to increase the diagnosis precision include ELISA test to detect titers of anti-bodies against bDD-treponemes in serum and milk samples (Gomez et al., 2014a, Frössling et al., 2017). However, the test is not commercialized and its price could be a limiting factor for their massive implementation. Moreover, these tests have a limited accuracy to distinguish cured cows among infected ones. Finally, spirochetes could be detected in lesion biopsies using silver staining technics (Figure 2).

Thus, regarding bDD diagnosis, if some improvement can still be achieved by visual inspection or ELISA methods, the main question today deals with the diagnosis of bacteria involved in bDD occurrence, persistence or virulence. Nevertheless, while spirochetes are consistently evidenced in bDD lesions, they are not alone and the numerous potential pathogens associated to treponemes in bDD raise the question of the precise etiology of bDD and its consequence on the elaboration of adequate control strategies.

2. bDD Pathogenesis and Etiology: Many findings, many new questions…*Treponema,* Poly-*Treponema,* and Poly-Microbial?

In the last forty years, bDD has been broadly studied and considerable advances in understanding the disease have been achieved. Nevertheless, the precise etiology remains unclear. The capacity of the disease to spread among animals and between herds evidenced their contagious nature. Therefore, since 1998 some studies have investigated the controlled induction of the disease (Read and Walker, 1998a; Gomez et al., 2012; Capion et al., 2013; Krull et al., 2016; Wilson-Welder et al., 2017). However, these investigations have failed to completely fulfill the Koch's postulates. In summary, according to the original Koch's postulates to impute the causality of a suspicious microorganism, firstly it must occur in every case of the disease; second, it must not be found in healthy organisms; and third, after the microorganism has been isolated from a diseased organism and propagated in pure culture, the suspicious pathogen must induce disease anew. In addition, a fourth post-Koch's postulate involving the reisolation of the pathogen from the experimentally infected host was established to confirm the pathogen causality. Thus, although the first postulate is completed because treponemes have been consistently identified and isolated from bDD lesions, the subsequent postulates remains unfilled. Indeed, the second postulate fails because bDD-treponemes are as well inhabitants of the bovine foot skin, and the second and third, face the inconvenient that the simply inoculation of treponemes does not result in the lesion occurrence and thereby the recovery of these pathogens is not possible. Nevertheless, despite the failures to completely fulfill the Koch's postulates, the disease had been successfully reproduced under controlled conditions (Read and Walker, 1998a; Gomez et al., 2012; Krull et al., 2016; Wilson-Welder et al., 2017). In these experiments, to successfully reproduce bDD lesions, foot suffering skin maceration were inoculated with macerates of bDD lesions. To attain the skin maceration, an artificial environment was induced in feet to assuring prolonged wet and restricted air conditions. The fact that the skin damage determines the successful reproduction of the disease, evidence a paradigm that overlaps the true etiology of the disease between the occurrence of the skin maceration and the establishment and proliferation of the pathogen. In addition, from the macerates used as inoculum, multiple treponemes species were isolated highlighting the importance of the multi-*Treponemal* component in the disease pathogenesis. Indeed, different *Treponema* species are recognized as the principal etiological agent involved in the disease (Wilson-Welder et al., 2013). Among them, *Treponema phagedenis* are the most common species isolated. However, other species such as *Treponema denticola, Treponema medium, Treponema refringens and Treponema putidum* are as well consistently involved in the disease (Klitgaard et al., 2008). As pointed before, treponemes are gram-negative bacteria's, characterized by their capacity of migration

and to produce encysted forms in adverse conditions (Döpfer et al., 2012a). Those encysted forms represent the proposed mechanism for the persistence of chronic lesions and the recurrence of active lesions (Figure 2).

It is important to remark that beyond bDD, the same treponemes have also been isolated in a number of other chronic infections in cattle, such as udder cleft dermatitis (Stamm et al., 2009), ischaemic teat necrosis (Clegg et al., 2016b), and some non-healing hoof disorders related to lameness such as toe necrosis, sole-ulcer, and white line disease (Evans et al., 2011). Likewise, beef feedlot cattle and dairy goat and sheep are facing a chronic infectious bDD-like disease (Sullivan et al., 2015; Sullivan et al., 2014, Kulow et al., 2017). All these conditions share the same risk factors, and several coincidences in their microbial composition have been described. Furthermore, clinically, all those bDD-like lesions lead to ulcerative and painful process related to lameness and productivity losses. Nevertheless, other species raised in intensive conditions such as Buffaloes seems unsusceptible to develop the disease (Guccione et al., 2016). Equally, many of the bDD treponemes are as well associated with different ulcerative diseases in other mammals, such as the polymicrobial periodontal disease of humans and dogs (Griffen et al., 2011; Abusleme et al., 2013; Nordhoff et al., 2008; Abusleme et al., 2013) , the porcine skin ulcers (Karlsson et al., 2013), the cankers in horses (Nagamine et al., 2005; Moe et al., 2010a; Sykora and Brandt, 2014), the genital chronic ulcerations in European wild hares (Lumeij et al., 1994), and the lameness of wild elk (Clegg et al., 2015). To remark, much of these lesions resemble the clinical and histological pattern founded in bDD.

Another factor blocking the complete understanding of bDD is concerning the unclear routes of transmission of the disease. Although, bDD it is categorized as an infectious disease based on their broad spread of the pathology across the herds and the cows and the responsiveness of diseased animals to antibiotics, the routes of transmission between individuals or from other environment sources remain unclear. The main bDD-Treopenemes have been isolated or identified in different sources, and therefore those sources are proposed as potential reservoirs and routes of transmission of bDD. Therefore, ulcerative – bleeding lesions (M2) are considered as a major source of bDD, and chronic lesions (M4) as a potential reservoir of the disease. Different studies have as well isolated the bDD-Treponemes from ruminal fluids and gastro-intestinal tissues, thereby saliva, feces, and manures represent potential reservoir and route of transmission (Evans et al., 2012; Klitgaard et al., 2014; Sullivan et al., 2015a;

Nascimento et al., 2015; Zinicola et al., 2015) Finally, the hoof-trimming tools have been as well pointed as a potential route of transmission of bDD pathogens (Sullivan et al., 2014a).

Moreover, the complexity of the bDD is increased by this polymicrobial nature (Figure 3 and Table 1). Indeed, from bDD lesions different bacteria have been isolated and then associated with the disease. Even more, coming back to the controlled studies looking for the disease induction, the macerate surely contained multiple bacteria apart from the isolated treponemes. The popularization of next-generation sequencing (NGS) technologies such as metagenomics analyses has open new perspectives for the recognition of bDD pathogens. NGS allows to explore and to study the complete microbial communities (microbiota). Across the different investigations on the subject, it is possible to corroborate that most of those others pathogens frequently involved in bDD are ubiquitous in the farm environment.

Table 1. Studies comparing the bacteria present in the skin of healthy feet against inactive and/or active digital dermatitis lesions, according to the methodology implemented for bacteria identification.

	Reference and Year	Methodology Implemented
1.	Döpfer et al., 1997	Immunofluorescence staining
2.	Moe et al., 2010	Immunohistochemical staining
3.	Brandt et al., 2011	Quantitative real-time PCR
4.	Yano et al., 2010	Sanger sequencing 16S rRNA Amplicon
5.	Berry et al., 2010	Bacterial aerobe and anaerobe Culture
6.	Santos et al., 2012	Sanger sequencing 16S rRNA Amplicon
7.	Rasmussen et al., 2012	Fluorescent in situ hybridization
8.	Knappe-Poindecker et al., 2013	Fluorescent in situ hybridization
9.	Krull et al., 2014	NGS*- Shotgun and 16S rRNA Amplicon
10.	Zinicola et al., 2015	NGS*- 16S rRNA Amplicon
11.	Nielsen et al., 2016	NGS*- 16S rRNA Amplicon

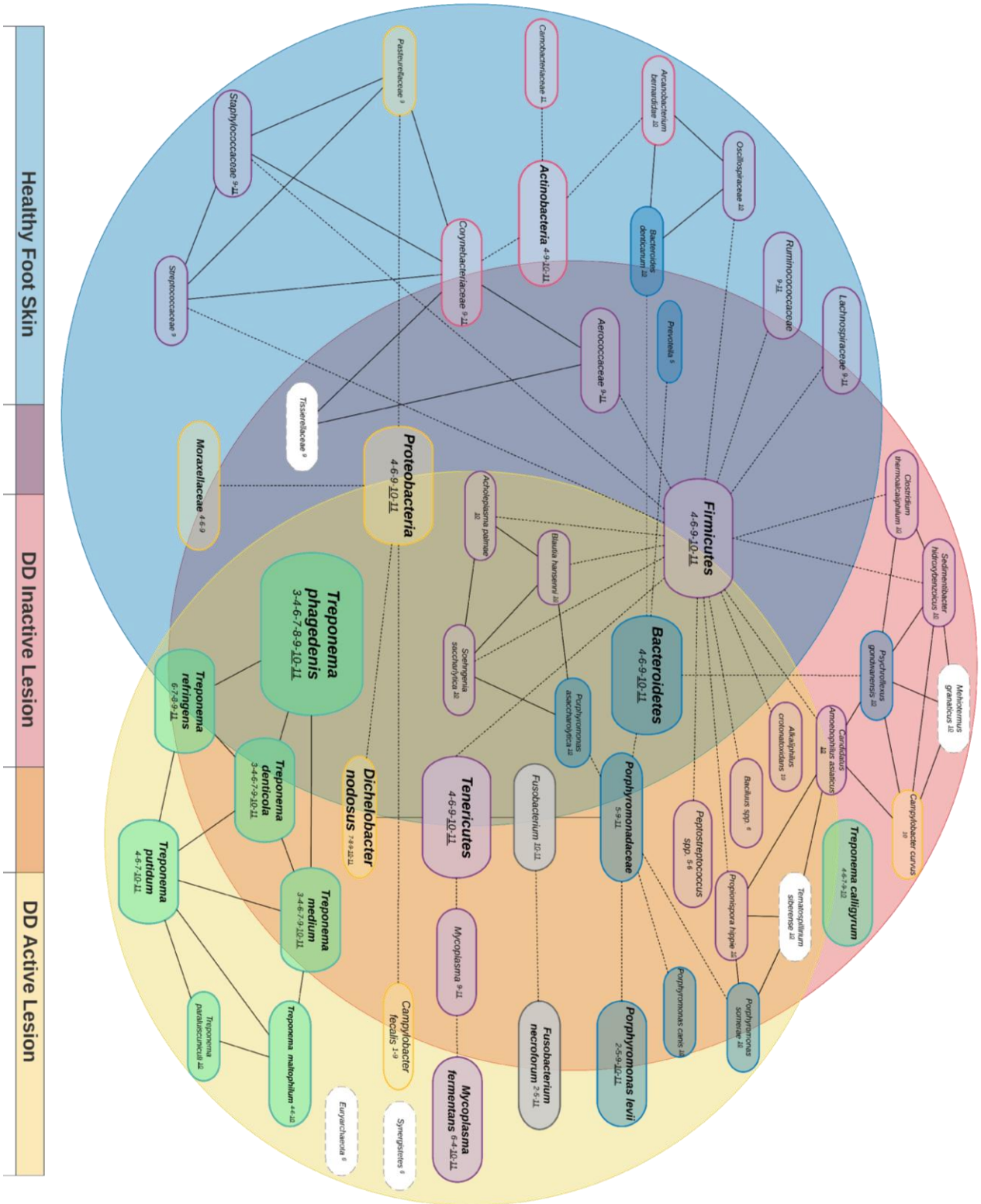

Figure 3. Microbiota representation of the healthy and diseased bovine feet skin according to the main findings of 11 studies comparing healthy samples against inactive and/or active bDD lesions. The circles represent the tree main bDD clinical stages, being bDD inactive lesion in red, the bDD active lesion in yellow, and the healthy skin in blue. The main bacteria present in the foot skin at each clinical stage are displayed, nodes of the same color are representative of the same bacteria taxon and the node sizes are proportional to the number of studies identifying the same taxon. Dashed lines link the associated taxonomy levels, and continued lines link specific relationship between microorganisms. Subscripts numbers are in reference to the studies included and presented in Table 1

Therefore, the putative incrimination of a specific bacteria for their simply presence seem to overestimate their role in the disease. In consequence, the inclusion of negative controls is essential when evaluating the putative potential of a microorganism. Nevertheless, across the bDD literature, only a few investigations have compared isolates from bDD lesions to negative healthy foot skin controls. Those studies have pointed again the importance of *Treponema spp.* (Brandt et al., 2011; Zinicola et al., 2015) and the potential connection between bDD infection and some others bacterial phylum, genera and species, such as *Firmicutes* (Santos et al., 2012), specifically *Mycoplasma* (Krull et al., 2014; Nielsen et al., 2016); some *Bacteroides* (Yano et al., 2010), specifically *Porphyromonas levii* (Berry et al., 2010); different *Proteobacteria*, such as *Campylobacter* (Döpfer et al., 1997), and *Dichelobacter nodosus* (Rasmussen et al., 2012; Knappe-Poindecker et al., 2013); and finally *Fusobacterium necrophorum* (Moe et al., 2010b). More precisely, Figure 3 summarizes the overall bacterial diversity of bDD lesions identified in these studies (Table 1). The diversity of colors among the bacteria represented in each skin stage reflect the bacterial diversity, thereby in active lesions, a reduced diversity is related when compared to inactive lesions and even more to the healthy skin. Inactive lesions resemble more healthy skin. The bacterial diversity decreases substantially between healthy and bDD lesions, as well than when compared inactive to active lesions. Indeed, the microbial communities of *Bacteroidetes*, *Firmicutes*, *Proteobacterias*, and *Actinobacterias* were gradually replaced by a larger number of *Spirochaetes*, reducing the overall diversity in the active stages of the disease. Together, all these findings have pointed up the poly-microbial component of bDD.

The farming conditions influence directly the complex environment in which animals coexist. Consequently, the feet skin and its commensal microbiota interact with the other multiple microorganisms present in the environment, some of them are pathogens, and all these bacterial communities are affected by the physicochemical characteristics of the environment, such as the pH, the temperature, the humidity, the presence of disinfectants or other antimicrobials, etc... Nevertheless, other factors might as well affect the skin microbiota, such as the usage of collective disinfectants or individual treatments, or the own immune response of an infected cow. This pattern where microbiota is disrupted by the interactions between multiple factors highlights the concept of bDD as a multi-factorial disease. Indeed, it has been proposed that the disruption of the skin microbiota might determine the treponemes proliferation and therefore the disease incidence.

To conclude, while NGS provides an opportunity to more deeply describe the bDD pathogenesis, bDD investigation raises specific questions. Unlike traditional, single species

infection models, causality with respect to infective microbiota might be evaluated in the multifactorial and polymicrobial background of the disease. Furthermore, the dynamic nature of bDD leads to establishing study protocols who include outcome measures across the time. Therefore, describing and understanding the dynamics in the microbiota composition over the course of the disease in different scenarios, such as for example, before and after treatment, could help to deeply investigate the true poly-microbial and multi-factorial components of bDD and understand its infection dynamics.

3. Risk factors for bDD occurrence and persistence:

The increasing number of epidemiological surveys about bDD made possible the recognition of several risk factors involved in the disease. Those risk factors could be gathered at 3 different levels: animal, individual level, and farm levels. Although this level structure enhances the coherence of this section, some of the factors explained below might overlap two or more of the levels.

3.1 Risk factors of bDD at the animal and individual levels

At the animal level, different studies have reported that primiparous animals were at higher risk of developing bDD (Somers et al., 2005; Rodriguez-Lainz et al., 1999), especially when they experience bDD before calving (Gomez et al., 2015b). Additionally, dry cows were reported at lower risk of developing bDD than lactating cows (Holzhauer et al., 2008), and the risk of occurrence and persistence of bDD lesions was found to increase in parallel to the milk production of the cow (Nielsen et al., 2012; Solano et al., 2016). Lastly, Holstein breed is consistently associated to an increased risk of bDD despite the over-representation of the breed and its high popularity in modern intensive dairy farming (Holzhauer et al., 2006; Relun et al., 2013b).

While the findings of epidemiological studies have revealed the importance of the risk factors previously described at animal level, others individual particularities, have been investigated and in this section were considered as individual-level factors. Thus, the individual susceptibility to bDD could be associated with any factor which could impact the own feet of an animal. Most of these factors are influenced by a genetic component, and therefore different studies have investigated this component as a single factor which could affect the bDD heritability or, by approaching potential configurations of a bDD phenotype. Some morphological factors have been proposed to play a role in the disease such as the conformational characteristics of the feet, the hoof, the skin and the hair follicles. However,

the most significant issue when studying such morphological factors raises from the fact that bDD lesions have been showed to alter the overall feet conformation itself (Gomez et al., 2015a). As a consequence, only longitudinal studies could determine the true causality of morphological characteristics in the disease. Nevertheless, even with a large variability, the genetic heritability for bDD has been evidenced across different studies ranging between 0.029 to 0.40 (van der Waaij et al., 2005; Onyiro et al., 2008; Perez-Cabal and Charfeddine, 2015; Schöpke et al., 2015). These findings open the perspectives of genetic selection in the bDD control, and this fact is already evidenced by breeding programs which offer genetic indicators for improving the resistance to lesions and hoof robustness (Gard et al., 2015). Otherwise, precise mechanisms of disease progression have been identified. Hence, the transcription of Interleukin 8 in keratinocytes was upregulated in bDD lesions suggesting that this chemokine involved in the inflammatory response and the keratinocytes proliferation play a major role in the disease pathogenesis (Refaai et al., 2013). Furthermore, 8 single nucleotide polymorphisms located in genes related to the skin proliferation and the immune response were identified exclusively in diseased animals (Scholey et al., 2012). Lastly, behavioral patterns which could influence the feet health, such as the fact that animals stand for long periods in a wet environment, or that nervous animals have trends to develop skin micro-wounds, represent somehow a potential risk due that both conducts favor the bDD development. Nevertheless, the current evidence about dairy cow behavior is exclusively related to non-infectious diseases (Proudfoot et al., 2010). However, at animal level, the putative impact of a specific microbiota of the skin in the prevention or persistence (or virulence) of bDD lesion remains unknown.

3.2 Risk factors of bDD at the farm level

Farm-level factors are related to those management practices which may impact the dynamics of the disease within a herd. Therefore, several studies have consistently evidenced the important relationship between bDD and unhygienic conditions. Among the studies, the unhygienic conditions seem to be mainly related to the time in which the feet of the cows are in close contact with the slurry and manures cumulated in the farm environment. The unhygienic conditions might be impacted by several factors such as, the structural design of the barn, the type of housing (Solano et al., 2016), the floor type of the barn (Wells et al., 1999), the type and frequency of floor cleaning, the type of scraping (Oliveira et al., 2017), the diet of the animals (Somers et al., 2005b), or the herd size (Holzhauer et al., 2006).

Likewise, access to pasture could help animals to avoid the continued contact with manures under circumstances in which grazing was longer than confinement duration (Read and Walker, 1998b; Rodriguez-Lainz et al., 1999), and where the pastures are not prone to simply expand the wet and muddy conditions of the barn (Oliveira et al., 2017). Consequently, as most of these factors will affect feet hygiene, measuring the feet dirtiness among a herd would indicate the hygienic status of a farm (Guatteo et al., 2013). The introduction of new animals into a herd represents a risk for bDD infection. Often evidenced by the introduction of replacement heifers infected with bDD (Rodriguez-Lainz et al., 1999; Oliveira et al., 2017), this risk factor probably represents the main transmission route for between herds contagion. Another important factor linked to farm practices include the hoof trimming which could improve the feet hygiene and enhance the lesion recovery by improving the conformation of the feet (Somers et al., 2005b; Relun et al., 2013b; Oliveira et al., 2017). Nevertheless, the trimming equipment was found as a potential reservoir of bDD-treponemes and therefore represents as well a risk factor (Sullivan et al., 2014a). Otherwise, it is suggested that herds supplemented with dietary minerals might enhance their immune response and the feet skin quality and therefore, this mineral supplementation represents a potential factor impacting the occurrence and severity of bDD lesions (Gomez et al., 2014b). However, there is still a lack of evidence on the subject.

Lastly, it is important to remark that the usage of collective disinfectants such as footbaths, and/or the individual treatment of ulcerative lesions might represent an important factor for the disease dynamic. Firstly, because, as pointed before, some of these practices such as footbathing aim at improving the feet hygiene and secondly because the healing and prophylactic proprieties of these strategies could improve the overall number of bDD active lesions and thereby reducing the prevalence of the disease, another risk factor recognized at farm level (Relun et al., 2013c). However, footbaths used in a wrong way could become slurry baths and therefore representing an unhygienic risk for animals.

Altogether, avoiding moist and unhygienic conditions in farms seems to be pivotal in the control of bDD. Thus, the barns restructuration or new designs of the buildings are often advised. However, few to none studies aimed at evaluating. As well, to control the spread of bDD pathogens-associated, the application of footbaths or others collective strategies to the entire herd are recommended. Nevertheless, the feasibility of the implementation of such strategies is often constrained by several reasons, such as for example: their time-consuming nature; the excessive cost associated with them; the unclear guidelines for their implementation; their toxicity; their complexity in terms of dilution and frequency of usage;

and the numerous required conditions which determine their efficacy, such the previous feet cleaning. Moreover, due to the often large differences between farms in their management practices, several risk factors might be associated with the bDD dynamics in a different level of importance. Hence, the control strategies implemented at each farm must be adaptable to their own risk factors, for instance, the herd size, the hygienic status or the bDD prevalence. Moreover, some risk factors are not consistent across studies (such as hoof trimming for instance) making difficult to elaborate control strategies. The need for assessing concomitantly the effectiveness of a new disinfectant for instance and the risk factors that could affect its efficacy is crucial.

4 Control strategies for bDD

Despite the notorious welfare impact and the economic importance of the disease in the dairy industry, to our knowledge, no eradication policies or measures are until the date imposed at regional or country level. Effective vaccine development has been unsuccessful (Berry et al., 2006; Fidler et al., 2012), mostly by barriers related to the precise recognition of the multiple pathogens involved in bDD and their pathogenicity mechanisms. Therefore, in the current scenario, two main reasons drive the prompt implementation of control strategies against bDD. Firstly, for welfare reasons, it is primordial to reduce the duration over which animals are lame and, secondly, for economic reasons to reduce the expenses linked to the disease by adopting appropriate and effective strategies.

As has been pointed in the precedent section, different studies have related the impact of multiple risk factors in the prevention and healing of bDD lesions in a relatively consistent manner. Therefore, strategies which limit those risk factors are described in this section as control strategies for bDD. Among them, treatment measures are advised for the collective prophylaxis of the entire herd in addition to the individual treatment of active lesions. They represent the most popular strategies currently implemented. Both strategies aimed at reducing the occurrence and/or the healing of active lesions and thereby controlling the spread of disease.

4.1 Footbaths & biocides: from laboratory assessment to field conditions…

The veterinary and livestock industry has been concerned by their responsibility in the growing resistance to antimicrobials of nosocomial, community-acquired and food-borne pathogens (Garcia-Migura et al., 2014; Sharma et al., 2018). In dairy farms, antibiotics are

used for multiple clinical purposes mainly of infection origin. In the case of digital dermatitis, antibiotics are only advised for the topical individual treatment of ulcerative lesions. However, even if the collective administration of antibiotics for bDD is currently banned, the over usage of antibiotics through footbaths still representing a major concern in dairy farming (Hyde et al., 2017). The reduction in the antibiotics usage in dairy farming has been largely supported by the implementation of measures to improve the biosecurity and hygiene of farms. Therefore, to enhance the hygiene of the feet and to limit the introduction and the spread of pathogens, the usage of disinfectant footbaths is advised for the bDD control.

Therefore, disinfectants represent in dairy farming an alternative to reduce the usage of antibiotics. Biocides encompass market chemical products with an antiseptic, disinfectant, and/or preservative activity.

Biocides are used for multiple purposes, such as for the disinfection of surfaces, to prevent or to limit the microbial infection of the skin, or to prevent the microbial contamination of pharmaceutical or cosmetic products (Mcdonnell and Russell, 1999). Their usage in footbaths targets the disinfection of the feet surface (hoof and skin). Nevertheless, the biocides most currently used in footbaths represent an environmental hazard (copper sulfate) or are unsafe for farmers (formaldehyde). Furthermore, biocides are challenged in field conditions against different levels of contamination.

The European legislation restrains the market of biocidal products for the veterinary usage of biocides tested under soil conditions (Regulation EU. No. 528/2012). Therefore, these biocide products shall demonstrate their bactericidal efficacy against *Enterococcus hirae, Proteus vulgaris, Pseudomonas aeruginosa* and *Staphylococcus aureus* after the exposure to 20g/L of organic matter, composed by 10g/L of yeast extract and 10g/L of bovine albumin (EN 1656). Nevertheless, the conditions in which these biocides are implemented in farms are largely distant from the laboratory environments. Indeed, the maximal levels of contamination of footbaths are uncertain and theoretically, these levels are highly dependent on the number of animal passages. Therefore, the guidelines for the usage of footbaths solutions are mainly referred to a renewal rate according to a certain number of animal passages. Thus, after a recent European directive, biocides products should confirm their efficacy according to their claimed guidelines (ECHA, 2017a). For the case of biocides used in footbaths, the bactericidal efficacy of the solution must be confirmed according to the renewal rates proposed for its usage. Therefore, the capacity of a biocide solution to support a claimed number of passages should be tested using the proportional concentrations of organic matter related to the number of passages claimed (“capacity test”). The implementation of these

regulations certainly leads to the development of more trustfully products with clear guidelines for their usage. Finally, it is important to remark that bacteria have the capacity to adapt rapidly to new environmental conditions and may survive exposure to biocides by using different resistance mechanisms (Russell, 2003).

Therefore, due to the intensive frequency and large quantities of biocides dispensed in dairy farming, the correct implementation and restricted usage of these disinfectants results crucial in the current context. Adequate studies aiming to assess the correct renewal rate and the effect of the increased amount of OM on the effectiveness of such biocides and footbath products are needed.

4.2 Footbaths and effectiveness for controlling bDD

Footbaths represent the large majority of the collective prophylactic strategies (CS). As mentioned before, its principle is based on the cleaning and disinfecting effect of topical disinfectants. Footbaths solutions aim to mitigate the effects on feet hygiene of the risk factors associated with the dirty and wet environments. Thus, the antimicrobial properties of those solutions plus the mechanical effect of walking through a bath might improve the foot hygiene. Footbathing, thereby reduce the risk of lesion occurrence and improve the lesion healing by limiting the microbial populations in the skin and the environmental spread of potential pathogens. The success of a footbath program is defined by their capacity to enhance the transition of the active lesions to the inactive lesions and by reducing the occurrence of new lesions, active or inactive. Some solutions as well argue to improve the hardness of the hoof (Fjeldaas et al., 2014) or to enhance the skin reparation by pro-inflammatory mechanisms (Smith et al., 2014). One of the main advantages of CS is the possibility of administering the solution to a large number of animals by a single effort, which represents a clear advantage in the context of bDD where within-herd prevalence is often high.

Although, several strategies of control focused in individual cases or in the entire herd are currently commercialized, for most of these products the evidence supporting their bactericidal efficacy against the main bDD-pathogens-associated is scarce and did not model the soil field conditions (Hartshorn et al., 2013). Moreover, the scientific evidence supporting their effectiveness is scarce and the conditions in which these strategies are studied in experimental settings are hard to extrapolate to field conditions. This represents a real challenge for the pharmaceutical industry to correctly promote and implement their product. Thus, after several years of research on the subject, a systematical evaluation of the scientific

literature on CS seems necessary to first identify the main gaps in the evidence supporting control strategies and secondly, to provide insights for the conception of clinical trials of high quality to evaluate bDD control strategies. Indeed, in the complexity of dairy farming, the effectiveness of such strategies is conditioned by several factors including: first, their correct usage, which is determined by the respect of the concentration and the renewal rates of the solution; second, the proper design of the bath, which may allow the complete immersion of the feet into the solution after a defined number of passages; and third, by the confirmed bactericidal efficacy of the solution, which in practice must remain effective under the farm and the skin environments plenty of organic matter and other contaminants.

Additionally, these strategies are perceived as time-consuming, and the cost of using a CS is considerable. Therefore, its usage represents a major concern regarding the perception of benefit which entails such effort for farmers (Relun et al., 2013b). Finally, another important concern facing CS entails the hazard occasioned by the substances used currently, more precisely the formaldehyde, which is a recognized carcinogenic (Cogliano et al., 2005), and the copper sulfate, which is considered as an important environment polluter (Ippolito et al., 2010). Therefore, developing and evaluating alternative strategies to avoid the usage of these substances is a priority to encompass the concept of "one health" in the context of bDD.

4.3 About individual treatment of bDD lesion

Fist we can notice that up to now there is no effective vaccine against bDD (Orsel et al., 2017). Otherwise, among the control strategies, the individual treatment of active lesions aims to enhance the transition of the active lesions to inactive lesions, and thereby reducing the charge and number of reservoirs. The products administered individually are mainly antibiotics including oxytetracycline and lincomycin, or non-antibiotic products such as salicylic acid (Schultz and Capion, 2013) or copper and zinc chelates (Dotinga et al., 2017). The healing rates of these treatments in controlled studies ranged from 9% to 93% (Apley, 2015; Krull et al., 2016a). This variability is mostly explained by the short periods of observation and the success definition between the studies. Although together this evidence supports the effectiveness of these strategies in a short-term, high recurrence of even 54% of the treated lesions were reported (Berry et al., 2012). Additionally, as pointed before, the individual treatment of a high infected herd could become easily an expensive and time-consuming strategy. Moreover, residues from these therapies can be potentially found in milk. In summary, individual treatments are useful to reduce pain caused by severe active lesions,

and in parallel reducing the spread of the disease by controlling active reservoirs. However, the recurrence or re-lapsing of the lesion is reported. Therefore, alternative substances to antibiotics must be explored for their potential effectiveness as individual treatments in a short term and long term clinical trials. Moreover, the massive and over-usage of antibiotics must be restricted by public health concerns. Indeed, even if the usage of antibiotics in footbaths remains anecdotal, their usage in sub populations could enhance the raising levels of antimicrobial resistance, and therefore their usage must be banned (Hyde et al., 2017). Lastly, the precise effect of such antibiotic treatment of the skin microbiota of the skin remains poorly investigated.

4.4 Beyond footbath and treatment for controlling bDD

Across the scientific literature, different studies have measured the impact of the exposure to multiple risk factors on bDD recovery and occurrence. The findings of these studies have opened the perspective for adapting multiple control strategies to each different farm scenario. These global approaches resemble more the complexity of field conditions where multiple risks are identifiable and different control measures are already implemented. Depending on the population concerned, these strategies could be focused on the specific group of animals at higher risk. Likewise, as risk factors vary over time, in critical periods the implementation or increasing the frequency of a specific strategy could potentially enhance their effectiveness. Farms with impaired designs of their barns and parlors lead often to unhygienic and wet conditions. For those recognized farm-level risk factors, different control strategies could be implemented at short, mid and long-term. In this case, the mid-long-term strategy will attempt to rebuild or restructure the barn and the parlor. Otherwise an integral strategy focused on the feet hygiene might include: the improvement on the frequency and manner at which the facilities are cleaned; the improvement on the renewal, the bed material or the usage of bed dryers; improvement or instauration of trimming practices; and, as noted before, the collective cleaning or disinfection of the feet (Relun et al., 2013b; Oliveira et al., 2017). Likewise, in farms with a high prevalence of the disease, strategies of early detection, early individual treatment, and preventive footbathing might improve the disease control in a short or midterm (Solano et al., 2017). Finally, at long-term, if individual factors are targeted to improve, strategies of genetic selection or the renewal of the entire herd could be considered. Nevertheless, the feasibility of implementing those long-term and complex strategies is often concerned, mainly for economic reasons without any evaluative support.

Additionally, independent of the clinical scenario of a particular farm, once the disease is recognized, strict and systematic cleaning of the trimming material is advised. Moreover, in some scenarios where the biosecurity represents the main risk factor regarding bDD, the establishment of biosecurity protocols is strongly recommended. Such protocols must precise the conduct to adopt by any visitor and the prophylactic measures that must follow new incoming animals, especially in herds renewed or enlarged frequently. Likewise, contact restriction between species could be protocoled due to the similarities of bDD with some diseases in other species (Wells et al., 1999; Rodriguez-Lainz et al., 1999).

It can be concluded that there is no gold standard strategy that leads to the eradication of bDD, thereby the simultaneous implementation of multiple measures seems to be the best current strategy for bDD control. Therefore, it is necessary to develop novel control strategies for bDD, feasible to implement and adaptable to the own farm characteristics. Such strategies (i) must be supported by a high quality of evidence, (ii) must include the instructions to adapt the regimes and doses of administration according to parameters easy to measure and to record in farms and (iii) must be safe for the environment, the animals, and the farmers. The integrality of this research questions might help to elucidated new insights about the etiology and the physiopathology of bDD, and therefore improve the strategies for its control.

C. Objectives

The main objective of this thesis is to generate knowledge about the effectiveness of a new footbath biocide solution for the control of Bovine Digital Dermatitis in dairy herds, and consequently investigate deeply the conditions which may determine the success or failure of such a control strategy.

More in details, the outline of this thesis will be:

Thesis component 1: Systematic review and Meta-analysis

Conduct a systematic review of the effectiveness of collective treatments for the control of the occurrence and persistence of bDD lesions.

Objective and expected results:

- Assess the evidence about the effectiveness of collective treatments.
- Evaluate the strengths and limitations of the different study designs to avoid such problems in future clinical trials.

Thesis component 2: Preliminary studies to determine the renewal rate of the biocide

Determine the parameters to adapt the renewal frequencies of a new biocide solution for the bDD control, according to best conditions for their implementation in field conditions.

2.1 Exploring the footbaths contamination by manure under field conditions.

Objective and expected results:

- Determine the range of contamination of footbaths, in terms of organic matter (OM) and microbial loads, according to the different number of cow passages and the hygienic status of the farms.
- Define a range of OM and microbial loads contamination, to test *in vitro* the stability and efficacy of the biocide solution against the main bDD associated-pathogens.

2.2 In vitro evaluation of the bactericidal efficacy of a new collective disinfectant solution under simulated soil conditions.

Objective and expected results:

- Assess the influence of OM and microbial loads, at different concentrations, simulated from field conditions, on the bactericidal activity of the biocide solution against the main bDD pathogens-associated.
- Define the renewal frequencies which assure the efficacy of the biocide solution, according to the number of cow passages and the hygienic status of farms.

Thesis component 3: clinical trial

Evaluate the effectiveness of a new footbath solution in the control of bDD under field conditions through a clinical trial taking into account other risk factors.

3.1. Evaluation of a biocide footbath solution in the prevention and healing of digital dermatitis lesions in dairy cows. A clinical trial

Objective and expected results:

- Evaluate under field conditions, in affected herds, the effectiveness of a new biocide solution at different regimens, in the collective prevention and treatment of bDD
- Providing advice and recommendations for elaborating control strategies including footbath.

3.2. Microbiota dynamics in the skin of feet affected by bovine digital dermatitis, before and after the implementation of footbath disinfectant practices

Objective and expected results:

- Contribute to the knowledge of bDD pathogenesis and etiology
- Investigate the putative interest of the foot microbiome diversity (Reduction of *Treponema* proliferation) as a valuable outcome for assessing the effectiveness of the biocide.

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Chapter 1. Introduction

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Chapter 2. Systematic Review and Meta-analyses

A. Effectiveness of collective treatments in the control of bovine digital dermatitis. Where is the evidence?

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Effectiveness of collective treatments in the prevention and treatment of bovine digital dermatitis lesions: A systematic review

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ABSTRACT

The collective treatment (CT) of an affected herd is commonly advised to control bovine digital dermatitis (DD). Several CT are commercialized, frequently without major evidence supporting their effectiveness. The objective of this systematic review was to evaluate the published evidence that supports CT in the treatment and prevention of DD lesions in dairy herds. Across the evidence, the main limitations in the studies design were identified and the possible sources of inconsistency were investigated. An extensive literature search of publications through electronic databases and gray literature was conducted between July 2015 and January 2016. Studies that did not include an untreated or placebo control group were excluded from the review. The literature search and screening process identified 13 publications with 24 treatment trial comparisons and 18 prevention trial comparisons. The published evidence included studies mostly considered to have a low or unclear risk of bias. Descriptive analyses were performed according to the prevention and treatment outcomes, and case and success definitions were identified for each study and summarized in odds ratios (OR). Pairwise meta-analyses were conducted according to the prevention and treatment outcomes, comparing directly the intervention used in each study, and ignoring any other differences in the intervention characteristics. The results of the meta-analyses indicated a low degree of heterogeneity across the evidence for the prevention outcome [I^2 = 0%, 95% CI: 0 to 37.2%, 95% prediction interval (PI): 0.72 to 1.74)] and a moderate degree for the treatment outcome (I^2 = 25.3%, 95% CI: 0 to 63%, 95% PI: 0.39 to 3.73). Similarly, appraisal of the graphical L'Abbé plot suggested a considerable degree of heterogeneity across the evidence for the treatment outcome. For both outcomes, the frequent small sample sizes of the trials indicate imprecision across the included studies. Additionally, for the treatment and prevention outcomes, an asymmetric funnel plot suggested possible publication bias. The overall quality of the evidence, for both outcomes (prevention and treatment), was therefore considered to be low, indicating that the true effect of CT may be substantially different from that estimated across the included studies. Consequently, this review and meta-analysis does not support an association between the CT considered in the review and a beneficial effect in the prevention and treatment of DD lesions. The effectiveness of CT therefore remains uncertain, and the epidemiological circumstances in which it can be useful must be investigated. These findings highlight the importance of developing high quality, controlled trials to evaluate the effectiveness of CT for DD control.
Key words: dairy cow, bovine digital dermatitis, collective treatment, meta-analysis, systematic review

INTRODUCTION

Bovine digital dermatitis (**DD**) is a multifactorial contagious disease, with worldwide distribution, characterized by painful and ulcerative lesions in the foot skin (Laven and Logue, 2006; Gomez et al., 2012). This condition is often associated with animal welfare concerns such as lameness (Bruijnis et al., 2012). Digital dermatitis is also related to economic issues such as reduced milk production, impaired reproductive performance, and increased risk of culling (Bruijnis et al., 2010; Ettema et al., 2010; Relun et al., 2013c). The disease affects 70 to 96% of dairy herds in Western Europe and North America, and the within-herd prevalence ranges from 5 to 30% among lactating cows (Brown et al., 2000; Holzhauer et al., 2006b; Cramer et al., 2008).

Despite more than 40 yr of research, the precise pathogenesis of the disease remains unclear. Nevertheless, the presence of specific *Treponema* species on feet suffering from cutaneous maceration is recognized as a major etiological component involved in the development of the disease (Gomez et al., 2012). Current control strategies aim to control the main risk factors of

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DD, such as moist and unhygienic conditions, to limit the spread of the infection. (Palmer et al., 2013; Relun et al., 2013c). These strategies rely particularly on the complementary use of individual medical topical treatment of active lesions and metaphylactic collective treatments (**CT**) of the entire affected herd. However, both approaches are time-consuming practices, presenting economic and environmental challenges for farmers and the veterinary industry (Relun et al., 2013b). Although antibiotics such as oxytetracycline and lincomycin are mainly used as individual treatments and their topical administration is considered effective (Apley, 2015), high rates of lesions recurrence (50%) are reported for some of these products (Berry et al., 2012). The use of antibiotics furthermore should be limited in order to decrease antimicrobial resistance and withdrawal periods for milk. Moreover, the collective administration of antibiotics is no longer advised and such practices are already banned by European Union policies.

Disinfectants such as formaldehyde and copper sulfate ($CuSO_4$) have been used in footbaths as the standard CT in the control of DD. However, formaldehyde is carcinogenic and $CuSO_4$ is toxic for the environment via accumulation in the soil (Ippolito et al., 2010). Moreover, a recent systematic review revealed that the effectiveness of $CuSO_4$ footbaths against DD is not adequately supported by the evidence (Thomsen, 2015). In addition, new evidence suggests possible genetic resistance to copper and zinc in microbiomes associated with DD lesions (Zinicola et al., 2015). Currently, several CT for DD are commercially available, most of which are supported by anecdotal evidence and a few by clinical trials (Laven and Logue, 2006). However, high variability in the efficacy of some of the products evaluated by scientific studies is perceived in practice (Relun et al., 2013b). Last, for most CT, their bactericidal efficacy against DD *Treponema* groups remains uncertain (Hartshorn et al., 2013).

In evidence-based veterinary medicine, randomized controlled trials (**RCT**) are considered the gold standard to guide treatment and prevention decisions. However, under certain circumstances, such as on commercial dairy farms, it can be difficult to conduct RCT for practical reasons. Consequently, part of the existing evidence about CT is based on non-randomized studies (Sargeant et al., 2014). The results of scientific studies on DD are furthermore often difficult to extrapolate to real conditions; this is most likely due to a lack of guidelines for CT use under diverse conditions (Relun et al., 2013b).

An assessment summarizing the scientific evidence concerning existing CT based on an objective procedure is therefore required to assist veterinarians and farmers in their DD control decisions. The main objective of the present systematic review was to evaluate the evidence supporting the use of CT in the treatment and prevention of DD to provide new insights into the design of high-quality DD control effectiveness trials. Data from multiple studies were combined through a meta-analysis to investigate the main sources of heterogeneity between studies and to calculate a summary effect estimate of the effectiveness of CT in the treatment and prevention of DD.

MATERIALS AND METHODS

The review was conducted following the guidelines proposed by Sargeant and O'Connor (2014) for systematic reviews in animal agriculture and veterinary medicine. A protocol was developed a priori that included a detailed description of the review process (Supplemental Data File S1; https://doi.org/10.3168/jds.2016-11875).

Search Strategy

The review questions were designed based on the evidence-based veterinary medicine concept of PICO terms: population (P), intervention (I), comparator (C), and outcomes (O) (Richardson et al., 1995). The study population of interest consisted of dairy cows, including heifers and lactating and dry cows. The intervention was CT, defined as the topical administration on feet of the same treatment (dose and frequencies) at a given time to 2 or more animals without restraining them individually. The comparators were parallel control groups of untreated animals (absence of CT) or groups treated with a water placebo. Two outcomes of interest were defined. The first involved prevention, where the outcome was the incidence, defined as the occurrence of new clinical DD lesions within the follow-up period. The second involved treatment, where the outcome was the healing of DD lesions, defined as the reduction of existent clinical DD lesions within the follow-up period. For both outcomes, the diagnosis and evolution of clinical lesions must be assessed by direct visual diagnosis and measured by an objective methodology (lesion score system). Two clinical questions were therefore defined as follows: "In dairy cows, are collective treatments more effective at preventing the occurrence of clinical lesions of bovine digital dermatitis compared to a placebo or the absence of any collective treatment?" and, "In dairy cows, are collective treatments more effective for the treatment of clinical lesions of bovine digital dermatitis compared to a placebo or the absence of any collective treatment?"

Separate database searches were conducted for both outcomes simultaneously across PubMed, CAB, and Web of Science (core collection) between July 2015 and January 2016. The research was restricted to papers published between 1974 (first official description of DD) and 2016. No language restrictions were fixed. The citations, title, and abstract were screened for relevance by the principal author.

PubMed database searches were conducted using the Medical Subject Headings (MeSH) terminology and Boolean operators in this order: Cattle, Foot Diseases/veterinary OR Digital Dermatitis/drug therapy OR Digital Dermatitis/prevention and control OR Digital Dermatitis/therapy AND Disinfectants/administration and dosage OR Disinfectants/therapeutic use OR Anti-Bacterial Agents/therapeutic use OR Copper Sulfate/therapeutic use OR Anti-Infective Agents, Local OR Copper/therapeutic use OR Probiotics/therapeutic use OR Zinc/therapeutic use OR baths/veterinary"[Mesh] OR Occlusive Dressings/veterinary OR Administration, Topical Foot Diseases/veterinary OR Digital Dermatitis/drug therapy OR Digital Dermatitis/prevention and control OR Digital Dermatitis/therapy. Additionally, a manual search of the gray literature was performed by the principal author on the principal proceedings on the subject: World Buiatrics Congress 2002–2014, International Conference on Lameness in Ruminants 2002–2013, Cattle Lameness Conference 2009–2015, European Buiatrics Forum 2009–2013, the Journées 3R (Rencontres autour des recherches sur les ruminants) 1994–2015, and the British Society of Animal Science Conference 1999–2015.

For the relevant citations identified, their title, abstract, and materials and methods were verified for eligibility by 2 of the authors, who worked independently using a screening tool designed for this systematic review. Studies were eligible for the synthesis if a positive answer was given to all 4 of the following questions:

(1) Does the study describe a primary research study?
(2) Does the study evaluate CT in dairy herds?
(3) Does the study include the visual and objective measure of the incidence (prevention) or healing (treatment) of DD lesion, or both, as an outcome?
(4) Does the study include a parallel untreated control (absence of any CT) or a placebo group (water)?

In case of discrepancy between the 2 authors concerned, a third reviewer resolved the conflict.

Data Collection Process

The information considered as relevant to extract for this review was determined by the research team with the advice and supervision of a statistician involved in the study (Supplemental Data File S2; https://doi.org/10.3168/jds.2016-11875). Information was extracted by the principal author; in cases where the study data seemed confused or inconsistent, the assistance of the review team was requested.

The relevant information from each study trial was extracted at 5 levels (publication, population, intervention, outcome, and study design). The publication level includes author information, citation details, year of publication, and publication source (i.e., databases or gray literature). The population level includes data relative to the breed and lactation stage of the cows, the housing and milking system, and the initial prevalence of the disease in the herd. At the intervention level, information was extracted about the products used in the experimental and comparison groups, the type of intervention used (i.e., footbath, split footbath, foam system, collective spraying), the doses and frequencies of administration and, when appropriate, the concomitant individual treatments used. The data extracted at the outcome level included information about the number of outcome events by group as a rate derived from the 2 × 2 contingency tables, the frequency by which the measurements were taken, the follow-up time (from the first to last observation), the diagnostic methodologies used, and the "outcome unit" assessed (foot, cow). For each study, case and success definitions of DD clinical lesions were identified according to the treatment and prevention outcomes measured. For 2 studies, results were time-to-event outcomes (Relun et al., 2012, 2013a). In those cases, the proportions of outcome events in the intervention and control groups were provided by the authors. In one study (Thomsen et al., 2008), the proportions of outcome events and the OR (95% CI) were combined for the overall intervention groups, because information about the number of events and subjects for each of the 3 intervention groups was unavailable.

Finally, at the study design level, information was extracted about randomization efforts, blinding of caregivers and observers, statistical methods used for analyses of the outcomes, handling of missing data, and the funding sources of the study. After the full-text assessment of the publication, the authors were contacted when some information was unavailable in the published paper.

The extracted data describing the effectiveness of the intervention were used to calculate the OR from the

event proportions (incidence or healing) between the CT group and control group.

Risk of Bias Assessment

The quality of the evidence included was assessed independently by 2 reviewers using a "Risk of bias tool" created for this study and based on the recommendations of the "Cochrane collaboration's tool" for assessing the risk of bias in RCT (Higgins et al., 2011).

At the study level, sources of bias were evaluated in 5 domains (selection, performance, detection, attrition, and other bias). The selection bias domain assesses the efforts implemented in the trials to randomize the subjects or to balance the baseline risk among the intervention groups. The performance bias domain considers the measures used to reduce the effects linked to possible over usage of co-interventions or overprotection of animals in trials when the participants (caregivers) were aware about the group allocation. The detection bias domain assesses the methods and the objectivity by which the clinical evolution of DD lesions was measured (lesion score system). The attrition bias domain considers the amount, nature, and handling of incomplete outcome data. Finally, the "other bias" domain considers the possible carryover effects in the trials. After describing each bias domain, a grade of high, low, or unclear risk of material bias for each domain was assigned. Unclear risk was considered when the information relative to a domain was insufficient and when the possible risk of bias had an unknown effect.

From these within-study assessments, a general appreciation of the quality of each study was summarized in 3 categories: low risk of bias in studies with at least 4 domains judged as low; unclear risk of bias in studies with all key domains judged between low and unclear; and high risk of bias was considered in studies that judged one or more key domains as high.

The "risk of bias tool" used in this systematic review was modified from the one planned in the protocol in an effort to broadly approach the main methodological bias present in DD trials.

Synthesizing the Overall Results

The analyses were performed using the "meta" package in R (Schwarzer, 2015; R Core Team, 2015). For each trial and outcome evaluated (prevention or treatment), the number of events were entered for both the untreated or placebo control group and the experimental intervention group. We ignored any differences in intervention type, dose, or duration of therapy and directly compared the intervention group to the untreated or placebo group in 2 different pairwise meta-analyses, one for the prevention and one for the healing of DD lesions. The publications where the data required for the quantitative synthesis were missing (e.g., number of events and subjects in the intervention and control groups) were excluded from the meta-analyses. Studies with multiple intervention group comparisons were combined to create single pairwise comparisons according to the type of CT application (i.e., spray and footbath). Therefore, in such cases, and according to the number of intervention groups formed, the control or placebo group was split into 2 or more comparisons (Higgins and Green, 2011). For the studies that reported multiple-outcomes observations during the follow-up period (Speijers et al., 2010; Relun et al., 2012, 2013a), only the data from the last observation session was included in the meta-analyses. The meta-analyses were performed by computing the study effect sizes in log OR and their 95% CI, using a random effects model (DerSimonian and Laird method, DL; DerSimonian and Laird, 1986), assuming that the intervention effects varied across the trials following a normal distribution. The individual study OR were weighted by the inverse variance, so large studies provided more information to the summary OR. However, in trials where "outcome units" were clustered by herd or by cow side (right or left) in the herd, effective sample sizes were adjusted by the intracluster (or intraclass) correlation coefficient (ICC; mean 0.3) obtained from previous DD scientific studies (Holzhauer et al., 2006a; Cramer et al., 2008). Forest plots were created, including the OR and the summary effect calculated and their 95% CI, with the size of the shaded box reflecting the relative contribution of each study to the summary OR.

Heterogeneity among studies was assessed using the L'Abbé plot, a graphical method that displays the relationship between baseline risk (baseline incidence rates and spontaneous healing rates) and intervention effectiveness across the trials (L'Abbé et al., 1987). On the graph, trials were plotted according to the beneficial superiority on the comparison of event proportions between the CT group and the control group, with point size being proportional to the size of the trial. The trials in which the beneficial effect was superior in the CT intervention group than in the control group were plotted between the y-axis and the line of equality. Those trials in which the beneficial effect was superior in the control group than in the CT intervention group were plotted between the x-axis and the line of equality. The locations of the different points or cluster formations in the graph were indicative of the level of agreement among trials. The Cochran's Q test was used to assess whether the variation in effect estimates were beyond

chance. Between-study heterogeneity was quantified by the Higgins statistic (I^2) with 95% CI and the tau squared (τ^2) calculation (Higgins and Thompson, 2002). Finally, to illustrate the amount of heterogeneity, the 95% prediction intervals (**95% PI**) for the summary effects were calculated (Borenstein et al., 2017).

The small study effects, which may be caused by publication bias, were investigated using funnel plots, evaluating their symmetry both visually and objectively with Harbord's test (Higgins and Green, 2011).

Finally, subgroup analyses, determined a priori, to investigate possible sources of heterogeneity were conducted for study design (RCT vs. any other design), initial prevalence (high prevalence >30% vs. low prevalence <30%), length of the study (>8 wk vs. up to 8 wk) and follow-up assessments (before and after vs. multiple assessments). Post hoc subgroup analysis only included "study limitations" (low risk of bias vs. unclear/high risk of bias). The conditions for subgroup analyses were slightly changed from the protocol to enhance group formation and then allow the statistical comparisons. The threshold values for comparison were changed for initial prevalence (from 15 to 30%) and for length of the study (from 12 to 8 wk).

RESULTS

Search Results and Study Selection

PubMed, CAB, and Web of Science databases searches yielded 65, 233, and 112 citations, respectively; 134 duplicates were removed. Six additional relevant publications were identified by manual searches. Taken together, 282 unique records were assessed for relevance

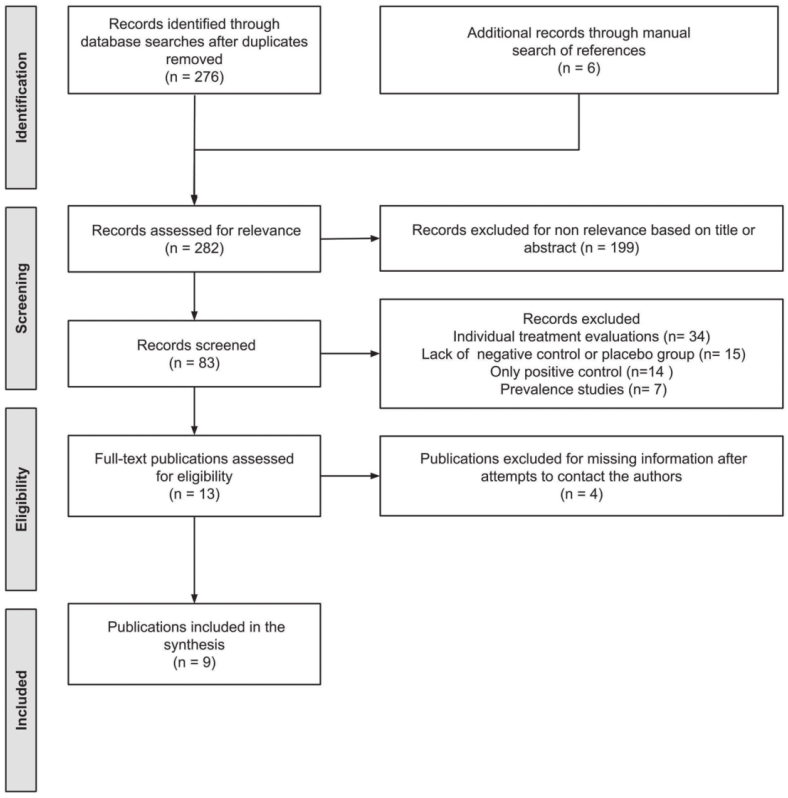

Figure 1. Summary of the search and selection process used to identify publications included in the systematic review.

Table 1. Descriptive summary of study design and population characteristics reported in 13 publications included in a systematic review assessing the effectiveness of collective treatments (CT) in the prevention and treatment of bovine digital dermatitis (DD) lesions

Study; country	Type of study[1]	Length of study[2] (wk)	Enrolled (no.)		Breed[3]	Outcome unit	DD initial prevalence (%)	Access to pasture during trial	Housing system	Milking system	Parity	Lactation stage (DIM)
			Animals	Farms								
Britt and McClure. (1998); United States	RCT	3	64	1	Hol	Cows	100	No access	Freestalls	Herringbone	1 (100%)	Unknown
Hernandez et al. (1999); United States	RCT	4.2	66	1	Hol	Cows	100	No access	Freestalls	Herringbone	<1 (50%) >1 (50%)	<190 (50%) >190 (50%)
Manske et al. (2002); Sweden[4]	NRCS	24	58	1	SRB/ SLB	Feet	54	Total access	Cubicles	Herringbone	1 (29–36%) >1 (64–71%)	<30 (18%) >30 (82%)
Fiedler (2004); Germany	RCT	12	55	1	BS	Cows	50–58	No access	Cubicles	Herringbone	Unknown	Unknown
Ishmael et al. (2005); United Kingdom	NRCS	8	60	1	Unknown	Feet	50	Unknown	Cubicles	Unknown	Unknown	Unknown
Bergsten et al. (2006); Sweden	NRCS	±16	279	1	SRB/ SLB	Feet	17	Grazing season	Cubicles	Rotary parlor	Unknown	Unknown
Klaas et al. (2008); Denmark[4]	CrT	4	114	1	Hol	Feet	24–28	No access	Cubicles	Automatic milking system	<2 (50%) >2 (50%)	<115 (50%) >115 (50%)
Thomsen et al. (2008); Denmark	CRT	8	1,200	4	Hol	Feet	21.8–22.7	Partial (2 wk)	Freestalls	Herringbone and Rotary parlor	Not recorded	Not recorded
Speijers et al. (2010); Northern Ireland	NRCS	5	118	1	Hol	Cows	59	Partial (2 wk)	Freestalls	Rotary parlor	<3 (50%) >3 (50%)	90–150 (100%)
Rasmussen et al. (2011); Denmark	NRCS	8	405	4	Hol	Feet	40	Unknown	Unknown	Unknown	Unknown	Unknown
Relun et al. (2012, 2013a); France	NRCS	24	4,677	52	Hol/ Norm	Feet	6–13	Grazing season (6 farms no access)	Cubicles/ Freestalls	Herringbone	1 (35%) 2 (27%) 3 (17%) >4 (20%)	<90 (35%) 90–150 (23%) >150 (43%)
Fjeldaas et al. (2014); Norway	RCT	12	45	1	NR	Cows	53–73	No access	Freestalls	Herringbone	Unknown	<150 (100%)

[1]RCT = randomized controlled trial; CrT = crossover trial; CRT = cluster randomized trial; NRCS = nonrandomized controlled study.
[2]Time from the first observation until the last observation.
[3]Hol = Holstein; SRB = Swedish red and white Holstein; SLB = Swedish Holstein; BS = Brown Swiss; Norm = Normande; NR = Norwegian Red.
[4]Studies where the observations were considered independent.

and, based on the title or abstract, 199 were excluded. Of the 83 relevant citations included for verification through the screening tool (title, abstract, and material and methods), 34 publications concerned evaluations of individual treatments, 29 publications lacked a comparative untreated or placebo control group, and 7 were observational prevalence studies. No discrepancies between the 2 reviewers were evidenced when using the screening tool. The full text of the remaining 13 publications was assessed through data extraction. After different attempts to contact the authors in cases of missing information, 4 additional publications were excluded and, finally, 9 papers were included in the quantitative synthesis (Figure 1).

Study Characteristics

Table 1 summarizes the main characteristics of the 13 relevant publications included in the systematic review. Five of the publications were retrieved from the gray literature and 8 from peer-reviewed journals. The year of publication ranged from 1998 to 2014. The majority of studies were undertaken in Europe, with only 2 in the United States. Six studies were RCT, including 1 crossover trial (CrT) and 1 cluster randomized trial (CRT). Six were nonrandomized controlled studies (**NRCS**), where subjects were allocated to interventions by nonrandomization methods. Before the start of the trials, initial DD prevalences ranged from 6 to 100% among the studies. The length of the studies (period of CT administration and follow-up) ranged from 2 to 24 wk. Only 4 studies performed multiple-outcomes observations during the follow-up period (Ishmael et al., 2005; Speijers et al., 2010; Relun et al., 2012, 2013a). The preventive crossover trial included in the review (Klaas et al., 2008) did not have a washout period; therefore, only information on the first period of this study was considered for each group of animals as a trial. Among the 13 publications included in the systematic review, 9 trials evaluated the preventive outcome assessing 18 comparisons, and 11 trials evaluated the treatment outcome assessing 24 comparisons. Nine of the publications used untreated control groups and 4 used water placebos as control groups.

The quality assessments are displayed in Figure 2. Within-studies assessments considered 5 studies to be at low risk of bias. Among them, unclear limitations were found in the selection and attrition bias domains. The remaining studies were considered to be at unclear (6) and high (2) risks of bias. In general, limitations were mostly related to randomization efforts (selection bias domain) and carryover effects (other bias domain), followed by the unclear risk of bias related to the studies' limitations in the handling of missing data (attrition bias domain) and the blinding of caregivers (performance bias domain).

Case and success definitions, proportions of outcome events (occurrence rates and healing rates), and the OR (95% CI) associated with each study are reported in Tables 2 and 3, according to the prevention or treatment outcomes. For 4 studies, data on the results of the trials' effectiveness were unclear in the publication, and it was therefore impossible to calculate the proportion of outcome events and the OR (Fiedler, 2004; Ishmael et al., 2005; Bergsten et al., 2006; Rasmussen et al., 2011). Among the prevention trial comparisons, 17 products were tested involving different disinfectants; 9 of these relied on copper bactericidal properties, 2 on glutaraldehyde, 2 on organic acids, 1 on sodium hypochlorite, 1 on NaCl, 1 on quaternary ammonium, and 1 on calcium hydroxide. Additionally, 2 prevention trial comparisons used water as the active CT. For the treatment outcome comparisons, 19 products were tested, with 9 based on copper, 4 on hydrogen peroxide, 2 on glutaraldehyde, and 1 on sodium hypochlorite. Two trials used water as the active treatment. Finally, an antibiotic was administered as a CT in only 2 studies, involving 2 treatment comparisons and 1 prevention comparison. The types of intervention used among the studies varied between footbath (7), spraying (4), foam

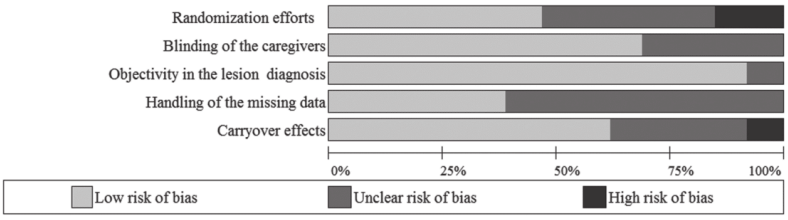

Figure 2. Graphical representation of the quality of the included studies, based on the risk of bias assessments.

Table 2. Evidence summary of collective treatments (CT) in the prevention of bovine digital dermatitis (DD) lesions: results are expressed as odds ratio (OR)

Study	Case definition	Success definition	CT strategy and regimen	Outcome proportion[1] (event/no.)	OR (95% CI)	Additional individual treatment
Manske et al. (2002)[2]	New cases were defined as lesions of active lesions (degrees 1 to 3) in feet without active lesions (degrees 0, 4, or 5) (Manske et al., 2002).	Prevention of new cases of active lesions (degrees 1 to 3) during the study period.	Footbath: water (within-cow control: split-footbath)	0.69 (38/55)[3]	Referent	None
			Footbath: acidified ionized copper 0.6% solution, 2 milkings/d, for 10 d, with 5.5 d in between.	0.69 (39/56)[3]	0.97 (0.43 to 2.18)	
Ishmael et al. (2005)	Not specified. Feet lesions were scored between 0 (absence of lesion) and 3, based on the lesion size, pain score, appearance and lameness.	Prevention of new cases throughout the study period.	No CT (untreated control)	Not specified	Referent	None
			Spray: oxytetracycline solution, 3 times a week	Not specified	—	
			Spray: NaCl 20%, 3 times a week	Not specified	—	
Bergsten et al. (2006)	Lesions were scored from 0 for no lesion to 2 for a severe lesion.	Absence of deterioration in feet with a score <2 at the beginning of the experiment.	Footmat: water (within-cow control: split-footbath)	Not specified	Referent	None
			Footmat: $CuSO_4$ 7%, after every milking (2/d)	Not specified	—	
			No CT (untreated control)	Not specified	Referent	
			Foam-system: tensides and paracetic acid, 2 milkings/d	Not specified	—	
Klaas et al. (2008)[2]	New cases were defined as active lesions (degrees 1 to 4), in feet previously scored as non-active lesions (degrees 0 or 5) (Manske et al., 2002).	Prevention of new cases of active lesions (degrees 1 to 4) during the study period.	No CT (within-cow control: split-footbath)	0.86 (33/38)[3]	Referent	Only in ulcerative lesions
			Footbath: CuSO, ZnSO, and organic acid solution, once weekly by 17 h	0.92 (35/38)[3]	0.56 (0.12 to 2.55)	
			No CT (within-cow control: split-footbath)	0.98 (30/34)[3]	Referent	
			Footbath: copper sulfate 7.5% solution, once weekly by 17 h	0.94 (32/34)[3]	0.46 (0.07 to 2.74)	
Thomsen et al. (2008)	New cases were defined as active lesions (degrees 1 to 4), in feet previously scored as non-active lesions (degrees 0 or 5) (Manske et al., 2002).	Prevention of new cases of active lesions (degrees 1 to 4) during the study period.	No CT (within-cow control: split-footbath)	0.91 (492/535)[3]	Referent	None
			Footbath: glutaraldehyde 1.5%, organic acids 2.1% and quaternary ammonium compounds 2%, 2 consecutive milkings, twice a week	0.89 (480/535)[3]	1.31 (0.86 to 1.99)	
Speijers et al. (2010)	Cows with early or acute classical ulcerative DD lesions (M1 or M2) on at least 1 hind foot were defined as cows having active DD lesions (Döpfer et al., 1997).	Prevention of the occurrence of DD active lesions (M1 or M2).	No CT (untreated control)	0.25 (10/39)[4]	Referent	Only in ulcerative lesions
			Footbath: $CuSO_4$ 5%, 4 consecutive milkings/wk	0.32 (12/37)[4]	0.71 (0.26 to 1.94)	
			Footbath: ClO 2% hypochlorite sodium, 4 consecutive milkings every week	0.27 (10/37)[4]	0.93 (0.33 to 2.58)	
Rasmussen et al. (2011)	Distinction between active and inactive lesions was not specified.	Prevention of new cases (active lesions). Feet lesions changing from inactive to active after 2 or 8 wk.	No CT (within-cow control: split-footbath)	Not specified	Referent	None
			Footbath: calcium hydroxide, 3 times a week for the first 2 wk; then twice a week.	Not specified	—	

Continued

Table 2 (Continued). Evidence summary of collective treatments (CT) in the prevention of bovine digital dermatitis (DD) lesions; results are expressed as odds ratio (OR)

Study	Case definition	Success definition	CT strategy and regimen	Outcome proportion[1] (event/no.)	OR (95% CI)	Additional individual treatment
Relun et al. (2013a)	First occurrence of an active DD lesions (M1 or M2) (Döpfer et al., 1997).	Prevention of the occurrence of DD active lesions (M1 or M2) over lesions considered non-active (M0 or M4).	No CT (untreated control)	0.82 (1,575/1,917)[3]	Referent	Only in ulcerative lesions
			Footbath: copper and zinc chelates 5%, 4 consecutive milkings every 4 wk	0.78 (1,125/1,434)[3]	1.26 (1.06 to 1.50)	
			Footbath: copper and zinc chelates 5%, 4 consecutive milkings every 2 wk	0.87 (931/1,063)[3]	0.65 (0.52 to 0.81)	
			Spray: copper and zinc chelates 50%, 2 milkings every 2 wk	0.86 (1,019/1,184)[3]	0.74 (0.60 to 0.91)	
Fieldaas et al. (2014)	Interdigital dermatitis and DD were recorded together as DD. Lesions were graded and recorded as not present (0), mild (1), moderate (2), or severe (3) (Sogstad et al., 2005).	Prevention of the occurrence of DD lesions (graded 1, 2, or 3).	No collective treatment (untreated control)	0.33 (3/9)[4]	Referent	Only before the start of the first trial
			Footbath: water after every milking (2 milkings/d)	0.77 (7/9)[4]	0.14 (0.01 to 1.16)	
			No collective treatment (untreated control)	0.77 (7/9)[4]	Referent	
			Footbath: copper sulfate 7% solution, 2 milkings day, every 2 wk	0.88 (8/9)[4]	0.43 (0.03 to 5.92)	
			No collective treatment (untreated control)	0.50 (2/4)[4]	Referent	
			Water flushing, after every milking (2 milkings/d)	0.57 (4/7)[4]	0.75 (0.06 to 8.83)	
			No collective treatment (untreated control)	0.81 (9/11)[4]	Referent	
			Water and glutaraldehyde flushing, after every milking (2 milkings/d)	0.87 (7/8)[4]	0.64 (0.04 to 8.61)	

[1]Outcome proportions: occurrence rates of DD lesions in each comparison group.
[2]Studies where the observations were considered independent.
[3]The outcome units were feet.
[4]The outcome units were cows.

Table 3. Evidence summary of collective treatments (CT) in the treatment of bovine digital dermatitis (DD) lesions; results are expressed in odds ratio (OR)

Study	Case definition	Success definition	CT strategy and regimen	Outcome proportions[1] (event/no.)	OR (95% CI)	Additional individual treatment
Britt and McClure (1998)	"More red" indicates continued lesion growth, "same" indicates no change in lesion color, "darker" indicates cessation of lesion growth, "new skin" indicates healing, and "no lesion" indicates completely healed.	Color change, to "darker," "new skin" and/or "no lesion" formation was considered the indicator of regression of the lesion.	Spray: distilled water daily for 21 d Spray: peroxyacetic acid 5.8% and hydrogen peroxide 27.5% undiluted solution daily for 21 d Spray: peroxyacetic acid 5.8% and hydrogen peroxide 27.5% 1:25 diluted solution daily for 21 d Spray: peroxyacetic acid 5.8% and hydrogen peroxide 27.5% 1:12 diluted solution daily for 21 d	0.71 (10/14)[2] 0.2 (1/5)[2] 0.46 (7/15)[2] 0.7 (14/20)[2]	Referent 0.10 (0 to 1.19) 0.35 (0.07 to 1.63) 0.93 (0.20 to 4.19)	None
Hernandez et al. (1999)	Lesions scores ranged from 0 to 2 (0 = no visible lesion; 1 = lesions <2.5 cm in diameter; 2 = lesion >2.5 cm in diameter)	Evolution of visible lesions (1–2) to score 0.	Spray: water[3] Spray: oxytetracycline solution[3] Spray: commercial formulation of soluble copper, peroxide compound, and a cationic agent solution[3] Spray: 5% copper sulfate solution[3] Spray: acidified ionized copper solution[3] Spray: hydrogen peroxide peroxyacetic acid solution[3]	0.20 (2/10)[2] 0.63 (07/11)[2] 0.78 (11/14)[2] 0.20 (2/10)[2] 0.09 (1/11)[2] 0 (0/10)[2]	Referent 7 (0.96 to 50.56) 14.66 (1.96 to 109.20) 1 (0.11 to 9.94) 0.40 (0.03 to 5.24) —	None
Manske et al. (2002)[4]	Active lesions (degrees 1 to 3) (Manske et al., 2002).	Evolution in feet lesions previously scored 1–3 (active lesions) to scores 0, 4, or 5 (non-active lesions).	Footbath: water (within-cow control: split-footbath) Footbath: acidified ionized copper 0.6% solution, 2 milkings day, for 10 d, with 5.5 d in between No CT (untreated control)	0.52 (12/23)[2] 0.83 (20/24)[2] Not specified	Referent 4.58 (1.18 to 17.67) Referent	None None
Fiedler (2004)	Not specified	Not specified	Foam-system: tensides and paracetic acid, 2 milkings/d for 1 wk every 2 wk for 8 wk; then 2 milkings/d for 3 consecutive days with 11 d between for 4 wk	Not specified	2.7 (0.99 to 7.35)[5]	
Ismael et al. (2005)	Not specified. Feet lesions were scored between 0 (absence of lesion) and 3, based on the lesion size, pain score, appearance and lameness.	Reduction on the lesion score of lesions. Degree not specified.	No collective treatment (untreated control) Spray: oxytetracycline solution, 3 times a week Spray: NaCl 20%, 3 times a week	Not specified Not specified Not specified	Referent — —	None
Bergsten et al. (2006)	Lesions were scored from 0 for no lesion to 2 for a severe lesion.	Improvement in feet with a score >0 at the beginning of the experiment.	Footbath: water (within-cow control: split-footbath) Footbath: $CuSO_4$ 7%, after every milking (2 milkings/d) No CT Foam-system: tensides and paracetic acid, 2 milkings/d	Not specified Not specified Not specified Not specified	Referent — Referent —	None
Thomsen et al. (2008)	Active lesions (degrees 1 to 4) (Manske et al., 2002).	Evolution in feet lesions previously scored 1–4 (active lesions) to scores 0 or 5 (non-active lesions).	No CT (within-cow control: split-footbath) Footbath: glutaraldehyde 1.5%, organic acids 2.1% and quaternary ammonium compounds 2%; 2 consecutive milkings, twice a week.	0.35 (28/79)[6] 0.26 (21/79)[6]	Referent 0.65 (0.33 to 1.30)	None

Continued

Table 3 (Continued). Evidence summary of collective treatments (CT) in the treatment of bovine digital dermatitis (DD) lesions; results are expressed in odds ratio (OR)

Study	Case definition	Success definition	CT strategy and regimen	Outcome proportions[1] (event/no.)	OR (95% CI)	Additional individual treatment
Speijers et al. (2010)	Cows with early or acute classical ulcerative DD lesions (M1 or M2) on at least 1 hind foot were defined as cows having active DD lesions (Döpfer et al., 1997).	Positive evolution of DD lesions. "Transition grade" (1) was assigned based on whether the DD lesions improved or (0) if deteriorated or did not improve from week to week.	No CT (untreated control)	0.10 (4/39)[2]	Referent	Only in ulcerative lesions
			Footbath: $CuSO_4$ 5%, 4 consecutive milkings every week	0.35 (13/37)[2]	4.73 (1.37 to 16.29)	
			Footbath: ClO 2% hypochlorite sodium, 4 consecutive milkings every week	0.13 (5/37)[2]	1.36 (0.33 to 5.54)	
Rasmussen et al. 2011)	Distinction between active and inactive lesions was not specified.	The change from active at first registration to inactive after 2 and 8 wk.	No CT (within-cow control: split-footbath)	Not specified	Referent	None
			Footbath: calcium hydroxide, 3 times a week for the first 2 wk and then only twice a week.	Not specified	—	
Relun et al. (2012)	Active DD lesions were defined as an early or acute DD stage (M1 or M2) on a hind foot (Döpfer et al., 1997).	Evolution of foot active lesions (M1 or M2) to non-active lesions (M0 or M4), during at least 2 consecutive visits.	No CT (untreated control)	0.84 (250/296)[6]	Referent	Only in ulcerative lesions
			Footbath: copper and zinc chelates 5%, 4 consecutive milkings every 4 wk	0.79 (200/252)[6]	0.70 (0.45 to 1.09)	
			Footbath: copper and zinc chelates 5%, 4 consecutive milkings every 2 wk	0.94 (186/197)[6]	3.11 (1.56 to 6.17)	
			Spray: copper and zinc chelates 50%, 2 milkings every 2 wk	0.95 (268/281)[6]	3.79 (2 to 7.18)	
Fjeldaas et al. (2014)	Interdigital dermatitis and DD were recorded together as DD. Lesions were graded and recorded as not present (0), mild (1), moderate (2), or severe (3) (Sogstad et al., 2005).	Positive evolution in the grade of disease of infected cows.	No CT (untreated control)	0.80 (8/10)[2]	Referent	Only before begin the trial
			Footbath: water, after every milking (2 milkings/d	1 (11/11)[2]	—	
			No CT (untreated control)	0.66 (6/9)[2]	Referent	None
			Footbath: copper sulfate 7% solution, 2 milkings/d, every 2 wk	0.69 (9/13)[2]	1.12 (0.18 to 6.93)	
			No CT (untreated control)	0.46 (7/15)[2]	Referent	
			Water flushing, after every milking (2 milkings/d	0.20 (3/15)[2]	0.28 (0.05 to 1.44)	
			No CT (untreated control)	0.50 (6/12)[2]	Referent	
			Water and glutaraldehyde flushing, after every milking (2 milkings/d	0.40 (6/15)[2]	0.66 (0.14 to 3.08)	

[1]Studies where the observations were considered independent.
[2]Once daily for 5 consecutive days, not treated for 2 d, and then treated once daily every other day.
[3]Outcome proportions: healing rates of DD lesions in each comparison group.
[4]The outcome units were feet.
[5]The outcome units were cows.
[6]Calculated from the direct conversion of a relative risk of 2.7 without any information about the number of outcome events.

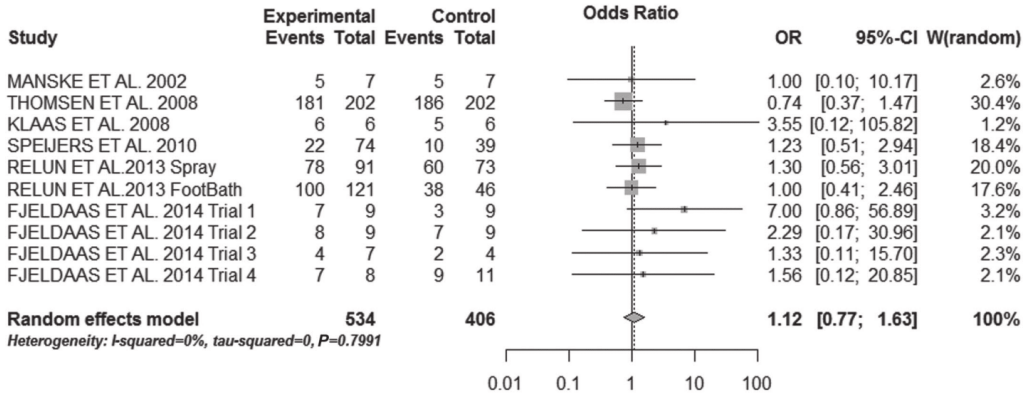

Figure 3. Meta-analyses forest plot of odds ratios (OR) and 95% CI for 10 trials (comparisons) investigating the effectiveness of collective treatments (CT), compared with no treatment or the use of a placebo, in reducing the occurrence of digital dermatitis (DD) lesions. Point estimates and 95% CI for each study are presented on each line. Relative weighting [W (random)] of each study is represented by the gray box surrounding the point estimate. Combined effect estimates (diamond) are presented at the bottom of the graph. Studies are listed chronologically by first author's last name and year only.

system (2), footmat (1), and automatic flushing (1). Only one study reported adverse events related to the collective administration of calcium hydroxide (Rasmussen et al., 2011). For the prevention of DD lesions, the OR ranged from 0.14 to 1.31. For the healing of DD lesions, the OR ranged from 0.10 to 14.66. In only 3 of the 18 prevention comparisons the null value was not contained within the OR 95% CI, whereas for the treatment outcome, in 5 of the 24 comparisons, the null value was not contained in the calculations.

Synthesis of Results

Prevention Outcome. For the 10 prevention comparisons included in the synthesis, the summary OR was 1.12 (95% CI 0.76 to 1.62; $P = 0.56$), suggesting that the uncertainty of the CT effect extends from no prevention to prevention (Figure 3). The visual appraisal of the L'Abbé plot suggests the absence of heterogeneity, with most of the plots displayed close or over the equality line, and a cluster formation on the extremes of the y- and x-axes (Figure 4A). The heterogeneity measures were consistent with the graphical findings [Cochran's Q test ($P = 0.79$); $I^2 = 0\%$, 95% CI: 0 to 37.2%; and $\tau^2 = 0$]. The calculated 95% PI ranged from 0.72 to 1.74. Subgroup analyses did not demonstrate any differences in the prevention effect (Table 4).

The funnel plot was slightly asymmetrical (Figure 4B), and suggested that larger trials were more likely to report effects closer to the null value (no effect). These findings were likewise confirmed by the Harbord's test ($P = 0.04$).

Treatment Outcome. For the 11 treatment comparisons included in the synthesis (Figure 5), the summary OR was 1.22 (95% CI: 0.73 to 2.01; $P = 0.44$). The L'Abbé plot displayed a dispersed pattern indicative of considerable heterogeneity (Figure 6A). The heterogeneity assessments suggested a small degree of heterogeneity between the included studies (Cochran's Q test, $P = 0.20$; $I^2 = 25.3\%$, 95% CI: 0 to 63%; $\tau^2 = 0.1779$). The calculated 95% PI ranged from 0.39 to 3.73. Subgroup analysis by study design (subgroup Cochran's Q test, $P = 0.02$) suggested a qualitative interaction in favor of NRCS design (OR = 1.99; 95% CI: 1.08 to 3.66). Likewise, subgroup analysis by follow-up assessment (subgroup Cochran's Q test, $P = 0.04$) suggested a qualitative interaction in favor of multiple-outcomes assessments (OR = 1.95; 95% CI: 1.03 to 3.68). The remaining subgroups assessed showed no association with the healing effect (Table 4).

The funnel plot was slightly asymmetrical (Figure 6B), suggesting possible publication bias. However, these findings were not confirmed by Harbord's test ($P = 0.55$).

Taken together, for the prevention and treatment outcomes, the heterogeneity assessments revealed an uncertain degree of inconsistency across the included evidence. Although the summary effect and the heterogeneity findings cannot be interpreted, they are presented as valuable information for the reader.

DISCUSSION

This systematic review summarized the body of current literature describing the effectiveness of CT in the treatment and prevention of DD lesions in dairy cattle. The evidence was supported by studies considered to be mostly at low and unclear risk of bias. The review results indicated a low degree of heterogeneity across the evidence for the prevention outcome. Nevertheless, for the treatment outcome, the considerable degree of heterogeneity across the evidence suggested the presence of inconsistency, indicating that the summary effect calculation is not sensible. Likewise, imprecision was suspected due to the frequent small samples sizes of trials and the fact that for most of the studies, when evaluating the prevention or the treatment outcome, the 95% CI were wide and included the null effect. Additionally, possible publication bias was considered for the treatment and prevention outcomes. The overall quality of the evidence for both outcomes (prevention and treatment) was therefore considered to be low, indicating that the true effect of CT may be substantially different from the summary effect estimated by the meta-analysis.

The broad literature search conducted in this review, including gray literature sources and the main databases of veterinary and animal science journals (Grindlay et al., 2012), led to a spectrum of available literature, reducing the selection bias in the review process. Gray literature sources are important to consider, especially in veterinary science, where a large part of the research is reported only through conference proceedings (Brace et al., 2010). However, manual searches were time consuming, the publications obtained for this review were mostly unclear or not sufficiently detailed for their data abstraction, and contacting the authors was difficult or unfeasible in some cases. Finally, and regardless of search strategy efforts, only a few studies were included in the summary and synthesis because of the limited number of interventional studies and clinical trials including an untreated or placebo control comparison group in their design. These findings coincide with the search results from a previous descriptive review study on the treatment and prevention of cattle lameness (Potterton et al., 2012), where the number of intervention studies and clinical trials was low and most of the papers on prevention were observational and analytic epidemiologic studies. The screening process targeted only trials evaluating the incidence and the healing of DD lesions, resulting in the non-inclusion of prevalence studies, which are possible sources of evidence in favor of CT usage. Nevertheless, because exposure and disease status are measured at the same time point in cross-sectional studies, it may not always be possible to distinguish whether the intervention preceded or followed the appearance of DD lesions, and thus the relationship between cause and effect remains unclear or is concealed among the possible effects of the risk factors associated with the disease.

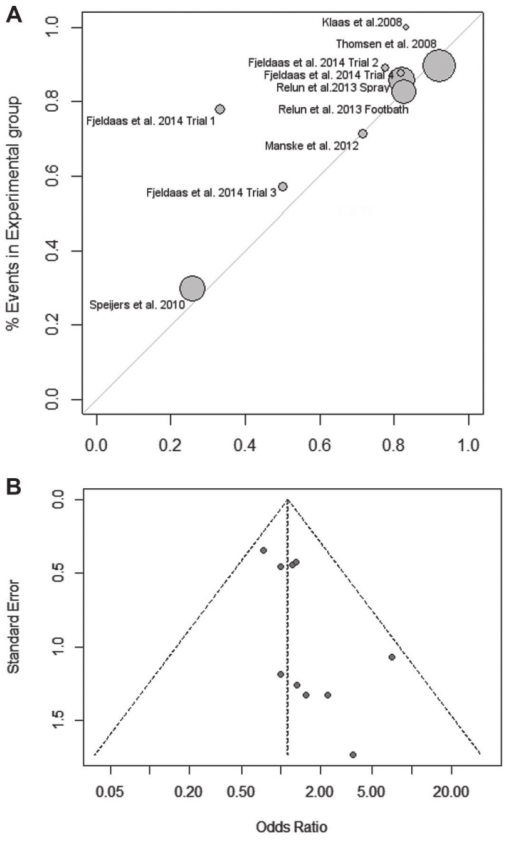

Figure 4. Heterogeneity assessments of the prevention outcome: (A) L'Abbé plot for trials evaluating the prevention of bovine digital dermatitis (DD) lesions; trials were plotted according to the beneficial superiority on the comparison of event proportions between the collective treatments (CT) group and the control group, with point size being proportional to the size of the trial; (B) funnel plot of the summary odds ratio (vertical dashed line) of studies involved in the prevention of DD lesions. Each trial is represented by a gray circle.

The main limitation of this meta-analysis, as with any other synthesis, was the differences across studies in terms of dairy cow populations, CT regimens, and outcomes definitions. Nonetheless, a comparable methodology for the outcome measure was used in the stud-

Table 4. Subgroup analysis assessing the effectiveness of collective treatments (CT) in the prevention and treatment of bovine digital dermatitis (DD) lesions

Subgroup[1]	Number of trials	Odds ratio	95% CI	*P*-value	Heterogeneity[2]		
					I^2 (%)	Between groups (*P*-value)	Within groups (*P*-value)
Preventive outcome							
Study design							
NRCS	5	1.19	0.73 to 1.93	0.47	0	0.67	0.73
RCT	5	1.01	0.55 to 1.83	0.96	13.2		
Initial prevalence							
Low (<30%)	4	0.96	0.61 to 1.52	0.88	0	0.26	0.84
High (>30%)	6	1.54	0.78 to 3.03	0.20	0		
Length of the study							
More than 8 wk	7	1.34	0.79 to 2.29	0.27	0	0.33	0.81
Up to 8 wk	3	0.92	0.54 to 1.57	0.78	0		
Follow-up assessments							
Only before and after outcome observations	7	1.04	0.59 to 1.84	0.87	0	0.76	0.72
Multiple outcome observations	3	1.17	0.71 to 1.94	0.52	0		
Study limitations							
Low risk of bias	3	0.94	0.59 to 1.49	0.81	0	0.20	0.87
Unclear/high risk of bias	7	1.59	0.81 to 3.09	0.16	0		
Treatment outcome							
Study design							
NRCS	4	1.99	1.08 to 3.66	0.02	0.4	0.02	0.48
RCT	7	0.77	0.43 to 1.38	0.48	0		
Initial prevalence							
Low (<30%)	3	1.27	0.54 to 2.98	0.56	55	0.14	0.88
High (>30%)	8	1.18	0.58 to 2.39	0.64	21.8		
Length of the study							
More than 8 wk	7	1.22	0.59 to 2.53	0.57	27.8	0.98	0.14
Up to 8 wk	4	1.21	0.53 to 2.74	0.64	41		
Follow-up assessments							
Only before and after outcome observations	8	0.81	0.45 to 1.44	0.48	0	0.04	0.41
Multiple outcomes observations	3	1.95	1.03 to 3.68	0.04	29.6		
Study limitations							
Low risk of bias	5	1.22	0.60 to 2.45	0.57	34.6	1.00	0.14
Unclear/high risk of bias	6	1.22	0.52 to 2.84	0.64	31.2		

[1]NRCS = nonrandomized controlled study; RCT = randomized controlled trial.
[2]I^2 = statistic that describes the proportion of total variation in study effect estimates that is due to heterogeneity.

ies included, and therefore we considered that the data across the studies could be combined to estimate CT effectiveness with more precision than in a single study. In the context of this review, the number of follow-up assessments and the length of the follow-up periods were considered to have an important influence on the precision of the effect estimate, especially given that the reported median time before the occurrence of a new DD lesion is 5 mo (Relun et al., 2013a; Krull et al., 2016), and that DD lesions can be completely healed within 1 mo (Holzhauer et al., 2008). The subgroup analyses that investigated the importance of these factors suggested a qualitative interaction in favor of studies using multiple-outcomes observations and for NRCS designs to evaluate the treatment outcome. However, the assessments of the subgroup analyses were limited by the small number of studies. Likewise, as different lesion scoring methodologies were used across studies, the case and success definitions were different. Therefore, to harmonize the results of future clinical trials, it is crucial to homogenize the classification system of lesions to set uniform objectives for control strategies. Independent of the method used to score DD lesions, chronic non-ulcerative lesions might be considered non-active stages and consequently a successful target stage. However, it is unknown whether these lesions are truly healed (Döpfer et al., 2012) or to what degree they represent a risk factor stage for the relapse of ulcerative lesions and spread of the disease.

The uncertain extension and degree of heterogeneity across studies guide us to approach the meta-analysis using a random-effects model. The frequent small size of the trials, probably due to practical, ethical, and financial reasons, represents a large part of the imprecision evidenced across the studies. Nevertheless, in contrast to what would be expected based on the low number of studies included and their small size, the confidence intervals for the summary effect estimates were relatively

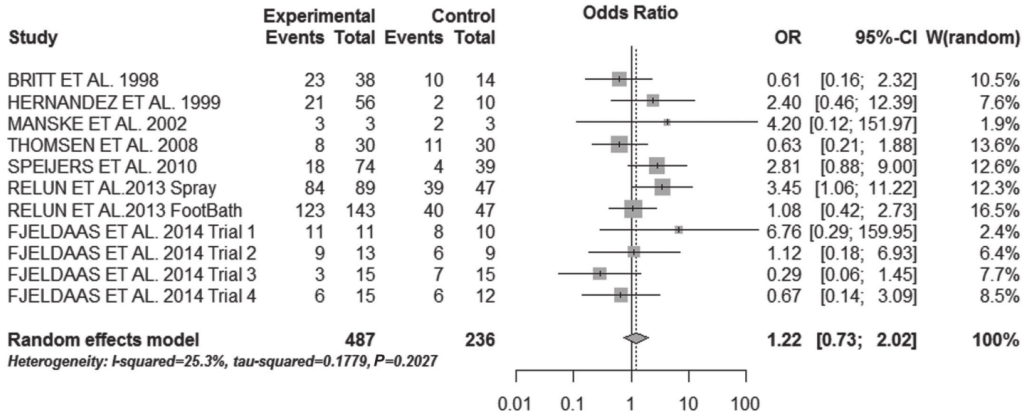

Figure 5. Meta-analyses forest plot of odds ratios (OR) and 95% CI for 11 trials (comparisons) investigating the effectiveness of collective treatments (CT), compared with no treatment or the use of a placebo, in healing digital dermatitis (DD) lesions. Point estimates and 95% CI for each study are presented on each line. Relative weighting [W (random)] of each study is represented by the gray box surrounding the point estimate. Combined effect estimates (diamond) are presented at the bottom of the graph. Studies are listed chronologically by first author's last name and year only.

narrow. An alternative to reduce sample sizes would be trials designed for paired within-cow comparisons. However, depending on the statistical methods used, these designs could entail some problems related to disease prevalence and the fact that both legs (treatment and control) must share the same lesion status, a requirement that leads to possible important losses of statistical power, or otherwise, to biased interpretations in the cases where the analysis ignores the dependence between the legs of the same cow. Performance and detection bias related to blinding were limited across the studies by the objective measure of lesion evolution and, in some cases, when co-interventions were adjusted for in the analyses.

Another limitation encountered in the synthesis process was related to water treatments used in placebo control groups that might have induced a beneficial effect on DD lesions by controlling feet hygiene, a risk factor associated with the spread of DD. This limitation leads to final interpretation bias. The correlation between the healing and the occurrence of DD lesions could entail some issues for studies that evaluate prevention and treatment outcomes in parallel. However, in such studies, the degree to which the CT effects could be over- or underestimated is uncertain because of the contagious dynamics of the disease. Across the studies included in the synthesis, some risks of carryover bias were evidenced, mostly because washout periods between trials were not feasible, probably for financial and practical reasons. The frequent attrition bias evidenced in some of the studies was the result of unclear methodologies for dealing with missing data or when imbalances generated by exclusions were not reported.

Different limitations were associated with the low number of studies included in the quantitative synthesis and, consequently, the insufficient statistical power for heterogeneity and publication bias tests. Nevertheless, the strategy implemented to assess heterogeneity across the evidence was to integrate visual and statistical methodologies to allow an integral approach to the evidence and avoid possible problems related to the small number of studies and statistical power. Therefore, even if statistical heterogeneity was barely evidenced, the L'Abbé plot allowed a broad heterogeneity assessment. Likewise, the calculated 95% PI for the treatment outcome was wider than the 95% CI, suggesting the presence of heterogeneity in the sample. Because of statistical considerations, publication bias was difficult to evaluate and cannot be excluded. In particular, the absence of intervention effect on both outcomes renders the appreciation of asymmetry difficult. Although the low number of publications may be explained by the difficulties in conducting effectiveness trials in veterinary science for ethical and economic reasons, most of the DD studies are sponsored by private funding, and possible negative results could remain unpublished, as in the case of human medical sciences (Hopewell et al., 2009).

The lack of scientific evidence supporting the effectiveness of CT found in this paper is in agreement with

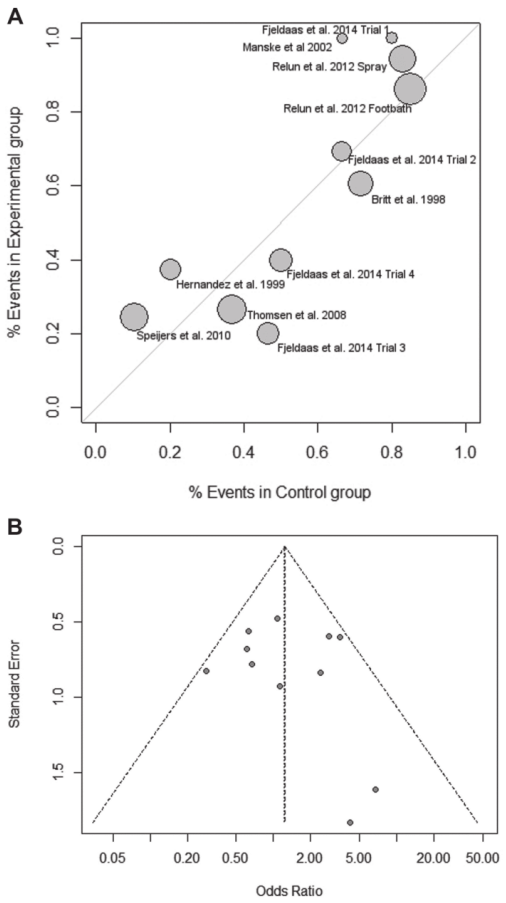

Figure 6. Heterogeneity assessments of the treatment outcome: (A) L'Abbé plot for trials evaluating the treatment of bovine digital dermatitis (DD) lesions; trials were plotted according to the beneficial superiority on the comparison of event proportions between the collective treatments (CT) group and the control group, with point size being proportional to the size of the trial; (B) funnel plots of the summary odds ratio (vertical dashed line) of studies involved in the treatment of DD lesion. Each trial is represented by a gray circle.

the conclusions of a previous review paper (Laven and Logue, 2006). Our findings highlight the constraints faced by collective intervention trials and point to the need for research into the development and design of high-quality protocols to evaluate the effectiveness of collective interventions. Based on our findings, we propose that future protocols for the assessment of the effectiveness of CT in the prevention or treatment of DD lesions must include the following key elements: (1) reduction of confounding and selection bias through randomization or other comparable methods; (2) negative untreated controls to compare with the experimental treatment (avoid placebo water controls); (3) an objective measure of DD lesion evolution, clearly describing case and success definitions, for the outcomes assessments; (4) multiple observations by trained assessors at intervals no longer than 1 mo within the follow-up period; (5) longer follow-up periods of at least 5 mo; (6) sample sizes determined for statistical power; and (7) co-interventions or other confounding variables (e.g., individual treatment of active lesions) that are adjusted for in the analysis.

CONCLUSIONS

Practitioners, animal health advisors, farmers, and the veterinary health industry must be informed that the preventive and treatment effectiveness of CT remains uncertain, and the epidemiological circumstances in which they can be useful must be further investigated. This systematic review and meta-analyses demonstrated that the number of studies was small and the quality of the evidence was low. A standardized protocol and high-quality clinical trials are urgently needed to investigate the effectiveness of CT in the treatment and prevention of DD lesions.

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B. Protocol of a systematic review and meta-analysis on the "Effectiveness of collective treatments in the prevention and treatment of bovine digital dermatitis lesions"

J. Dairy Sci. 100:7401–7418
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Supplemental File S1

Protocol of a systematic review and meta-analysis on the "Effectiveness of collective treatments in the prevention and treatment of bovine digital dermatitis lesions".

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Collective treatments (CT) currently are advised as part of the control strategies for Bovine Digital Dermatitis (DD). However, the effectiveness of these CT and the optimal conditions for their use seems unclear. Therefore, the aim of this study is to evaluate and summarize the evidence about the effectiveness of CT in the prevention and treatment of DD clinical lesions. We will perform a systematic review to identify and evaluate the scientific studies supporting the use of CT. A systematic literature search will be conducted in the specialized grey literature sources, and in the PubMed, CAB Abstracts and Web of Science databases. We will describe the general information of the studies with regard to populations, interventions, outcomes, comparisons and study designs. The results of each study will be summarized in numbers needed to treat (NNT). Additionally, if appropriate, a meta-analysis will be conducted to determine the effectiveness of the CT. The heterogeneity and inconsistency across the included evidence will be assessed by the graphical appraisal of the L'Abbé plot and the calculation of the Cochrane's Q and the Higgins (I2) statistical tests. Risk of bias for each study will be assessed and the overall quality of the evidence will be summarized.

The results of this systematic review will provide a synthesis of the evidence concerning CT for the prevention and treatment of DD clinical lesions. The main limitations in the design of effectiveness trials for DD control will be identified to provide new insights into the conception of high quality trials to support DD control strategies.

MATERIALS AND METHODS

Formulation of the clinical question

The study population of interest will be dairy cows in lactation. The interventions will be CT, defined as the implementation of a same treatment at a given time to two or more animals. The comparisons will be animals collectively treated with a placebo (water) or which received no CT. The outcomes of interest for respectively

J. Dairy Sci. 100:7401–7418
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prevention and treatment will be the occurrence and persistence of DD lesions.

Clinical question

In dairy cows, are collective treatments more effective at reducing the occurrence and persistence of clinical DD lesions compared to a collective placebo treatment or the absence of any collective treatment?

Evidentiary search strategy

The PubMed, CAB and Web of Science (Core collection) databases will be searched between July 2015 and March 2016. The research will be restricted to papers written in English, French, German, Portuguese and Spanish languages, and published between the year 1974 (first official description of DD) and 2016.

Medical Subject Headings (MeSH) will be identified for the PubMed database searches. Additionally, a manual search of the grey literature will be performed on the principal proceedings of the subject, World Buiatrics Congress 2002-2014, International Conference on Lameness in Ruminants 2002-2013, Cattle Lameness Conference 2009-2015, European Buiatrics Forum 2009-2013, the "Journées 3R" (Rencontres autour des recherches sur les ruminants) and the British Society of Animal Science Conference 1999-2015.

Study selection process

For the citations identified in the search process, their title and abstract will be screened for relevance by the principal author. For the relevant publications identified, their title, abstract and materials and methods will be verified for eligibility by two of the authors through a screening tool designed for this systematic review. Studies will be eligible for the synthesis if all 4 of the following questions receive a positive answer:

1. Does the study describe a primary research study?
2. Does the study evaluate CT in dairy herds?
3. Does the study include the occurrence and/or persistence of DD lesions as an outcome?
4. Does the study include an untreated or a placebo control group against the intervention?

In case of discrepancy between the two authors concerned, a third reviewer will resolve the conflict.

Collection data from relevant studies

The information considered as relevant to extract for this review was determined by the research team with the advice and supervision of a statistician. Information will be extracted by the principal author, in cases where the

J. Dairy Sci. 100:7401–7418
https://doi.org/10.3168/jds.2016-11875

study data seemed confused or inconsistent, the assistance of the review team will be request.

The relevant information of each study will be extracted at 5 levels. The publication level includes author information, citation details, year of publication and publication source (i.e., databases or grey literature). The population level includes data relative to the breed and lactation stage of the cows, the housing and milking system and the initial prevalence of the disease in the herd. At the intervention level, information will be extracted about the intervention products used in the experimental and comparison groups, the type of intervention employed (i.e., footbath, split footbath, foam system, collective spraying), the doses and frequencies of administration and, when appropriate, the concomitant individual treatments used. The data extraction at the outcomes level will include the information about the measure of the outcomes, as the outcomes rates recorded in a 2x2 contingency tables, the outcomes measure frequencies and follow-up lengths, the diagnostic methodologies employed and the "outcome unit" assessed (foot, cow). Finally, at the study design level, information will be extracted about the randomization efforts, the blinding of care-givers and observers, the statistical methods employed for the analyses of the outcomes, the handling of missing data, and the funding sources of the study. The authors will be contacted in cases where some information is unavailable in the publication paper.

Outcomes assessment

The primary aim of this study will be to evaluate the effectiveness of CT in the treatment and prevention of DD clinical lesions. Therefore, treatment and prevention outcomes will be assessed separately. The treatment outcome will be considered as the positive effect of an intervention on the reduction in the persistence and/or relapse of DD lesions within the follow-up period of the experiment. The prevention outcome was defined as the positive effect of an intervention to limit or delay the occurrence of new DD lesions within the follow-up period of the experiment. Case and success definitions of DD clinical lesions will be identified for each study according to the treatment and prevention outcomes measured.

Risk of bias assessment

To assess the methodological quality of the studies included, an adapted tool (Higgins et al., 2011) designed to examine in a systematic manner the risk of bias will be used. The tool will evaluate 4 domains inside each study. The first domain is relative to selection bias, specifically the randomization procedures. The second domain, confounding bias, includes the blinding of the outcome observer. The third domain approaches the handling of incomplete outcomes data. The fourth domain considers the impact of missing information in the publication paper, the use of non-validated outcome measures and the possible carryover effects present in the trials.

J. Dairy Sci. 100:7401–7418
https://doi.org/10.3168/jds.2016-11875

Authors will evaluate each publication assigning a judgment of high, low or unclear risk of bias for each risk domain. Discrepancies among the reviewers will be solved by discussion. A study will be considered a "low risk of bias" when most of the domains' criteria were considered to be at a low risk level. Studies where all 4 domains were considered between "low or unclear risk of bias" will be awarded a "moderate risk of bias". Finally, for studies where "high risk of bias" in one or more domains was determined, a general "high risk of bias" will be awarded.

Quantitative data synthesis

A formal statistical combination of the data extracted from the studies will be performed if the review team considers that the definitions in the diagnosis and the clinical evolution of DD clinical lesions are comparable between the different studies. Otherwise, if the data are unsuitable for a quantitative synthesis, a descriptive review to summarize the evidence will be performed.

The data for statistical analysis will be extracted into an Excel file. If suitable, the meta-analyses will be performed using the "meta" package in R (Schwarzer, 2015; R Core Team, 2015). For each trial and outcome evaluated (prevention and/or treatment), the 2x2 contingency tables formed from the event rates reported on each comparison group will be used to compute the study effect sizes in Odds Ratios (OR) and their 95% CI.

Heterogeneity among studies will be assessed visually using the L'Abbé plot (L'Abbe et al., 1987), and objectively by calculating the Cochran's Q test and the Higgins statistic (I2) and its 95% CI (Higgins and Thompson, 2002). Additionally, to address potential heterogeneity and inconsistency across trials, we will perform a subgroup analysis. This will include "study design" (Randomized controlled trials vs. Any other designs), "initial prevalence" (High prevalence > 15% vs. Low prevalence < 15%), "length of the study" (More than 12 weeks vs. Up to 12 weeks) and "follow-up assessments" (Before and after vs. Multiple assessments). The possibility of publication bias will be investigated using funnel plots.

The overall quality of the evidence will be assessed from the review findings and the judgments made about the risk of bias, the indirectness, the inconsistency, the imprecision and the publication bias across the studies.

This systematic review is expected to provide practitioners, animal health advisors, farmers and the veterinary health industry an interpretable evidence summary about the effectiveness of CT. The farmers and their advisors could implement better strategies to control DD according to the particular conditions of their herds. Moreover,

J. Dairy Sci. 100:7401–7418
https://doi.org/10.3168/jds.2016-11875

the findings of this review might help researchers in the conception of high quality effectiveness trials to support new control strategies against the disease.

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J. Dairy Sci. 100:7401–7418
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Supplementary Table S2. Data extracted from publications investigating the effectiveness of collective treatments (CT) in the treatment and prevention of bovine digital dermatitis (DD) during a systematic review.

Level I – Publication
First author
Citation details
Year of publication
Publication origin (Indexed or grey literature)
Level II – Population
Country
Number of cows enrolled
Number of farms enrolled
Breed
DD Initial prevalence
Access to pastures during trial
Housing system
Milking system
Parity
Lactation stage (DIM)
Level III - Intervention and Comparison groups
CT, doses and frequencies
Type of intervention evaluated
Length of administration
Concomitant individual treatments used
Level IV – Outcomes
Outcome measured
Number of events in control and intervention groups
Case definition
Success definition
Method to measure the outcome
Follow-up length
Outcome unit
Level V – Study Design
Randomization efforts
Blinding of the outcomes
Loss to follow-up information
Type of study
Funding sources

Chapter 3. Preliminary studies to determine the renewal rate of the biocide.

A. Current recommendations for footbath renewal rates: The need for adaptation?

Submitted to:

Animal: An International Journal of Animal Bioscience
06 October 2017.

Chapter 3. Preliminary studies to determine the renewal rate of the biocide.

Description de la contamination des pédiluves par les matières organiques en conditions d'élevage bovin laitier

Assessment of footbath contamination by dairy cattle organic matter under field conditions

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INTRODUCTION

Parmi les mesures de maîtrise médicales des boiteries infectieuses des ruminants, les pédiluves contenant un désinfectant représentent une alternative permettant de traiter au même moment un nombre important d'animaux. Mais les conditions de mise en œuvre de ces pédiluves sont cruciales. En effet, les substances actives utilisées peuvent être exposées à différentes quantités de matière organiques (MOs) et de conditions physico-chimiques qui pourraient altérer leur activité bactéricide. Ainsi, en pratique, une fréquence de renouvellement des pédiluves tous les 150 à 200 passages est préconisée (Holzhauer et al., 2004), mais sans preuve majeure dans la littérature ni prise en compte de la propreté des animaux rentrant dans ce pédiluve. Cette étude visait à déterminer dans des conditions de terrain les variations de volume, température, pH, charge microbienne et quantités de MOs dans des pédiluves après un nombre croissant de passages de vaches.

1. MATERIEL ET METHODES

L'étude a été réalisée dans 5 élevages de l'ouest de la France. Les élevages sélectionnés devaient (i) être en zéro pâturage afin d'éviter le nettoyage naturel des pieds au pâturage, (ii) être en système logettes où la propreté des pieds est attendue plus dégradée et variable qu'en système aire paillée, (iii) avoir au moins 50 vaches, traites deux fois par jour, afin d'homogénéiser la période d'échantillonnage à 24 heures. Une notation de la propreté des pieds de toutes les vaches en lactation permettait d'estimer un degré de propreté globale du troupeau (Guatteo et al., 2013). Un pédiluve rempli d'eau a été placé à la sortie de la salle de traite pendant 24 heures. Les températures ambiante et du pédiluve ont été contrôlées en continu à travers un dispositif de capture embarqué. Après homogénéisation du pédiluve, 3 échantillons (1L/échantillon) ont été prélevés à différents endroits après les passages des vaches (avant premier passage puis toutes les 50 vaches jusqu'à 200), afin d'analyser l'évolution du pH, de la teneur en MOs (Dean, 1974) et de micro-organismes revivifiables (Blumenthal et al., 2000). Le volume d'eau dans le pédiluve a été contrôlé après le passage des vaches. Enfin, le nombre de vaches qui ont déféqué dans les pédiluves a été enregistré afin d'identifier la source de contamination du pédiluve (pieds et/ou fèces).

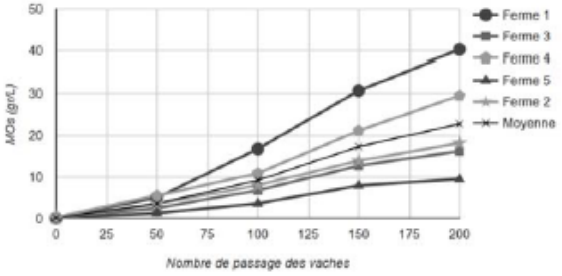

Figure 1 : Evolution de la teneur en MOs selon le nombre de passages de vaches.

2. RESULTATS

Les résultats indiquent que le contenu de MOs augmente principalement, avec le nombre de passages d'animaux, et dans une moindre mesure, avec le nombre de défécations dans le pédiluve. Cette augmentation a été indépendante du degré de propreté globale du troupeau, indiquant que le nombre croissant de passages a influencé la teneur de MOs dans les pédiluves et non l'état de propreté des pieds. Les quantités de MOs ont varié entre 0,056 g/L avant le premier passage jusqu'à 40,3g/L après la dernière mesure (Figure 1). Les quantités de micro-organismes revivifiables après différents nombres des passages varient entre 1,2 x 10^3 et 8 x 10^7 CFU/mL. Pendant le passage des vaches dans le pédiluve, le changement maximal de température à l'intérieur du pédiluve a varié selon les élevages entre 1,3°C à 2°C avec une variation maximale de 5°C par rapport à la température ambiante. Le pH, a varié de façon inconstante (0,71 à 1,85) Ces légères variations, normalement, ne sont pas susceptibles d'impacter les produits désinfectants. Enfin, en moyenne 7% de vaches ont déféqué dans les pédiluves pendant leur passage et le volume d'eau a diminué notablement, de l'ordre de 40 à 50%, après 200 passages (Figure 2).

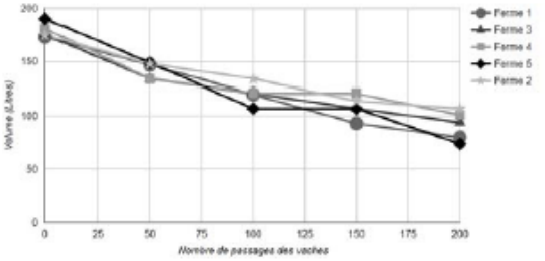

Figure 2 : Evolution du volume total selon le nombre de passages de vaches.

3. DISCUSSION

Réglementairement (EU, 2012), les désinfectants avec une indication bactéricide doivent supporter jusqu'à 20g/L de contamination par des MOs. D'après nos résultats, cette mesure correspond au passage de 100-150 vaches, ce qui correspond au taux de renouvellement historiquement conseillé. A condition toujours, que le volume résiduel du désinfectant dans le pédiluve arrive à couvrir tout le pied. Ainsi, un renouvellement après un nombre supérieur de passages peut altérer l'efficacité des substances désinfectantes contenues dans les pédiluves.

CONCLUSION

Afin de s'approcher des conditions de terrain, de futures études doivent intégrer dans leur design toutes les différents variables physico-chimiques qui peuvent influencer l'efficacité bactéricide des désinfectants.

Les auteurs remercient les éleveurs ainsi que les vétérinaires ayant contribué à la réalisation de cette étude.

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Chapter 3. Preliminary studies to determine the renewal rate of the biocide.

Current recommendations for footbath solutions renewal rates: The need for adaptation?

J.M. Ariza [1,2,a], N. Bareille [1,a], K. Oberle[2,b] and R. Guatteo[1,a]

Abstract

Footbaths represent a potentially useful strategy for the prevention of claw infectious diseases by treating a large number of animals concomitantly. Nevertheless, under field conditions, footbath solutions are exposed to increasing number of animal passages and therefore, to different volume losses and concentrations of manure contamination which could alter their presumed bactericidal activity. Across increasing number of cow passages, the organic matter (**OM**) concentration, the microbial loads, and the residual volumes were assessed in 6 commercial farms. The results indicate that the OM concentration and microbial loads increased linearly with the number of passages of animals, and with the number of defecations in the footbath. No differences between the farm's feet hygiene status and the OM concentration or microbial loads were detected, suggesting that probably the increasing number of cow passages and defecations influenced more the contamination of footbaths than the hygiene of the feet. However, in all the farms the volumes decreased drastically after 200 cow passages (50%). The OM concentrations after 150 and 200 cow passages reached the regulatory concentrations in which disinfectant products should demonstrate to still be effective (20g/L-1), and coincide with the often advised renewal rates. Nevertheless, taken together, these results suggested that beyond the concentration of OM contamination, to ensure the topical action of a footbath treatment, the renewal rates must be mainly adapted according to the footbath remaining volume, as the entire foot should be covered by the footbath solution. The findings of this study indicate the

importance of the footbath designs for the successful implementation of these strategies in practice.

Keywords: Footbath, Dairy cattle, Organic matter, Contamination, Renewal rates.

Implications

Footbaths represent a potentially useful strategy for the prevention of claw infectious diseases. Nevertheless, footbaths solutions are exposed to contamination and losses of volume after the cow passages. Across increasing number of cow passages, the organic matter concentration, the microbial loads, and the residual volumes were recorded in footbaths. The results suggested that beyond the concentration of organic matter contamination, to ensure the action of a footbath treatment, the renewal rates must be mainly adapted according to the footbath remaining volume, as the remaining solution should cover the entire foot. This volume capacity problem seems inherent to the footbaths currently commercialized.

Introduction

In ruminants, claw infectious diseases such as digital dermatitis and footrot, are important conditions associated with lameness, decreased production, and thereby economic and welfare concerns (Clifton and Green, 2016; Bruijnis *et al*., 2012; Relun *et al*., 2013b). A classical strategy for the control of such diseases is the usage of disinfectant footbaths. Footbaths solutions, in theory, limit the spread of infectious diseases by their bactericidal properties and therefore this practice potentially might improve the prevention and healing of foot lesions. However, in practice, footbaths are implemented empirically at different frequencies and renewal rates (renewal of

the entire footbath solution), as a possible consequence of the unclear guidelines for its usage (Relun *et al*., 2013a). Thus, even if footbaths solutions are frequently advised to be renewed every 150 to 200 cow passages, no major evidence in the scientific literature supports this renewal rate or take into account the hygiene of the feet of the animals passing through the footbath. The designs of the commercial baths for footbathing includes diverse dimensions and forms, sometimes largely different from the advised footbath dimensions (Cook et al., 2012). In practice, footbaths solutions are exposed to increasing concentrations of manure contamination. Manure is incorporated into footbaths by animal defecations or carried by the animal feet. Therefore, the organic matter **(OM)** concentrations could hugely differ from farm to farm depending on their management practices and their impact on the feet hygiene. The concentration of OM and the microbial loads contained in manure can alter the bactericidal efficacy of the active compounds of footbath solutions (Hartshorn *et al*., 2013). The European legislation restrains the market of disinfectant products for the veterinary usage (Regulation EU. No. 528/2012). Therefore, these biocide products shall demonstrate their bactericidal efficacy against *Enterococcus hirae, Proteus vulgaris, Pseudomonas aeruginosa and Staphylococcus aureus* after the exposure soil conditions (20g/L of OM) (EN 1656). Nevertheless, the conditions in which these biocides are administered in farms through footbaths are maybe largely distant from the laboratory environments. Therefore, the guidelines for the usage of footbaths solutions are mainly referred to a renewal rate according to a certain number of animal passages. Thus, after a recent European directive, biocides products should confirm their efficacy according to their claimed guidelines (ECHA, 2017). For the case of biocides used in footbaths, the bactericidal efficacy of the solution must be confirmed according to the renewal rates

proposed for its usage. Therefore, the capacity of a biocide solution to support a claimed number of passages should be tested using the proportional concentrations of organic matter related to the number of passages claimed ("capacity test"). Finally, as claw infectious lesions are often located between the lower metatarsus-metacarpus and the digit (Read and Walker, 1998; Angell et al., 2015), footbath solutions should cover the entire foot to allow their bactericidal effect. In practice, it is unclear the degree in which the physical effect of the cow passages could alter the residual volume of the footbath solutions and, therefore their presumed efficacy. The objective of this study was to investigate under field conditions how footbath solutions might be impacted by the increasing number of cows passages, in terms of residual volume, microbial loads and OM concentrations, and thereby how these factors might affect the renewal rates of footbath solutions.

Material and methods

Study population

The study was carried out in 2016, in 6 dairy cattle farms from western France (638 lactating cows). Each farm was visited once during a period when cows were housed without access to pastures to minimize the potential season effect on the feet hygiene. To reduce the stress and therefore the number of defecations produced by the first implementation of footbaths, in all 6 farms routine footbathing was practiced.

The main characteristics of the 6 farms included in the study are presented in Table 1.

Feet hygiene assessments

The feet hygiene (metatarsus and digit (phalanges)) of the lactating cows was assessed by scoring the dirtiness present at both hind feet of each animal during the milking previous to the first footbath sampling, using a 3-point nominal scale, varying from clean (score 1) to dirty (score 3) (Figure 1). For each cow, the higher foot score was retained. The farms were classified according to the overall percentages of feet hygiene score in the following 3 hygienic status: clean (≥ 50% of animals in score 1), fair (≥ 50% of animals in scores 1 and/or 2), and dirty (≥ 50% of animals in score 3).

Footbaths assessments

The footbaths were filled with water sourced from the farm and their initial volume was calculated. In farms with milking parlors (n=5), the footbath was placed at the exit. In farms with automatic milking systems (**AMS**), all the animals were grouped beside the bath before being forced to pass through it all at once, as the usual management. To evaluate the variation in the OM concentration, in each farm the footbath solution was sampled 3 times at 3 different sites (500mL/sample) after every 0, 50, 100, 150 and 200 cow passages, the content of the footbath was homogenized by agitation. Microbial load's measurements were only performed for the samples related to 0, 50, 150 and 200 cow passages. Concomitantly with each sampling, the remaining liquid depth of the footbath was recorded. Likewise, the number and moment (before 0, 50, 100, 150 or 200 cow passages) in which the cows defecated into the footbath were recorded to identify the main source of contamination (foot dirtiness and/or feces). The cow passages were recorded by simple observation. Two investigators participate in the recordings. The first investigator exclusively recorded the number of cow passages and sampled the footbath. The second

recorded the defecations, the pH and the temperature of the footbath. The samples were stored in plastic bottles at 4°C and delivered to the laboratory for further analysis (within 24 hours max.). Depending on the herd size of each farm, a different number of milkings was necessary to reach the 200 cows passages. When footbaths contents stayed more than 12 hours, control samples were taken immediately before and after the stand period to determine the potential impact of the stand time on the OM concentration.

The "Weight Loss-on-Ignition 550°" (Houba et al., 1997) method was used to estimate the OM concentration of footbaths samples after 0, 50, 100, 150 and 200 cow passages. The method consists in drying the samples at 105°C to remove moisture, a procedure that enables the dry matter estimation. Then, the dry sample is heated at 550° C for 2 hours to decompose the OM but not the carbonates. Before ignition the sample contains OM, but after ignition, all that remains is the mineral portion. The OM concentration in the samples was calculated by the difference in the weight before and after ignition, in grams by liter.

Microbial loads were determined by the counting of heterotrophic bacteria through the pour plate method with yeast extract agar at 30°C for 48 h (ISO, 1999). The colonies present in each plate were counted to estimate the number of colony forming units (CFU) present in 1 mL of sample. Plates with >300 cfu, in the highest dilutions used, were considered to have a number of viable bacteria greater than the limit of reliable quantification and were therefore expressed as an approximate value.

Data analysis

According to the data collected in this study, the statistical analyses were performed using R software (R Core Team, 2017). Significance was set at $P < 0.05$. First, descriptive statistics were computed calculating the volume reduction and the average contamination on footbaths, in terms of OM concentration and microbial loads. Second, a non-parametric Kruskal-Wallis rank sum test was used to determine the differences between the hygienic status assigned to the farms and the average OM and microbial contamination. Finally, non-parametric Kendall's Tau-b correlation analyses estimating the strength, direction, and significance of the relations between the increasing number of cow passages and the number of cows that defecated into the footbaths, the residual volume, and the average microbial and OM contamination in the footbaths were conducted. The residual volumes calculated were corrected to account for the liquid removed during the sampling.

Results and Discussion

In this study 3 different milking system were represented, 5 farms had milking parlors (3 herringbones (# 2, 5 and 6), 2 carousels (# 1 and 4)) and 1 (# 3) had an AMS. Excepting the AMS farm, the cows were milked twice a day in the milking parlors farms. The average herd size was 106 cows and therefore the number of milkings to complete 200 cow passages varied between 2 and 3. Only the Farm 5 has less than 100 animals (74 cows). All 6 farms used plastic commercial footbaths, two (# 1 and 6) used split footbaths and the rest of them used conventional footbaths. The hygienic status of the farms was considered as "dirty" in 2 farms (# 1 and 3), as "fair" in 3 farms (# 4, 5 and 6), and as "clean" in only one of the farms (# 2).

Table 1 *Main characteristics of the 6 farms included in the study.*

Farm ID	Milking System	Herd Size	Hygienic status-Overall feet hygiene (%) [1]	Type of Footbath	Footbath Dimensions (cm)[2]
Farm 1	Carousel	107	*Dirty* 1=0 2=17 3=83	Split footbath	228x58x13
Farm 2	Herringbone	120	*Clean* 1=67 2=32 3=1	Conventional	184x73x13
Farm 3	AMS[2]	100	*Dirty* 1=0 2=27 3=73	Conventional	184x73x13
Farm 4	Carousel	118	*Fair* 1=12 2=60 3=28	Conventional	184x73x13
Farm 5	Herringbone	74	*Fair* 1=22 2=51 3=27	Conventional	150x110x12
Farm 6	Herringbone	119	*Fair* 1=28 2=47 3=25	Split footbath	228x58x15

[1] Percentages of animals scored according to the feet hygiene (1=Clean, 2= Fair, and 3=Dirty). The hygienic status given to each farm is indicated in italic letters.

[3] *Dimension (length by width by length)*

[3] *AMS= Automatic milking system*

The OM concentration increased linearly with the number of passages of animals (r_T = 0.78, P < 0.0001) (Figure 2, A), and with the number of defecations in the footbath (r_T = 0.68, P < 0.0001). However, no differences between the farm's feet hygiene status and the OM concentration were detected (*P*= 0.76), suggesting that probably the increasing number of cow passages and defecations influenced more the OM concentration in footbaths than the feet hygiene. The footbaths OM concentration before the cow passages was on average 0.1 g/L (0.1 SD) and 21.2 g/L (11.2 SD) after complete 200 cow passages (Table 2). European policies (European Parliament, 2012) standardize disinfectants with a bactericidal indication in veterinary products which support contamination concentrations up to 20 g/L-1 of OM (Fig 1, A). According to our results, this concentration corresponds to the passage of 150-200 cows, which is the renewal rate often advised in practice. Nevertheless, due to the restricted indoor conditions of this study, the level of contamination found in the footbath samples might largely differ from footbaths administered to grazing herds where the cow feet might be benefited by the natural cleaning effect of the pasture contact. Similarly, the type of facilities, the floor scrapping method, the bedding material used and the cow's diet might influence the cow's hygiene and thereby the contamination of footbaths. Therefore, future studies are needed to investigate the impact of these factors on the footbath contamination. Otherwise, no major differences were recorded in any of the farms when comparing the average OM concentrations before and after a stand time of more than 12 hours (-0.91 g/L (2.06 SD)) (Data not shown). Nevertheless, the number of passages before the stand time, the stand times, and the temperatures during the stand time were highly variable between farms, restricting comparative analyses or any inferences with the small number of samples collected.

The microbial loads of footbaths increased as well with the number of cow passages (r_T = 0.59, P < 0.001), and the number of defecations. (r_T = 0.49, P < 0.001). Otherwise, no differences between the farm's feet hygiene status and the microbial contamination were detected (*P*= 0.59). Depending on the herd size, a different number of milkings were needed to complete 200 passages (Table 1). Typical French farms with herds of less than 60 animals require at least 4 milkings (2 days) to complete 200 passages and then renew the footbath solution. Therefore, the changes in the microbial loads and OM after the exposition to different stand times and temperatures could be another factor of special interest to study in future studies. From the temperature and the pH recorded (Table 2), only slight variations were evidenced across the increasing number of cow passages. Nevertheless, depending on the intrinsic properties of each footbath solution, these slight variations usually are not likely to impact their efficacy. The bactericidal efficacy of disinfectants might be impacted by pH changes (Mcdonnell and Russell, 1999). Consequently, in theory, the water used in the farms to prepare the footbath solutions might impact the pH and thereby impact their efficacy depending on the characteristics of each product. Therefore, before the footbaths implementation, the water source properties require special consideration.

Table 2 *Footbath recordings across increasing number of cow passages.*

Footbath Recordings							
Farm ID (# cow passages)	Volume (L)	Liquid Depth (cm)	Number of Defecations	Organic Matter Concentration (gr/L) [1]	Microbial Loads (Log_{10} cfu/mL)	Temperature (°C)	pH
Farm 1							
0	173	13	0	0.08 (0.03)	-	12.5	6.7
50	147	11	5	4.95 (0.56)	6.60	13.7	7.7
100	120	9	12	16.72 (2.09)	-	15.5	7.6
150	93	7	18	30.75 (5.11)	7.17	16.1	7.4
200	80	6	22	40.34 (6.6)	7.23	16.7	7.2
Farm 2							
0	175	13	0	0.06 (0.01)	4.98	12.9	7.5
50	148	11	4	3.46 (0.78)	6.44	13.7	7.2
100	134	10	6	8 (0.72)	-	14.3	7.2
150	114	8.50	10	13.86 (1.55)	7.30	13.0	7.0
200	107	8	15	18.86 (1.55)	7.17	13.9	6.8
Farm 3							
0	175	13	0	0.10 (0.005)	3.83	13.5	6.3
50	134	10	0	2.45 (0.22)	6.32	14.1	7.8
100	121	9	2	6.75 (0.78)	-	14.8	8.2
150	107	8	2	12.66 (0.28)	6.04	12.4	8.1
200	94	7	2	16.2 (0.28)	6.25	12.9	8.1
Farm 4							
0	181	13.50	0	0.30 (0.01)	4.74	13.3	7.0
50	134	10	7	5.47 (0.78)	6.59	13.7	7.6
100	121	9	15	10.84 (0.66)	-	14.8	8.0
150	121	9	23	21.11 (1.97)	6.89	13.9	8.0
200	101	7.50	24	29.55 (2.20)	6.90	14.1	8.5
Farm 5							
0	190	12	0	0.05 (0.01)	3.07	13.6	5.8
50	149	9	2	1.28 (0.19)	6.43	12.9	6.9
100	107	6.50	4	3.54 (0.33)	-	12.5	7.6
150	107	6	4	7.91 (4.41)	6.69	8.2	7.3
200	74	4.50	5	9.46 (1.98)	7.43	8.6	7.5
Farm 6							
0	199	15	0	0.13 (0.01)	6.41	17.4	6.7
50	148	11	2	2.4 (0.17)	6.84	20.5	8.1
100	121	9	2	8 (0.34)	-	18.3	8.5
150	101	7.50	2	9.1 (1.22)	7.49	20.6	8.3
200	81	6	3	13.66 (1.56)	7.77	21.3	8.3

[1] Standard deviation is given in parentheses

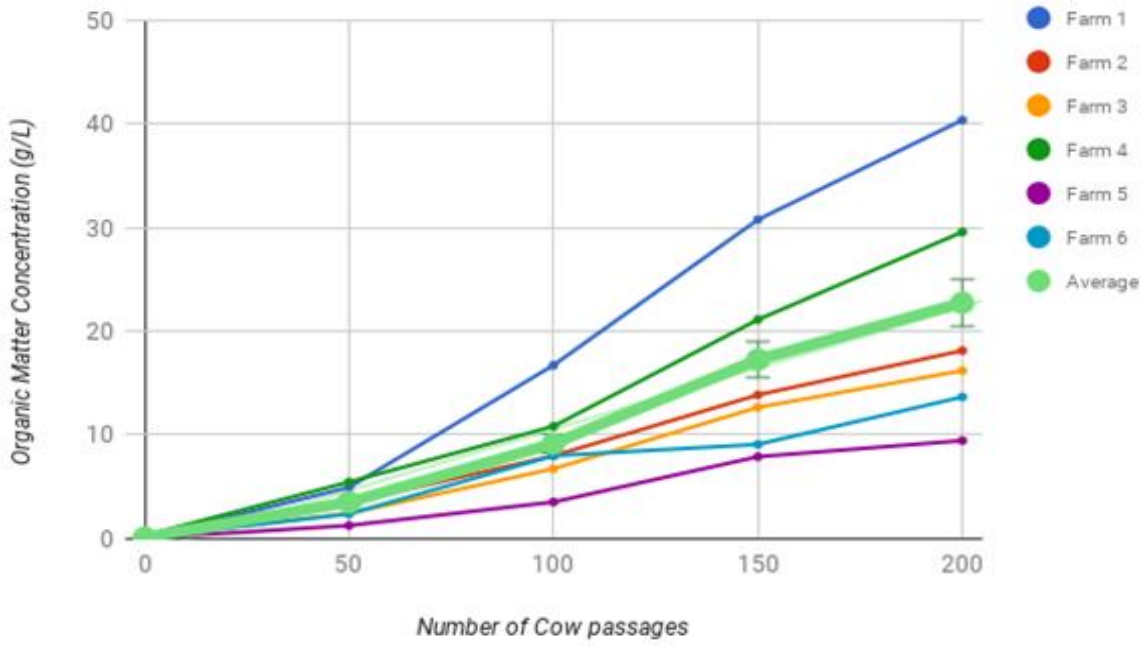
From the 1200 cow passages recorded, only one cow urinate and on average over 200 passages 6% of cows defecated in footbaths. These findings are similar to the results of Manning *et al.* (2016) were the concentrations of contaminants in the prewash bath increased in parallel with the cow passages, and the defecation rate inside the footbaths over 100 passages was about 5.4%. Otherwise, the defecation rates of other studies were very low (Fjeldaas *et al.,* 2014). The stress generated by implementing a footbath can notably influence the defecation rates, especially when footbaths are administered for the first time. Therefore, the renewal rates might have to be adapted during the first administrations. During the visits, it was remarked that several animals defecated in the alleys between the milking parlor and the footbath location. Hence, in future studies, others variables such as the footbath passage flow, the walking distance between the parlor and the footbath and the number of defecations before the footbath passage might enhance the understanding of the footbath contamination.

Footbaths of different types and dimensions were used among the farms, leading to a relatively broad range of initial water volumes (between 173 and 190 L) (Table 1). Interestingly, the reduction of the residual volume was highly correlated to the increasing number of cow passages ($r_T = 0.86$, $P < 0.0001$). Moreover, after 200 cow passages the water volumes decreased drastically (Average reduction= 50% (9% SD) and below the height required to cover entirely the feet (Average liquid depth= 6.5 cm (2.06 SD)) (Fig 1, B). Contrary to the size recommendations of 3m long, 0.5m wide and 0.28m step-in height (420 L) (Cook *et al.,* 2012), the footbaths of this study were of small dimensions (Average 182.16 L (10.32 SD)). One factor that would

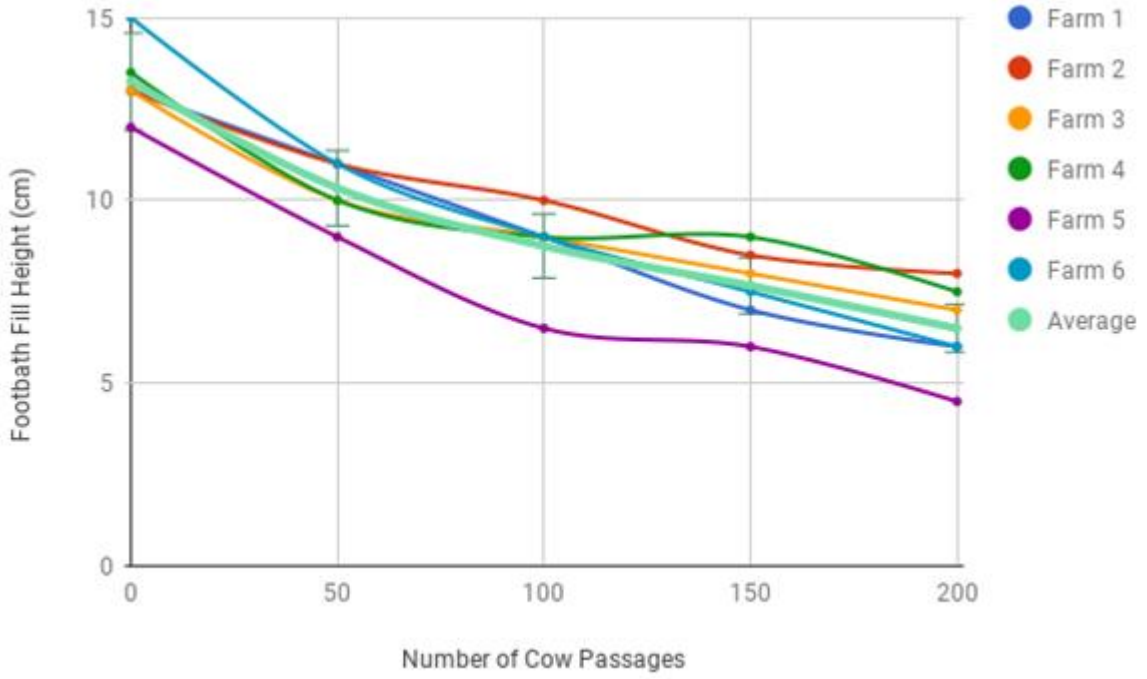
seem to intuitively impact the loss of fluid from the footbath would be the liquid splashing out of the bath as the animal passes through. Therefore, it is probable that in baths with a lower wall height in relation to the liquid depth, the losses would be greater. This hypothesis seems to be confirmed by our findings, wherein all the farms the baths were filled to the top, the wall heights were under the size recommendations (Average 13.25 cm (0.98 SD)) (Figure 2, B), and barely enough tall to cover the average height of a cow foot (Average digit-metacarpal condyle length = 18.67 cm) (Muggli *et al*., 2016). The large volume losses recorded in this study are contrary to the findings of similar studies, which used larger footbaths where the volumes were unchanged or possibly replaced by manure, urine, and dirt (Holzhauer *et al.,* 2004). Disinfectant losses and volume replacements could lead to transforming the footbaths into slurry baths. The close and frequent contact of the feet with slurry might alter the skin permeability, and increase the risk of infection (Palmer *et al.,* 2013). Altogether, these findings reflect a volume capacity problem inherent to the baths studied, and indicate the importance of following the recommended dimensions when implementing footbaths treatments. Based on our findings and according to the bath dimensions of the farms studied, to ensure at least the partial covering of the digit ((Average 2^{nd}-3^{rd} phalanges length = 6.98 cm) (Muggli *et al*., 2016)) by the footbath solution, the content of the bath should be renewed after every 100 passages on average corresponding to an average liquid depth of 8.75 (1.17 SD) (Figure 2, B).

Figure 1 Changes in the organic matter (OM) concentrations (A) and residual volumes (B) of footbaths after increasing number of cow passages (B).

A.



B.



This study reported the concentrations of OM and microbial loads contaminating the footbath solutions of six different farms after exposure to increasing number of cow passages. Further studies over longer periods, using a large sample including different housing systems and farm managements are needed to determine with precision how footbath solutions are contaminated under field conditions. Furthermore, due to the known impact of contaminants over disinfectant solutions, future in vitro studies evaluating footbath solutions should incorporate into their design all the different physicochemical variables which might affect their bactericidal efficacy in practice (ECHA, 2017).

Conclusion

In summary, the results of this study suggested, that the concentrations of OM contamination reached after 150 and 200 passages match with the regulatory concentrations in which disinfectant products should demonstrate to still be effective ($20g/L^{-1}$), and coincide with the often advised renewal rates. Nevertheless, the significant reduction in the footbath solution volume after the increasing number of cow passages, highlight the importance of adapting renewal rates according to the remaining volume as the disinfectant solutions administered through footbaths should cover the entire foot to guarantee its topical action. The findings of this study indicate the importance of the footbath designs for the successful implementation of these strategies in practice.

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Chapter 3. Preliminary studies to determine the renewal rate of the biocide.

B. In vitro evaluation of the bactericidal efficacy of a new footbath biocide under simulated field conditions

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Chapter 3. Preliminary studies to determine the renewal rate of the biocide.

In vitro evaluation of the bactericidal efficacy of a new footbath biocide under simulated field conditions

ABSTRACT

Lameness is one the main concerns facing modern dairy farming. Welfare concerns, antibiotics misuse, and economic losses are associated with lameness in dairy herds. Footbathing practices are a common strategy for the control of infectious lameness, allowing the topical treatment of a large number of animals at the same time. Nevertheless, in practice, footbaths solutions are challenged importantly against increasing amounts of contaminants mainly originated from the animal defecations and the feet dirtiness. Therefore, the contamination levels should determine the frequencies for the renewal of footbathing solutions. Currently, the footbath solutions used in dairy farms often lack of evidence supporting their in vitro efficacy when approaching real conditions. Besides, the most common solutions used in footbaths are related to environmental hazards (copper sulfate) or to cancerogenic risk for humans (formaldehyde). Consequently, biocide solutions, such as the broad-spectrum bactericide Pink-Step™, represent a nontoxic and biodegradable alternative for the collective footbathing. The objective of this study was to develop an *in vitro* protocol for the evaluation of the bactericidal efficacy of Pink-Step™ after the exposure to different contamination levels which mimic the levels of contaminants found in field conditions. For this purpose, organic matter (OM) and mesophilic bacteria (MB) were used at the level concentrations related to 0, 100 and more than 200 cow footbath passages. Subsequently, before the bactericidal activity measurements, two filtering methodologies were compared to determine the best method to reduce the quantities of MB present in samples after the exposition. Thereafter, the bactericidal activity (NF EN 1040 standard) was measured through a quantitative suspension test, after having separated the of Pink-Step™ solution from the MB using a 0.22µm filter and the OM using a paper filter. Results have shown that after the exposure of Pink-Step™ to several densities of MB its bactericidal efficacy was unaffected. Otherwise, after the exposure of Pink-Step™ to OM, preliminary results highlighted the negative and important effect of this parameter on the bactericidal activity. Hence, the densities of *Enterococcus hirae* at low OM quantities (0.10g/L) were totally reduced, were reduced by 3-Log at moderate OM quantities (9.0 g/L) and conversely at high quantities (40.0 g/L) the densities were reduced only by 1.89 Log. After the exposure to the moderate OM quantities (9.0 g/L), a shift phase of the solution was evidenced suggesting possible

interactions between OM and the surfactants components of Pink-Step™. This *in vitro* study for a first time approached the bactericidal efficacy of a footbath solution according to the contamination challenge expected in field conditions. To enhance the specificity of this methodology, further studies must include the pathogenic bacterias responsible for hoof infectious diseases.

INTRODUCTION

Lameness in ruminants together with mastitis represents the main issue facing the modern livestock industries (Algers et al., 2009a). Ruminants experiencing lameness episodes might reduce their productive and reproductive performances (Fourichon et al., 2000; Wassink et al., 2010). When a large part of the herd is lame, important quantities of antibiotics may be dispensed in farms (Hyde et al., 2017). Likewise, routinely several disinfectants are administered collectively in massive quantities (Relun et al., 2013b). Furthermore, the most severe cases of lameness could lead to the premature culling of the diseased animals (Cramer et al., 2009). Additionally, lameness is considered as a painful condition which may induce negative changes in the behavior of cows entailing a recognized welfare detriment (Walker et al., 2008, Bruijnis et al., 2012;). Intensive farming entails the high concentration of animals in the same space and thereby promoting a wet and unhygienic environment. Such conditions are recognized as the main risk factors which may lead to the feet skin damage, and the subsequent occurrence and spread of a broad range of infectious diseases, such as the case of interdigital dermatitis heel horn erosion (IDHE) (Somers et al., 2005b), bovine digital dermatitis (bDD) in cattle (Gomez et al., 2012), or the ovine foot root (FR) (Green and George, 2008) and the contagious ovine bDD in sheep (CObDD) (Dickins et al., 2016). Most of these diseases share a multifactorial and polymicrobial etiology.

Among the control strategies for infectious claw diseases, disinfectant footbaths are frequently advised. These practices allow the topical administration of a disinfectant solution collectively and concomitantly to the entire herd. However, farmers perceived footbathing practices to be insufficiently effective and as well expensive and time-consuming (Relun et al., 2013b). The effectiveness of footbathing practices is determined by several factors. Hence, in practice, footbaths are challenged by the defecations and feet dirtiness carried by the walking through animals (Chapter 3.1). Therefore, several degrees of contamination may importantly affect the bactericidal efficacy of footbath solutions and thereby alter the renewal frequencies of the solutions. Furthermore, the standard disinfectants most frequently used and considered as effective, such as formaldehyde and copper sulfate (CuSO4) are unsafe for

people and for the environment, respectively (IARC, 2006; Ippolito et al., 2010). Currently, several collective disinfectants are commercialized, however, there is lack of evidence supporting the effectiveness of such strategies (Ariza et al., 2017). Additionally, and contrary to the current European guidelines for biocides (ECHA, 2017a), for the large part of the commercial disinfectants used in footbaths, their bactericidal properties in increasing soil conditions mimicking those encountered under fields conditions, and against the main pathogens involved in claw diseases remains poorly studied and therefore uncertain.

Therefore, the development of safe and effective footbath solutions and protocols adaptable to the particular farm challenges seem a priority. To achieve this goal, the methodologies implemented in the preclinical research must approach the field conditions in terms of contamination to support their expected effectiveness. Therefore, the main objective of this study was to develop an *in vitro* protocol simulating the field conditions in order to evaluate the effect of increasing quantities of contaminants (organic matter (OM) and mesophilic bacteria (MB)) on the bactericidal efficacy of the new footbath biocide Pink-Step™ (Qalian, Neovia group, France). The methodology implemented in this study allowed determining the renewal frequencies for the biocide solution. The quantities of contaminants used were established according to a previous field study that determined a range of levels of OM and MB recorded in footbaths after increasing number of cow passages in.

MATERIALS AND METHODS

Tested product

The footbath biocide, named Pink-Step™(Qalian, Neovia group, France), is composed by lactic acid (30%; weight/weight) and glycolic acid (10%; weight/weight) as active substances, anionic and non-ionic surfactants and other excipients as a dye. The recommended concentration for their usage is 5% (vol./vol). At this concentration, the bactericidal efficacy of the solution has been confirmed using a standardized test for high levels of soiling (serum albumin bovine 10g/L and yeast extract 10g/L) (NF EN 1656) as recommended by the European regulation EU n°528/2012. The bactericidal effect of the solution is supported by the optimal association between its active and surfactants components. Furthermore, the lactic and glycolic acids are confirmed biocides.

Footbath challenge: Bacterial strains and contaminants used

Mesophilic bacteria chosen for the *in vitro* efficacy evaluation of the product were *Enterococcus hirae* (CIP 58.55) and *Pseudomonas aeruginosa* (CIP 103467) equally distributed for each preparation (1:1). The artificial organic matter was prepared using an iso concentration (1:1) of standard preparations of serum albumin bovine (ACROS / Thermo Fisher Scientific, Belgium) and yeast extract (AES laboratory, France). Both compounds are recognized as organic contaminants by European standards and their implementation in this study allowed the standardization of the methodology. The densities of MB and quantities of OM used in the current study were determined from the findings of a previous field study. On the mentioned study, the OM and MB of six footbaths placed in 6 different dairy farms were recorded after 0, 50, 100, 150 and >200 cow passages (Ariza *et al.*, submitted). Therefore, a range of contamination levels was elaborated from this data to simulate the contamination levels in this *in vitro* study (Table 1).

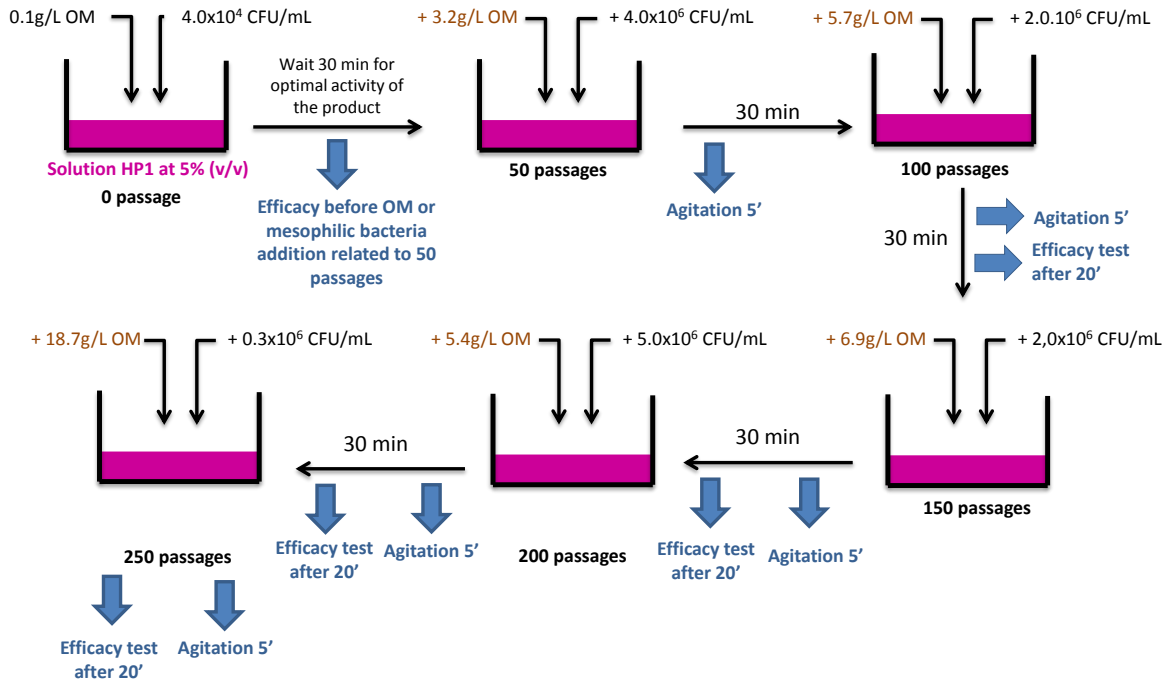
Table 1. Footbaths contamination levels under field conditions according to the number of cow passages, in terms of mesophilic bacteria (MB) and organic matter (OM).

Contamination Level	**Number of cow passages**	**MB densities (**CFU/mL)	**OM concentration (**g/L)
Initial	0	$4x10^6$	0.10
Moderate	100	$6.04x10^6$	9
High	>200	$1.33x10^7$	40

Thereafter, in the laboratory, the field conditions were mimicked using two different 10L-footbaths, one for the OM and the other for the MB. Both footbaths were filled with the Pink-

Step™ solution at 5% concentration. Progressively, the preparations of OM or MB were added to the footbath until reaching the level concentrations established in table 1 (Figure1).

Figure 1. Representation of the in-vitro protocol for mimic the field condition in which increasing numbers of cows passages condition the contamination levels of the footbaths.



Organic matter (OM) or mesophilic bacteria (MB) in number of Colony Forming Unit. (CFU/mL) were added over the time in order to mimic increasing numbers of cow passages (0,100 and more than 200 passages).

Contaminants separation

After the exposition of the Pink-Step™ solution to MB, two different methods of separation were tested to determine the best methodology to separate the contaminants from the Pink-Step™ solution in order to allow the successful evaluation of the bactericidal efficacy of the product. Therefore, a centrifugation method (3,500 rpm during 10 min) was compared to a filtration method using a 0.45µm filter. For each method, a mixed culture of *Enterococcus hirae* and *Pseudomonas aeruginosa* (10^8 – 10^9 CFU/ml) was diluted until obtaining the inoculum densities corresponding to the different number of cow passages. Each inoculum was prepared in peptone water – NaCl and the bacterial counts were performed on TS medium (Tryptone Casein Soja) incubated at 30°C for 24h.

For the OM, to avoid the filter clogging and to simulate the gravity process that follows OM in footbaths, a paper filter was used for the separation.

Evaluation of bactericidal activity of the Pink-Step™ solution after exposure to mesophilic bacteria or organic matter

As described above, the disinfectant was exposed to different densities of MB or quantities of OM. After this exposure, the filtrate was collected and used for the evaluation of the bactericidal activity according to the NF EN 1040 standard for a contact time of 5 minutes at 10°C. Briefly, this standardized method consists in the exposure of the biocide solution to a defined bacterial suspension. Thereafter, the mixture is maintained at 10 °C for 5 min. At the end of this contact time, an aliquot is taken and the bactericidal activity in this portion is immediately neutralized or suppressed by dilution-neutralization.

A suspension of *Enterococcus hirae* was prepared for this test. This specific bacteria was previously considered for the Pink-Step™ solution as the most resistant by a precedent study (data not shown). The bactericidal efficacy of the solution was considered if a 5-log reduction was reached after the exposure to contaminants.

RESULTS

MB separation

After the complete evaluation of both methodologies, the filtration (0.45µm filter) showed to perform a better separation of the MB than the centrifugation. Less than $1.90x10^{2}$ CFU/mL were detected after the filtration of an initial inoculum of $5.85x10^{6}$ CFU/mL compared to centrifugation which obtained only a reduction of $2.5x10^{5}$ CFU/mL. Finally, in order to improve the filtering process, the diameter of 0.45µm was compared to a 0.22 µm diameter. After the exposition to an inoculum of $3.20x10^{7}$ CFU/mL, the 0.22 µm filter less $1x10^{1}$ CFU/mL were detected resulting in an increased performance compared to the $7x60^{7}$ CFU/mL detected after the 0.45 µm filtering.

Bactericidal activity of Pink-Step™ solution after exposure to increasing densities of mesophilic bacteria

Results showed that exposure to several mesophilic bacteria has no effect on disinfectant efficacy at 5% (Table 2). After every MB exposure for 5 minutes at 10°C, the bacterial densities retrieved were below the detection limit ($1.40x10^2$ CFU/mL), highlighting that the efficacy of Pink-Step™ remained unaffected after the bacterial contamination challenge.

Table 2. Bactericidal efficacy of Pink-Step™ against *Enterococcus hirae* after exposure to mesophilic bacteria (MB). [1]

Contamination Level	**Before Pink-Step™ exposure**	**After filtering and Pink-Step™ exposure for 5 minutes at 10°C**	
	***Mesophilic bacteria* (CFU/mL)[2]**	***Enterococcus hirae* density (CFU/mL)**	**Bacterial density reduction (log10)**
Initial (0 Passages)	$1.81x10^7$	$<1.40x10^2$	> 5.40
Moderate (100 Passages)	$7.35x10^7$	$<1.40x10^2$	> 5.40
High (>200 Passages)	$4.70x10^4$	$<1.40x10^2$	> 5.40

[1] Filtration using a 0.22µm filter

[2] Mesophilic bacteria is a mix (1:1) of *Enterococcus hirae* and *Pseudomonas aeruginosa*

Bactericidal activity of Pink-Step™ solution after exposure to increasing quantities of organic matter

After exposure of Pink-Step™ solution to the lowest quantities of OM (0.10 g/L), a bactericidal efficacy was recorded with a reduction superior to 5.26-Log of *E. hirae*. Contrarily, after the exposure to 9.0 g/L, corresponding to approximately 100 cow passages, a reduction of 3.07-Log of *E. hirae* was reported indicating the inefficacy of the Pink-Step™ solution. Finally, after the exposure to the highest quantities of organic matter (40.0 g/L), only a reduction lower than 1.89-Log of *E. hirae* was noticed indicating again the inefficacy of the solution under those conditions mimicking more than 200 cows passages. After the moderate OM exposition (9.0 g/L), a shift phase of the solution was noticed (Table 3).

Table 3. Bactericidal efficacy of Pink-Step™ against *Enterococcus hirae* after exposure to organic matter (OM). [1]

Contamination Level	Before Pink-Step™ exposure		After filtering and Pink-Step™ exposure for 5 minutes at 10°C	
	OM concentration (g/L)	***Residual MB* (CFU/mL)**	***Enterococcus hirae* density (CFU/mL)**	**Bacterial density reduction (log10)**
Initial (0 Passages)	0.10	$2.55x10^7$	$<2.25x10^4$	> 5.26
Moderate (100 Passages)	9	$2.55x10^7$	$2.18x10^4$	3.07
High (>200 Passages)	40	$2.55x10^7$	$>3.30x10^5$	< 1.89

[1] Decantation and filtration using a paper filter

[2] Organic matter is composed of a mix (1:1) of Bovine Serum Albumin and Yeast Extract.

DISCUSSION

The findings of this study revealed a reduction in the bactericidal efficacy of Pink-Step™ solution after tested against increasing levels of organic matter which mimic field conditions. Additionally, after the exposure to increasing densities of MB, the Pink-Step™ solution remained effective. This investigation as well enabled the establishment of a standardized methodology for mimicking in vitro the field conditions in which the footbath solutions must remain effective and propose therefore a frame for further assessment of different biocides. The impact of OM in the bactericidal efficacy of footbath solutions evidenced in this study, highlight the important challenge that footbath solutions must overpass to achieve effectiveness under field conditions. Therefore, to enhance the effectiveness of footbaths their implementation must encompass multiple conditions, such as the respect of the renewal rates (<100 passages), the correct design of the bath and more importantly the improvements in the feet hygiene of the herd.

This investigation succeeds the *in-vitro* efficacy evaluation of a footbath solution by an original approach that mimicked the field conditions in which these products are truly

implemented in farms. The originality of this study which by mimicking the field conditions succeeded to standardize a methodology for the evaluation of disinfectants used in footbaths was limited by (i) the unconsidered mineral part within the organic matter and (ii) the potential effects of the association of organic matter and bacteria in the same preparation.

Although the limitations of footbathing practices have been pointed in precedent studies (Cook et al., 2012; Chapter 3.1), the evidence supporting the bactericidal efficacy of footbath solutions under soil conditions remains scarce. A recent study has approached the subject using OM as the limiting parameter for disinfectant efficacy in footbaths (Hartshorn *et al.*, 2013). Nevertheless, and contrary to our study, this study used cow manures (10% and 20%) in which the organic part is highly variable and dependent on the diet of the animals. Moreover, manure was autoclaved at 134°C which obviously sterilized the sample, and on the other hand, could decrease the organic content with the thermal degradation, a phenomenon previously described in other studies (Russell et al., 1974; Papadimitriou, 2010). Although the findings of this study revealed a small impact of MB on the efficacy of Pink-Step™ solution, it is possible that MB impacts other products and therefore their evaluation must be advocated in any efficacy test. After the exposure to moderate quantities of OM the bactericidal efficacy of the solution was reduced (3.07-Log), and a shift phase of the solution was noticed. However, a previous study has as well demonstrated that partitioning of surfactants and organic matter may interfere with the activity of active substances (Hammer et al., 1999). The formation of micelles between a specific ratio of anionic surfactants and organic matter could trap organic acids leading to a decrease in the bactericidal activity.

In this study, the main pathogens involved in claw infectious disorders of ruminants were not explored. Nevertheless, the design of the protocol implemented may allow testing the bacteria species concerned. Although several etiologies have been identified as the main causative agents of a particular pathology, these same pathogens could be involved in some degree in the development of other different claw pathologies. For example, *Fusobacterium spp* and *Dichelobacter spp* which are involved in both claw diseases, bDD and IDHE (Knappe-Poindecker et al., 2014), and as well in FR and CObDD (Moore et al., 2005). Therefore, further studies evaluating the efficacy of footbath solution must scope multiple pathogens, taking into account the different levels of contaminants.

Altogether, the results of this chapter (3.1 and 3.2) allowed the determination of the renewal rates of the Pink-Step™ disinfectant products in cow footbaths. Therefore, according to this preliminary study, the Pink-Step™ solution requires being renewed at least every 100

passages. Further studies using this methodology to test disinfectant products against the different pathogens associated with infectious lameness in ruminants may enhance the strategies of control and improve the welfare and the economic benefits of farmers.

From the findings of this investigation a renewal rate each every 100 passages was implanted in the clinical trial exposed in the next chapter (Chapter 4.1)

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Chapter 3. Preliminary studies to determine the renewal rate of the biocide.

Chapter 4. Clinical Trial

A. Evaluation of a biocide footbath solution in the prevention and healing of digital dermatitis lesions in dairy cows. A clinical trial.

Evaluation of a biocide footbath solution in the prevention and healing of digital dermatitis lesions in dairy cows. A clinical trial.

FRENCH ABSTRACT

La dermatite digitée demeure actuellement la principale maladie responsable de boiteries chez les vaches laitières du fait de la difficulté de sa maitrise. L'objectif principal de cette étude était d'évaluer l'efficacité préventive et curative d'une nouvelle solution biocide biodégradable (Pink-Step™ Qalian) applicable en pédiluve vis-à-vis de lésions de dermatite digitée (DD). L'étude a été menée dans le cadre d'un essai clinique, dans lequel les pieds postérieurs des vaches de chaque ferme ont été alloués de façon randomisée soit au groupe témoin (aucun traitement), soit à l'un des deux différents régimes de traitement collectif par pédiluve (régime intensif ou modéré). L'essai a porté sur 1036 vaches (2072 pieds) provenant de 10 troupeaux laitiers situés dans l'ouest de la France où la DD était endémique. Des bi-pédiluves ont été placés à la sortie de la salle de traite de chaque ferme, permettant d'administrer spécifiquement la solution de biocide d'un côté et d'utiliser l'autre côté comme groupe témoin négatif (split design). Afin d'explorer la meilleure fréquence d'administration, deux groupes avec différents régimes d'administration de pédiluves ont été conçus. Le groupe modéré consistait en une administration 2 jours par semaine le premier mois, puis tous les quinze jours le deuxième mois, puis une fois par mois jusqu'à la fin de l'essai. Le groupe intensif consistait en une administration 2 jours par semaine les 2 premiers mois, puis tous les 15 jours jusqu'à la fin de l'essai. Les deux régimes ont été administrés pendant 140 jours, et les pieds ont été évalués pour le diagnostic des lésions de DD au moins une fois par mois dans la salle de traite. Des modèles de survie emboîtés ont été utilisés pour estimer le risque relatif (exprimé en Hazard Ratio) des régimes de pédiluve et d'autres facteurs de risque concomitants au moment où les lésions de DD sont apparues ou ont guéri. Le risque de survenue de lésions de DD était augmenté de façon importante principalement par la mauvaise propreté des pieds au niveau de la vache (HR = 1,69, IC 1,21-2,39) et au niveau de la ferme (HR = 2,06, IC 1,44-2,94). Par contre, les résultats indiquent l'efficacité curative de Pink-Step™ au régime intensif dans l'amélioration de la guérison des lésions de DD (HR = 1,79, IC 1,12-2,88). Le temps de guérison était également amélioré pour les lésions inactives (HR = 2,19, IC 1,42-3,37). Inversement, le temps de cicatrisation a été retardé pour les pieds étant parés (HR 0,41, IC 0,26-0,62), chez les vaches présentant une lésion controlatérale (HR 0,32, IC 0,22-0,46) ou en fin de lactation (HR 0,61 CI 0,43-0,85), et finalement, dans les fermes avec un effectif

important (> 100 vaches) (HR = 0,48, IC 0,34-0,67). Ces résultats renforcent le rôle crucial de l'hygiène dans la dynamique de la DD et soulignent l'importance de mettre en œuvre simultanément plusieurs mesures de contrôle, telles que des améliorations hygiéniques dans les bâtiments, la détection précoce des lésions et l'utilisation correcte des traitements individuels et collectifs. En conclusion, la mise en œuvre des pédiluves Pink-Step™ représente une stratégie prometteuse pour réduire la persistance des lésions de DD dans les troupeaux affectés.

ABSTRACT

The main objective of this study was to evaluate the effect of the implementation of different footbathing practices using a new biocide solution (Pink-Step™, Qalian, Neovia group, France) in the healing and occurrence of bovine digital dermatitis (bDD) lesions. The investigation was conducted through a controlled within cow clinical trial in which the hind feet of cows from each farm were allocated either to the control group or to one of two footbath regimen groups. The trial involved 1036 cows (2072 feet) from 10 dairy farms located in western France where bDD was endemic. Split footbaths were placed at the exit of the milking parlor of each farm, allowing the biocide solution to be administered to one side of the cows while using the other side as a negative control. According to the frequency of administration, footbaths regimen groups were moderate (MR = 2 days every week for the first month, then every fortnight for the second month, and then once a month) or intensive (IR = 2 days every week for the first 2 months, and then every fortnight). Both regimens were administered during approximately 140 days, and feet were evaluated for the presence of bDD lesions at least once a month in the milking parlor. Nested survival models were used to estimate the relative impact of the footbath regimens and other concomitant risk factors on the time that bDD lesions occurred (preventive effect) or healed (healing effect). No preventive effect of the Pink-Step™ solution was evidenced during the trial. The risk for bDD occurrence was increased importantly by poor feet cleanliness at both the cow (HR = 1.69, CI 1.21–2.39) and farm level (HR = 2.06, CI 1.44–2.94). Otherwise, the results indicate that Pink-Step™ footbaths used in an intensive regimen is effective in improving the healing of bDD lesions (HR = 1.79, CI 1.12–2.88). The time to healing was improved as well in inactive lesions (HR = 2.19, CI 1.42–3.37). Conversely, the time to healing was delayed in feet receiving hoof-trimming (HR 0.41, CI 0.26–0.62), in cows which either have a contralateral lesion (HR 0.32, CI 0.22–0.46) or were in late lactation (HR 0.61 CI 0.43–0.85), and finally, in farms with larger herds (>100 cows) (HR = 0.48, CI 0.34–0.67). These findings reinforce

the crucial role of hygiene in bDD dynamics and highlight the importance of implementing multiple control measures simultaneously, such as hygiene improvements in the barn, early detection and treatment of bDD lesions and the correct usage of individual and collective treatments. The implementation of Pink-Step™ footbaths represents a promising strategy for reducing the persistence of bDD lesions in affected herds.
Key words: Bovine digital dermatitis, biocide, footbaths, clinical trial, risk factors.

INTRODUCTION

Bovine digital dermatitis (bDD) is currently the principal cause of infectious lameness in dairy cows. bDD raises important economic, public-health and animal-welfare concerns. Indeed, bDD is associated with different challenges such as reduced farmers' incomes, increased use of antibiotics and, more importantly, lameness and thereby animal pain, impaired milk production and reproduction (Relun et al. 2013; Ettema et al. 2010; Bruijnis et al. 2010). bDD can affect 96% of herds and between 7% to 30% of cows within a herd (Solano et al., 2016; Cramer et al., 2008). The disease is characterized by the occurrence of ulcerative lesions in the skin of the interdigital cleft which may persist or evolve to chronic forms (Read and Walker, 1998). Diseased cattle act as reservoirs and thereby as potential sources for outbreaks (Döpfer et al., 2012). Although the precise cause of bDD is not completely elucidated, it is known that farming practices impact notably the environment in which the disease is established (Somers et al., 2005). The bDD is considered to be a multifactorial disease consistently associated with unhygienic and wet conditions which mainly alter the integrity of feet skin. Nevertheless, to accomplish the development of clinical lesions, the presence of specific Treponema species on feet suffering from cutaneous maceration is essential (Gomez et al., 2012). Consequently, control strategies aim to limit exposure to factors which might impact the spread of bDD. In practice, the control of bDD frequently relies on the individual treatment of active lesions and on the collective administration of disinfectant solutions through footbaths. However, evidence supporting the effectiveness of collective solutions remains scarce, mainly due to the small samples and design weaknesses that have limited existing studies (Ariza et al., 2017).

The banning of antimicrobial use in footbaths is a priority to respond to a growing antimicrobial resistance threat at human and animal levels (Holzhauer et al., 2017; Hyde et al., 2017). Moreover, other common products used in footbaths represent in some cases an

environmental risk, such as copper sulfate (Ippolito et al., 2010), or a harmful practice for farmers, such as formaldehyde, which has been recognized as cancerogenic (Cogliano et al., 2005). Several footbaths solutions that claim to be effective and safe are currently available on the market without major scientific evidence supporting these claims (Ariza et al., 2017). Furthermore, the guidelines for the implementation and use of some of the currently available footbath solutions entail several limitations because they are not adapted multiple farming scenarios. Indeed, significant differences between farms, for example, in terms of hygiene, housing system, herd size or lameness prevalence, may have an important impact on the implementation and effectiveness of footbathing practices (Relun et al., 2012; a Relun et al., 2013), and often this impact is ignored in controlled trials (Ariza et al., 2017). Additionally, the increasing bDD prevalence and the development of non-healing lesions are raising concerns that highly pathogenic or resistant strains are (Evans et al., 2011). Therefore, new solutions for the collective disinfection of feet must consist of efficient and safe practices that can be easily adapted to the complex setting of each farm. Pink-step™ (Qalian, Neovia group, France) is a biocide that represents a potential alternative for bDD control. This a safe and biodegradable disinfectant solution of confirmed efficacy under soil conditions designed for the footbath administration.

A clinical trial was developed to investigate the effectiveness of this new footbath biocide solution in preventing the occurrence of bDD lesions and in enhancing the healing of existent bDD lesions. Therefore, the main possible risk factors present at the cow and farm levels were concomitantly included in the trial analyses.

MATERIAL AND METHODS

This investigation is reported following the recommendations of the CONSORT statement, extension to within-person trials (Pandis et al., 2017). All procedures were carried out under the agreement of the Ethics Veterinary Committee in Clinical Research and Epidemiology from the Veterinary School of Nantes, France (CERVO, France) (registered number: CERVO-2016-12-V.)

Trial design

The trial was designed to be a controlled within cow clinical trial in which the hind feet of cows from each farm were allocated either to the control group or to one of two footbath regimen groups using Pink-step™.

Study population

The trial was conducted on 10 dairy farms in western France from October 2016 to June 2017. Farms were selected from a list provided by hoof-trimmers and veterinarians according to the trial protocol (Supplementary material S1). These farms were known to have experienced bDD for over at least two years. However, none of the farms had administered footbaths during the two months preceding the trial. The herds were composed of Holstein cows to reduce the potential effects of breed on bDD. Cows were milked in a rotatory or conventional milking-parlor (location for bDD scoring). Additionally, to minimize possible imbalances between farms, after the pre-study visits, only farms with a herd prevalence $\geq$ 15% of active bDD lesions were included. Farmers milked on average 90 cows (range: 45–145) twice a day. Cows were mostly housed in cubicles (9 farms), and only one farm had no access to pasture during the spring and summer seasons.

Footbath regimen groups and concomitant treatments

The footbathing procedure consisted in placing a footbath at the milking parlor exit and administering a disinfectant solution over a complete 5-month period. A split walk-through footbath was used to administer the disinfectant solution. The footbath consisted of 2 baths separated by a grill which partially avoids contamination of the footbath by cow feces (Intra-Bath™, Intracare). The disinfectant solution administered, named Pink-step™, was a new biocide with recognized in-vitro efficacy. Pink-step™ solution is composed of lactic acid (30%; v/v) and glycolic acid (10%; v/v) as active substances, anionic and non-ionic surfactants, and other excipients as a dye. The dose recommended by the manufacturer for footbathing was a 5% (v/v) solution in water. The split footbath made it possible to concurrently administer the Pink-step™ solution in one side of the footbath whilst the other side of the footbath remained empty and was used as a control. The feet of the lactating cows enrolled in the trial thus were allocated to three different groups, consisting of two different regimens of footbath administration frequencies and the empty bath (control group). The Moderate Regimen (MR) was planned to resemble current farm practices and consisted in footbath administration for 2 days (4 consecutive milkings) every week for the first month, then every fortnight for the second month, and then once a month until the end of the trial.

The Intensive Regimen (IR) aimed to evaluate the possible advantages of increasing the frequency of footbathing over time, and consisted in footbath administration for 2 days every week for the first 2 months, and then every fortnight until the end of the trial. Finally, to avoid possible interpretation bias due to interactions of placebo (water) effects in bDD lesions, the control group consisted in an empty bath. For both regimens studied, the biocide solution was expected to be renewed every 100 cow passages following the guidelines for the use of the solution.

Individual concomitant treatments were allowed during the trial resembling real field conditions. Therefore, and for ethical and welfare reasons, during the trial farmers were expected to individually treat all ulcerative-active bDD lesions which they themselves detected, using 2 applications of oxytetracycline (30 mg/ml) (Oxytetrin™, MSD) 2 days apart, regardless of the group assigned for the trial.

Follow-up, data collection, and outcomes measures

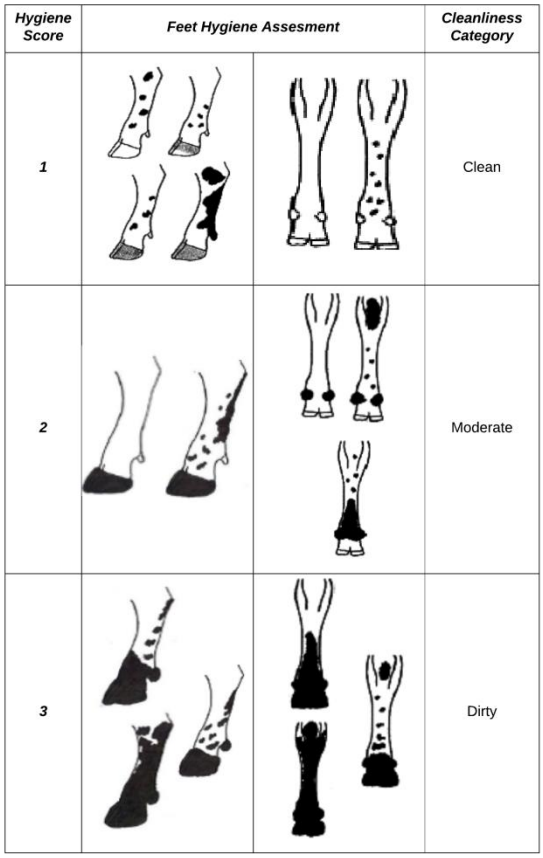
Farms were visited by 3 investigators trained through practical lessons to fill out the questionnaires and conduct the overall feet scoring. Each visit followed 3 steps: (1) scoring the hind feet of all lactating cows for bDD and feet hygiene during milking, (2) checking compliance with the protocol, and (3) checking any changes in herd management practices. The investigators filled a questionnaire which included all of the covariates presented in Table 1. Baseline records on the prevalence and other covariates of the participant farms were recorded during pre-study visits performed before the start of the trial.

Digital dermatitis status was assessed during milking using the methodology described by Relun et al. (2011). The hind feet of all lactating cows were washed using tap water before the examination. The hind feet then were recorded according to the M scoring system, modified from Döpfer et al. (1997) and Berry et al. (2012). In this system, the M0 stage corresponds to healthy feet without bDD lesions; M1 is considered as an early-stage ulcerative lesion (0–2 cm diameter); M2 represents painful ulcerative lesions with a diameter >2 cm; M3 is the healing stage with a lesion covered by a scab; M4 is the chronic stage characterized by dyskeratosis or surface proliferation; and M4.1 consists in a chronic lesion with a small area of ulceration. In addition, lesion scores were gathered into 2 different categories, inactive lesions (M3 - 4) and active lesions (M1 - 2 - 4.1). Otherwise, feet dirtiness (tarsus (hock); metatarsus and digit (phalanges)) of the entire herd was assessed in the milking parlor prior to

washing the feet for the bDD lesion diagnosis. The hind feet of each cow were scored using a 3-point nominal scale, varying from clean (score 1) to dirty (score 3), as described by Guatteo et al. (2013) (Figure 1). For each cow, the higher foot score was retained. The first scoring was performed immediately before the start of the administration of footbaths. Consecutive visits at intervals no longer than 30 days were performed during the trial period. Additionally, for ethical and welfare concerns, farmers were informed about the overall prevalences of bDD lesions with a delay of one week, but without any precision of the affected animals in order to avoid influencing the owners' perception of the study protocol or their decision-making process for the individual treatment of ulcerative lesions.

Two different outcome measures were recorded on each foot of the observed cows. A primary outcome studied the healing effect and evaluated the healing of bDD lesions, measuring the time in days to heal a bDD lesion counting from the first date of observation until the first date without any bDD lesion. The secondary outcome studied the preventive effect and evaluated the delay in the occurrence of bDD lesions, counting the time in days from the first observation of a foot without any bDD lesion until the first date of occurrence of a bDD lesion.

Figure 1. Scoring grid to assess feet cleanliness.

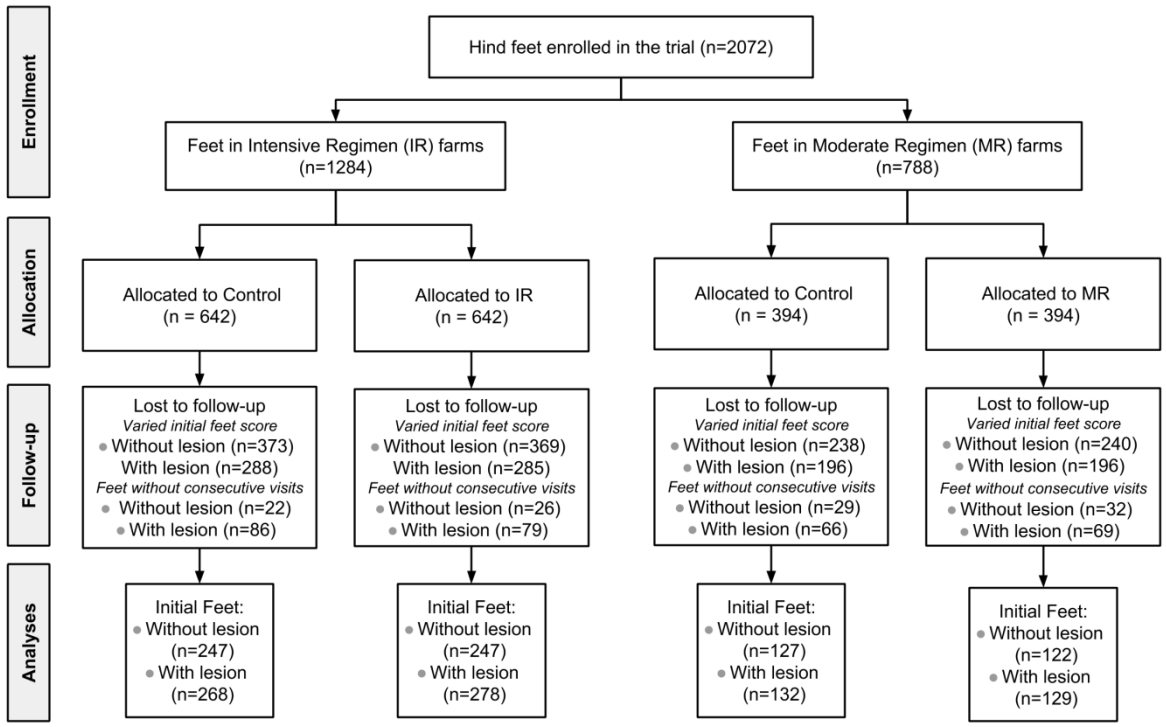


Sample size

Sample sizes were calculated for both outcomes studied using the formula for sample size estimation in clinical trials with clustered survival times as the primary endpoint (Xie and Waksman, 2003). The sample size implemented in the trial was based on the preventive outcome because the detectable differences in the target effect between the treatment and control group were smaller (10%) compared with the healing outcome (20%). Therefore, a larger number of animals was needed to achieve statistical power for the preventive outcome than the healing outcome. Due to the lack of previous data to account for the within cow correlation, a classical intra-

cluster correlation coefficient (ICC) was set at 0.05 (Adams et al., 2004). The number of farms included was calculated based on the average French herd size of 60 lactating cows and on the occurrence rates (4 cases for 100 feet-months at risk) reported in a previous study following a similar approach (Relun et al., 2013). Therefore, with a type I error risk of 0.05 (α=0.1), at least 264 cows by each footbath regimen were necessary to guarantee 80% power (β=0.2) to detect the target difference between control feet and footbath feet, leading to the recruitment of 10(2x5) farms.

Figure 2. Flow diagram of the feet included in the clinical trial. From the enrollment until the final analyses.



Treatment group allocation

The side allocation (left or right) of the control group was balanced between the farms. Masked envelopes containing the side allocation were prepared and chosen randomly just before the first footbath administration. During the recruitment process and before any lesion scoring, half of the farms were allocated to the IR according to the farmers' willingness to spend more time administering the footbaths. The footbaths were administered by the farmers and therefore they were aware of the side containing the biocide product. Likewise, due to the pink color of the biocide substance, it might be possible that investigators were aware of the feet being treated during the trial.

Data Analysis

All data were initially entered into a Microsoft Access database (Microsoft Corp., Redmond, WA). New covariates were built from the raw data using R (R Core Team, 2017). The effectiveness of both treatment regimens was evaluated on the clinical healing of bDD lesions and the reduced occurrence of bDD lesions through a survival analysis with a hind foot as the statistical unit. Survival analyses were carried out using the Frailtypack package in R (Rondeau et al., 2017). Nested survival models (Rondeau et al., 2012) were applied including a nested random effect for cows grouped in farms to adjust for clustering within observations, thereby feet in the models were considered as independent observations clustered at the farm level and subclustered at the cow level. Factors considered as potential effect modifiers of the healing and the occurrence of bDD lesions at the herd, cow and feet levels were included as covariates in the models (Table 1). The temporality between exposure and the outcomes studied was taken into account in the models, and when recurrent events were recorded time-dependent covariates were constructed. The survival analysis was planned in 3 steps: (i) treatment regimens and all covariates were tested in univariate analyses. Those covariates which contributed to the model at a 20% significance level were selected for multivariate analysis (Dohoo et al., 2003). (ii) The proportional hazards assumption and the goodness-of-fit of the final model were checked by graphic procedures and the Schoenfeld residuals test (Schoenfeld, 1982). (iii) The multivariate models were checked for confounding for every covariate by backward stepwise with footbath regimen group forced into the model. Confounding was assumed to occur when the estimates changed by more than 20%.

For the purpose of analysis, when evaluating the healing outcome, and to ensure the true healed status of a lesion, only feet initially scored with an active or inactive lesion were considered to be healed in the models if in subsequent visits an M0 (“Healthy stage”) score was noted on at least 2 consecutive visits. Likewise, for the outcome evaluating the occurrence of bDD lesions, only feet conserving the same M0 score during the 2 consecutive initial visits were included, ensuring the real absence of any lesion. The occurrence of a lesion was considered in the model if the included feet suffered any bDD lesion (active or inactive) during the trial period on 2 consecutive visits, to ensure the true lesion occurrence in the feet. For both outcomes, feet with visits spaced more than 45 days were removed from the analysis. Results of the models are presented as hazard ratios (HR) with their respective confidence intervals (CI), estimated for each covariate from the hazard function by taking the

exponent of the estimates of effects. Therefore, the HR calculated for the preventive outcome measures the instantaneous risk for a foot free of lesion to experience a bDD lesion being treated with one of the regimens versus being untreated. Meanwhile, the HR calculated for the healing outcome measures the instantaneous risk for a foot with a lesion to become healed being treated with one of the regimens versus being untreated. Finally, for the outcome evaluating the bDD occurrence, HR measures the instantaneous risk for the occurrence of a lesion in a foot being treated with one of the regimens versus being untreated.

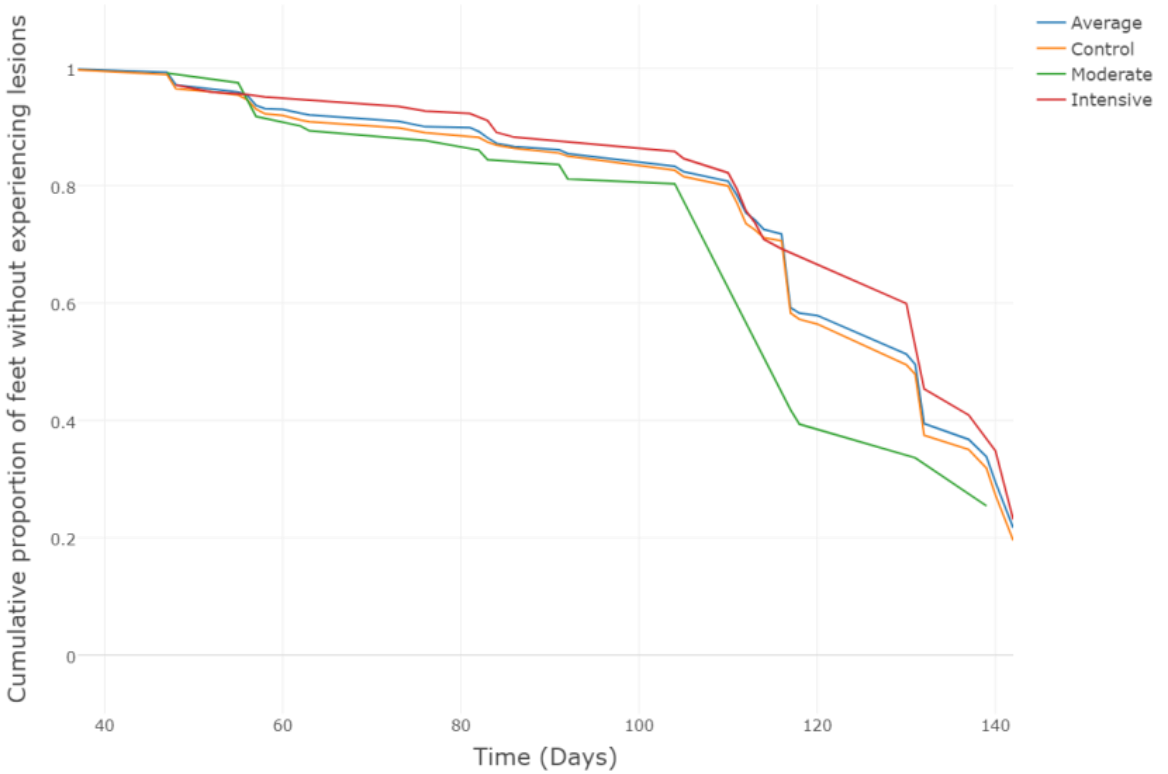
RESULTS

Farms were recruited between October and December 2016. For each farm, hind feet were inspected between 1 and 6 times at a median frequency of 30 days from January to June. Table 2 presents the main characteristics of the farms before the regimen side allocation. The baseline characteristics of the feet are summarized in Table 3. In total, 2,072 feet were allocated into one of the three groups, precisely, 394 in the MR group, 634 in the IR group, and 1,036 in the control group (Figure 2). During the follow-up period, there were no deviations from the trial protocol or adverse effects reported or observed.

Preventive Effect

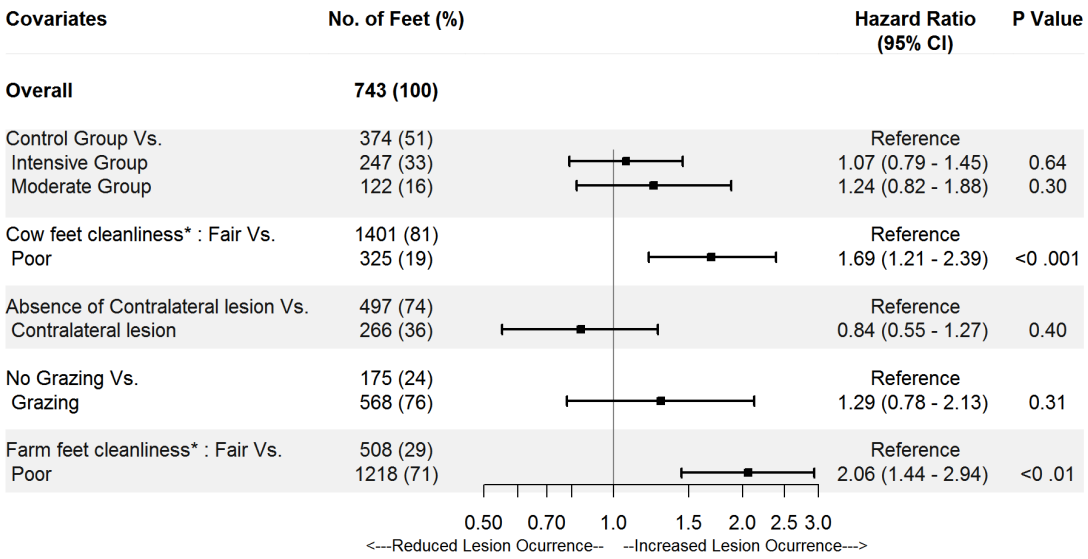
At the start of the trial, 852 hind feet (41%) out of 2,072 hind feet (1,036 cows) were free of active bDD lesions during two consecutive visits. Then, 109 feet were excluded from the dataset because their visits were spaced by more than 45 days. Finally, 743 hind feet of 468 cows from 10 herds were included in the analysis (Figure 2). Among these cows,only 275 cows shared the same bDD-free score in both hind feet at the start of the trial. Significant baseline differences were found between the feet allocation groups for cow feet hygiene, initial prevalence, proportion of heifers, herd size and farm feet hygiene covariates (Table 3).

Figure 3. Cumulative proportion of feet without experiencing digital dermatitis (bDD) lesions over time according to the three allocation groups and the overall average, respectively (moderate, intensive and control).



Among all of the feet included in the analyses, 161 (21%) experienced a bDD lesion during the trial period. Inactive lesions (142) were more prone to occur than active lesions (19). The median time before the occurrence of a bDD lesion was 80 days (37 to 142 days), and the mean incidence rate was 6 cases for 100 feet-months at risk (Figure 3). After the analyses, only poor feet cleanliness at cow level and at farm level covariates were significantly associated with a high risk of bDD occurrence in the multivariable analysis (Figure 4). No preventive effect of Pink-Step™ was evidenced during the trial. None of the other covariates included in the multivariate model or their interactions were statistically significant in the multivariable model. Finally, the estimated variance of the cluster effect at the farm level was 0.0073 (SE: 0.0032), and at the cow level 3.65 (SE: 0.69).

Figure 4. The effect of footbath regimen adjusted for herd, feet, and cow characteristics on the first occurrence of digital dermatitis (bDD) lesion in the nested survival model including observations on 743 hind feet from 468 cows from 10 French dairy herds involved in a clinical trial.

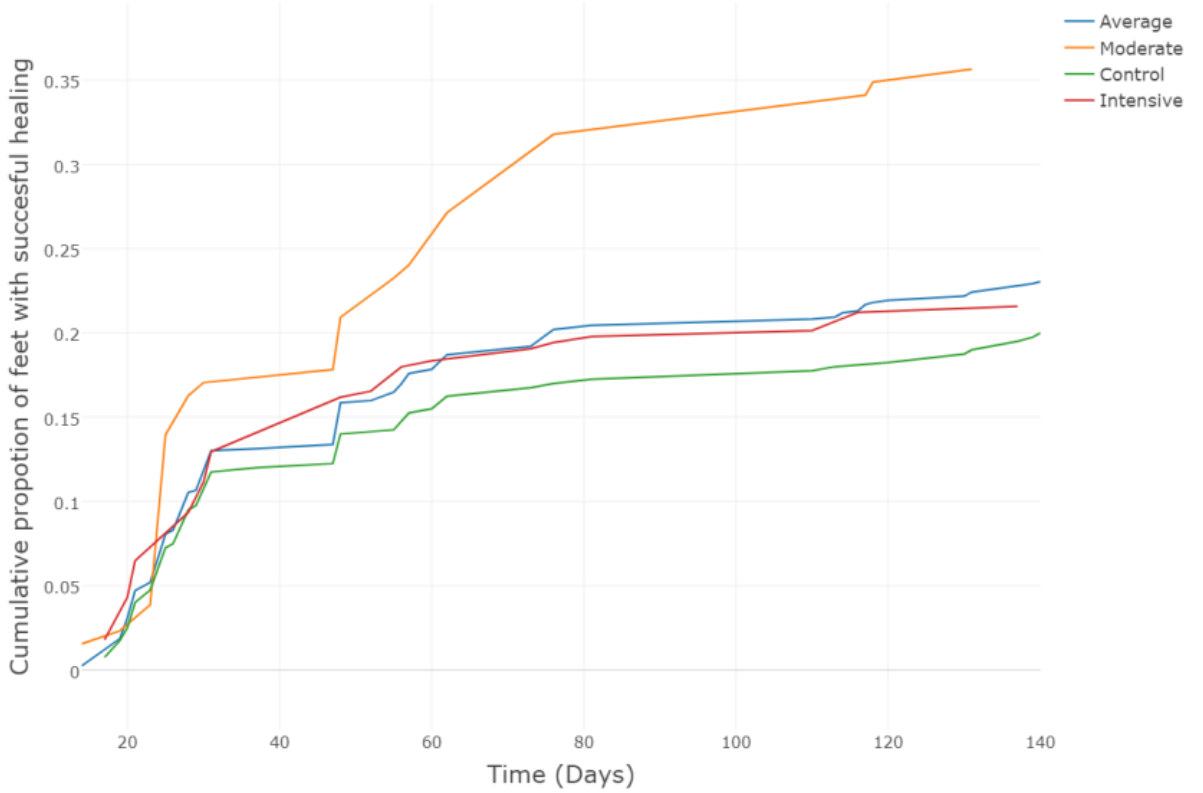


* Time-dependent covariates; ” number of feet “are transitions between categories during the follow-up trial period.

Healing Effect

Initially, 1,107 hind feet (53%) out of 2,072 hind feet (1,036 cows) were affected with active or inactive bDD lesions. However, of these feet, 300 were excluded from the dataset because their visits were spaced by more than 45 days. Therefore, 807 hind feet of 508 cows from 10 herds were included in the analysis (Figure 2). Among these cows, only 299 had bDD lesions on both hind feet at the start of the trial. Significant baseline differences were found between the feet allocation groups for cow feet hygiene, preventive hoof-trimming, initial prevalence, proportion of heifers and herd size (Table 3).

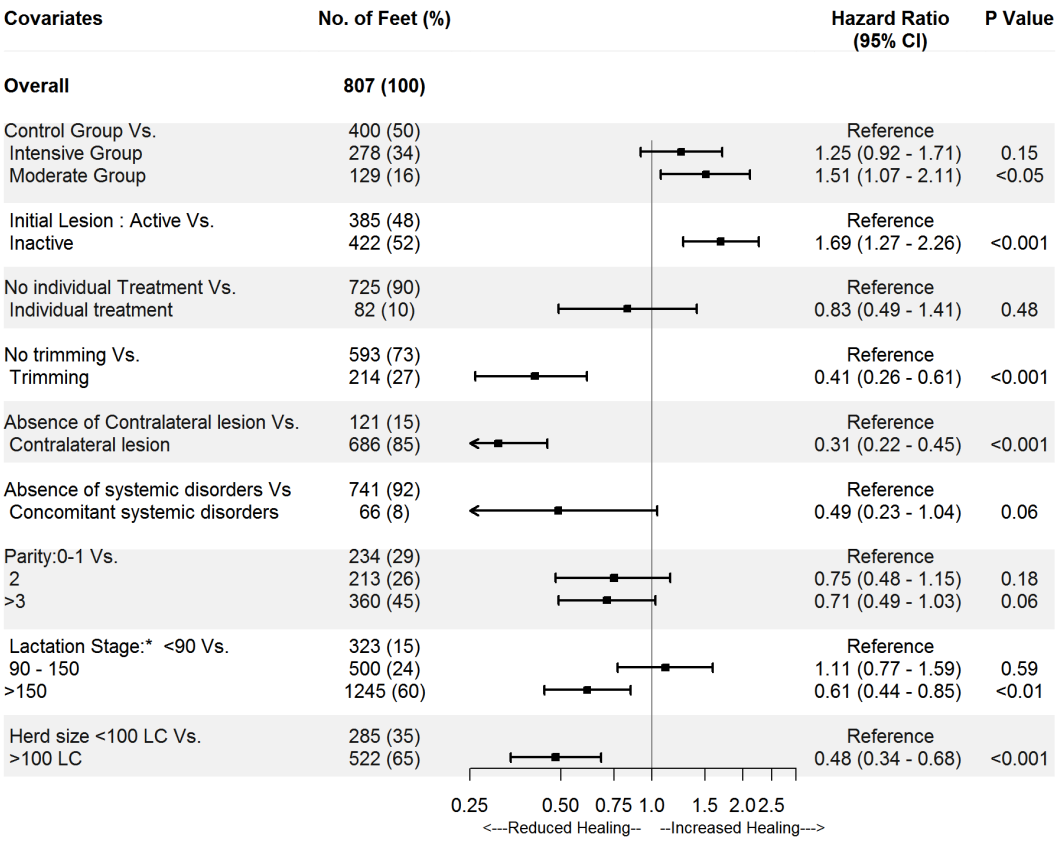
Figure 5. Cumulative proportion of feet with successful healing of digital dermatitis (bDD) lesions over time according to the three allocation groups and the overall average, respectively (moderate, intensive and control).



Among all of the feet included in the analyses, 186 (23%) achieved the healing either of an active lesion (74) or an inactive lesion (112). The median time before the healing of a bDD lesion was 46 days (30 to 140 days), and on average the healing rate was 5% between two visits. According to the group allocation, the mean healing rates by month were 4%, 8% and 5% in respectively the control, MR, and IR (Figure 5). From the multivariate analyses, six covariates were significantly associated with the risk of bDD healing (Figure 6). Thus, the time to heal was improved in inactive lesions and by the use of Pink-Step™ footbaths in IR. Otherwise, in feet trimmed during the trial period, the time to heal was reduced. Lesions in cows which either had a contralateral lesion or were at late lactation were identified to be at a higher risk of persisting. Moreover, in larger herds, the risk of lesion persistency was increased. A single interaction between the initial lesion aspect and the allocation footbath group was detected, indicating that feet with an active lesion in the IR group have a reduced time to heal compared to active lesions on feet allocated to the MR and control group. None of the others covariates included in the multivariate model or their interactions were

statistically significant in the multivariable model. Finally, the estimated variance of the cluster effect at the farm level was 0.0073 (SE: 0.0032), and at the cow level 1.52 (SE: 0.28).

Figure 6. The effect of footbath regimen adjusted for herd, cow, and feet characteristics on the healing of digital dermatitis (bDD) lesions in the nested survival model of the observations of 807 hind feet from 508 cows from 10 French dairy herds involved in a clinical trial.



* Time-dependent covariates; "number of feet" are transitions between categories during the follow-up trial period.

DISCUSSION

The findings of this investigation indicate that the collective disinfection of herd feet using Pink-step™ footbaths significantly improved the healing of bDD lesions when administered at an intensive frequency. The time to healing of bDD lesions was increased importantly in feet with active lesions, in trimmed feet, in cows in late lactation, in cows with contralateral lesions, and especially in larger herds. Otherwise, the occurrence of bDD lesions was mainly affected by feet cleanliness at the cow and farm level, and no preventive effect of the footbath solution (Pink-step™) was evidenced.

The originality of our investigation was to conduct a controlled clinical trial allocating into the same cow the control and the intervention groups. This approach allowed the drastic reduction of more than 3 times the sample size of the trial, which is an important limitation in veterinary studies (Ariza et al., 2017). Furthermore, all of the feet shared the same risk whatever group they were allocated to (control or footbath), therefore reducing the farm effect The findings of this investigation also enabled the estimation of the correlation between the feet of a same cow regarding the bDD status, a parameter which has been largely suspected but not until now reported. This highlighted the importance of developing and implementing collective prophylactic strategies. The trial was conducted on 10 farms in an effort to encompass the diversity of local herd management practices. Likewise, the multiple observations recorded over a long trial period were conceived to increase the precision of the measurements and to capture differences in the farming environment over time (Ariza et al., 2017). Therefore, due to both this and the dynamic nature of bDD, the trial was designed for a survival analysis, which enables one to adjust for covariates that change over time, such as feet cleanliness or the lactation stage. Additionally, the nested survival model used for the analyses accounted for the heterogeneity caused by unmeasured covariates at the farm and cow level in the same model. In turn, due to the high prevalence of bDD lesions and the high frequency of observations planned, a scoring methodology which had no impact on daily farming practices had to be adopted even if it was less accurate than bDD scoring on restrained cows in a trimming chute (Se $\geq$0.90; Sp $\geq$ 0.80) (Relun et al., 2011). Nevertheless, the inter-digital space remains hard to approach using this methodology and therefore score misclassifications might lead to underestimating the hazards for all the covariates (Dohoo et al., 2003). Additionally, although the two investigators received the same lesion scoring training, our trial protocol failed when accounting for the inter-observer agreement. However, each the farm was followed completely for a single investigator the random farm effect might have reduced this bias effect. To reduce this risk of over or underestimation evidenced by the diagnosis of "M1" or "M3" stages (Cramer et al., 2017), for the data analyses the "M5" stages were gathered into active, inactive and healthy stages and consequently, the healing or occurrence of bDD lesions was mainly determined by the presence or the absence of a healthy stage. In contrast with prior studies, and to avoid a potential overestimation of the footbath effect, the "M3" and "M4" stages were considered in the models as a diseased status. This original approach is one of the important features of the current trial. Finally, evaluating in commercial farms a footbath solution which its confirmed efficacy was tested mimicking field

conditions, might have enhanced the likelihood for evidence the effectiveness of the product by their implementation under this optimal usage conditions.

Few high-quality trails have reported footbath solutions to be effective (Thomsen, 2015). To our knowledge, the only peer-reviewed controlled trial that found good results was that of Solano et al., (2017), which used standardized footbaths of copper sulfate at 5% weekly. Other studies also have reported successful results using copper sulfate at 5% for the healing of bDD lesions, however, the solution was administered by collective spraying (Relun et al., 2012). The scarce evidence related to footbaths may indicate the difficulties entailed in the design and evaluation of such clinical trials, or the small effect of footbaths in practice when farm conditions are far from ideal for their implementation. The present trial also reports a beneficial effect of footbaths using a safe and a biodegradable solution. Beneficial effects were only evidenced in the healing of bDD lesions. Beyond the bactericidal effect of footbaths on bDD lesions, a potential mechanism of Pink-step™ for improving skin healing may be the presumed dermal regenerative effects of the glycolic acids present in the biocide solution (Green et al., 2009). Otherwise, it is important to note that due to the design and duration of this trial, it was not possible to record lesion recurrence, a phenomenon already described in individual treatment trials which should be of interest when evaluating the long-term effectiveness of footbaths. Therefore, future studies must focus on the possible recurrence or recrudescence of bDD lesions and the effective healing of active lesions. The increased intensity in footbathing has previously been noted as beneficial by other studies (Holzhauer et al., 2012; Solano et al., 2017). These benefits were also evidenced in this study by the healing rates recorded during the first months and the healing efficacy evidenced by the IR group. The frequencies implemented in the current trial changed over the time expecting to resemble the field conditions in France in which footbaths are empirically used, stayed that the footbaths usage is reduced during the summer season. Therefore, additional studies are necessary to clarify the relation between the intensity of footbathing, the influence of seasons, and the effectiveness of such measures. Moreover, as noted in a previous investigation, future studies must implement standardized footbath dimensions to ensure the optimal performance of the disinfectant products studied and to enable reliable and comparable results (Solano et al., 2017).

Table 1. A detailed description of the covariates investigated during the clinical trial.

Level	Covariates	Group	Definition
Feet	Footbath regimen group allocation	Control Moderate Intensive	Allocation group.
	Initial Score	M4 – 3 M1-M2-M4.1	Foot score recorded at the analyses inclusion according to Berry et al. 2010.
	Hoof-trimming	Yes No	Feet which have received a hoof-trimming during the follow-up period until censoring.
	Individual Treatment	Yes No	Feet which have received a treatment during the trial period until censoring.
Cow	Lesion on the contralateral foot	Yes No	The presence of a lesion in the contralateral foot during the follow-up period until censoring.
	Cow parity at the start of the trial	≥ 3 2 0-1	Number of calving's at the start of the trial follow-up.
	Milk yield potential	Low (<36.4) Moderate (36.4-42.2) High (>42.2)	Based on the milk-production yield recorded during the preceding lactation to the trial start. The values were adjusted by parity.
	Concomitant Disease	Yes No	Cows experiencing a concomitant systemic disease during the trial period until censoring.
	Lactation Stage[a]	DIM <90 DIM 90–150 DIM ≥150	Days in milk across the follow-up visits.
	Cow Feet hygiene[a]	Fair (< 2) Poor (≥ 2)	Score of the cow feet hygiene across the follow-up visits.
Farm	Preventive Hoof-trimming	Yes No	Farms which have practiced a hoof-trimming for a large part of the herd at least once during the 2 previous months or during the trial.
	Initial Prevalence	< 25 25-35 >35	Prevalence of active lesions at the pre-study visit.
	Heifer Proportion	< 10% > 10%	Heifer proportion introduced in the herd during the trial period.
	Herd size[b]	< 100 LC > 100 LC	Average number of lactating cows during the trial period.
	Farm feet hygiene[a]	Good < 1.5 Fair to Poor ≥1.5	Average scoring of the herd feet across the follow-up visits.

[a]Time-dependent covariates
[b]LC=Lactating Cows

An association between footbathing practices and a reduced risk of bDD occurrence (preventive effect) was not evidenced in this trial. The lack of effectiveness of both footbathing regimens to prevent bDD lesions might be related to the weak effect of the footbath solution, which was probably as well inferior to the expected effect calculated for sample size necessary for the trial. Likewise, as the correlation between feet was unknown

before this trial, the current sample size was probably inferior to that needed to evidence small effects on bDD occurrence. Additionally, in this trial all of the feet were cleaned for the scoring at each visit, and therefore every healthy foot periodically received what can be perceived as a preventive intervention. Consequently, the disinfectant efficacy of a solution over an already cleaned foot might be imperceptible, in other words, the preventive effectiveness of disinfectants might be roughly equivalent to regular cleaning with water (Thomsen et al., 2012). Furthermore, as the time to healing was enhanced for inactive lesions compared to active lesions, footbathing practices might act as a potential protective measure, controlling bDD reservoirs and the recrudescence of their lesions. One of the important limitations of this trial was related to the bias produced by the absence of investigator blinding during the follow-up. Although an objective methodology was implemented to score the lesions, investigators could not be blinded to the footbath allocation of feet due to the distinctive pink color of the solution studied. Future clinical trials in bDD control should aim to blind the investigators to ensure an objective assessment of the lesions.

Otherwise, the split-body design of this trial might lead to some limitations. Although all of the feet had the same baseline probability of developing or healing a bDD lesion, significant differences between the allocation groups were detected after randomization. The imbalances between the baseline characteristics of the feet groups highlight the importance of considering confounding by adjusting for all potential effect modifiers in the data analysis. Another limitation related to the split-body design is the possible carry across effects within feet. On one hand, the pathogens in untreated feet might have remained undisturbed during the trial and thus may have increased the infection pressure in the environment. On the other hand, the disinfectant effect of footbaths might have reduced to an important degree the densities of environmental pathogens, enhancing as well the healing rates and decreasing the risk of lesion occurrence in untreated feet. In both scenarios, an under-estimation of the true effect of

footbaths was expected. However, these methodological limitations inherent to this design might be compensated by the reduced sample size of the design. In this trial, a low between-cow variance (random effect at the farm level) reflected the homogeneous sample analyzed, being coherent with the strict inclusion parameters applied. Otherwise, the high within-cow variance (random effect at the cow level) estimated suggests a greater correlation of the survival times for feet belonging to the same cow. This strong correlation was evidenced, for example, by the reduced risk of bDD healing in cows which have contralateral lesions. Future studies implementing the split-body design must consider the implications of this choice, such as establishing an appropriate data analysis and including a smaller number of farms but ones with larger herds.

The individual treatment of active lesions was scarce in the data set. Although their effectiveness is supported by scientific literature (Apley, 2015), in this trial the time to healing was not improved by individual treatments. A possible explanation may lie in how the farmer's decision to treat was altered by the trial environment which involved a close follow-up by the veterinarians involved in the trial. Similarly, it may be possible that only the most severe cases of bDD capture the attention of farmers, and such lesions are frequently less responsive to treatment (Evans et al., 2011). Another explanation for the lack of efficacy of Oxytetracycline treatment involves an incorrect or incomplete implementation of the protocol (Sawant et al., 2005; Relun et al., 2013a). Likewise, during the trial, the feet were trimmed mainly for therapeutic reasons instead of prophylactic reasons. Therefore, the healing benefits of trimming might be missed in those severe cases which persisted longer than the trial period. Similarly, the presumed preventive influence of trimming was not evidenced in the trial, probably because the dirty conditions have a larger impact on the lesion occurrence than the prophylactic measures implemented in the included farms.

Table 2. Main farms characteristics before side randomization

Allocation	Farm ID	Lactating Cows	Heifers	Initial bDD prevalence (%)[a]	Housing system	Global Feet Hygiene[b]	Grazing Practices	Milking system	Preventive Hoof Trimming Practices	Number of feet included in the healing outcome (%)	Number of feet included in the preventive outcome (%)
Moderate Regimen	1	45	14	36	Cubicles	2.00	Yes	Conventional	Yes	59 (7)	26 (3)
	2	115	37	18	Cubicles	2.42	No	Conventional	Yes	75 (9)	118 (16)
	3	47	13	47	Cubicles	2.00	Yes	Conventional	Yes	32 (4)	21 (3)
	4	78	26	33	Cubicles	2.38	Yes	Conventional	Yes	75(9)	43 (6)
	5	57	22	21	Free-stalls	2.30	Yes	Conventional	No	20 (3)	41 (5)
Intensive Regimen	6	85	33	40	Cubicles	2.31	Yes	Conventional	Yes	99 (12)	45 (6)
	7	145	49	59	Cubicles	2.18	Yes	Rotary	Yes	180 (22)	99 (13)
	8	105	35	30	Cubicles	2.11	Yes	Conventional	Yes	81 (10)	123 (17)
	9	123	38	31	Cubicles	2.02	Yes	Rotary	Yes	103 (13)	138 (19)
	10	99	37	20	Cubicles	2.03	Yes	Conventional	No	83 (11)	89 (12)
Summary[c]	10	899 (90)	304 (30)	34 %[d]	Cubicles =9 Free-stalls=1	2.17[d]	Yes=9 No=1	Conventional=8 Rotary =2	Yes=8 No=2	807 (78)	743 (74)

[a]Prevalence of active lesions at the pre-study visit
[b]Average feet hygiene score (1-3) among the animals recorded at the pre-study visit
[c] Total count and mean proportion in parenthesis, unless otherwise specified.
[d]Total average

As has been consistently noted in other epidemiological studies (Relun et al., 2013b), poor feet hygiene was confirmed as the most important factor influencing bDD lesion occurrence at both the cow and farm level. However, although previous studies have suggested that poor feet hygiene might delay the healing of bDD lesions (Relun et al., 2012), we were unable to identify a relation between feet cleanliness and time to bDD healing. Experimental studies have confirmed that dirty and wet environmental conditions are the main determinants for the occurrence of bDD lesions (Gomez et al., 2012). Similarly, field studies have identified different factors which may alter environmental hygiene and thereby increase the risk of bDD, such as housing in cubicles, grooved concrete floors, and reduced manure scraping rates (Somers et al., 2005; Barker et al., 2009; Oliveira et al., 2017). In practice, feet hygiene is a measure of the impact of several factors that condition the farming environment in which

cows stand. Therefore, as the bDD lesions and the treatments implemented were centered at the foot level, focusing cleanliness observations on the foot rather than the leg might improve the precision concerning the association of the different factors which may affect feet hygiene and bDD lesions (Guatteo et al., 2013). Otherwise, other studies have reported an important seasonal effect on the risk of bDD (Argaez-Rodriguez et al., 1997) and other feet disorders (Murray et al., 1996). Although this factor is mostly related to a limited access to pasture, this association was not evidenced in the present study in relation to grazing practices or their impact on feet cleanliness and bDD healing or occurrence.

Beyond the heterogeneity between the farm characteristics and their herd management factors, the heterogeneity within cows indicates that other factors affecting the bDD dynamics differ between cows and their feet. Further investigation at the cow level therefore might enhance current understanding of bDD, highlighting, for example, the role of skin microbiota or the immune response in the disease outcome.

CONCLUSION

This investigation confirmed that multiple factors interact in the dynamics of bDD lesions determining their occurrence and persistence. Strategies to control the disease therefore must rely on the simultaneous implementation of multiple measures for improving feet environment and for reducing the severity and the presence of infected cows. The results of this study revealed the utility of footbathing practices for improving the time to healing of bDD lesions when the Pink-step™ solution was administered at an intensive frequency. Finally, to limit bDD lesion occurrence, trial findings confirmed the crucial importance of implementing efficient measures to improve feet hygiene.

Table 3. Feet baseline characteristics according to the allocation group, the covariates recorded and the outcomes of interest.

Covariates	Group	*Healing Outcome*					*Preventive Outcome*				
		Control (%)	Intensive (%)	Moderate (%)	Total (%)	p-value (Chi ^{2})	Control (%)	Intensive (%)	Moderate (%)	Total (%)	p-value (Chi ^{2})
Initial Score	M4 – 3	214 (53)	135 (49)	73 (57)	422 (52)	0,25 (2.73)	-	-	-	-	-
	M1-M2-M4.1	186 (47)	143 (51)	56 (43)	385 (48)						
Cow parity	1	115 (28)	83 (30)	36 (28)	234 (29)	0,50 (3.33)	189 (50)	124 (50)	60 (49)	373 (50)	0,88 (1.15)
	2	103 (29)	68 (25)	42 (33)	213 (26)		83 (22)	49 (20)	29 (24)	161 (22)	
	≥ 3	182 (45)	127 (45)	51 (39)	360 (45)		102 (27)	74 (30)	33 (27)	209 (28)	
Milk Yield Potential	Low (<36.4)	101 (26)	63 (23)	42 (34)	206 (27)	0,21 (5.76)	132 (35)	77 (31)	46 (38)	255 (34)	0,56 (2.96)
	Moderate (36.4-42.2)	157 (41)	120 (45)	49 (40)	326 (42)		112 (30)	78 (32)	30 (25)	220 (30)	
	High (>42.2)	126 (33)	86 (32)	32 (26)	244 (31)		130 (35)	92 (37)	46 (37)	268 (36)	
Lactation Stage	DIM <90	109 (27)	77 (28)	37 (29)	223 (28)	0,70 (2.15)	143 (39)	93 (38)	43 (35)	279 (38)	0.90 (1.02)
	DIM 90–150	85 (21)	58 (21)	20 (15)	163 (20)		61 (16)	45 (18)	20 (16)	126 (17)	
	DIM ≥150	206 (52)	143 (51)	72 (56)	421 (52)		168 (45)	107 (44)	59 (48)	334 (45)	
Cow Feet hygiene	Fair (< 2)	315 (78)	215 (77)	103 (80)	633 (78)	0,82 (0.37)	283 (76)	206 (83)	78 (64)	567 (78)	≤0,001 (17.29)
	Poor (≥ 2)	85 (22)	63 (23)	26 (20)	174 (22)		91 (24)	41 (17)	44 (36)	176 (22)	
Preventive Hoof-trimming	Yes	328 (82)	240 (86)	97 (75)	665 (82)	≤0,05 (7,62)	318 (85)	200 (81)	110 (90)	628 (84)	0,06 (5.42)
	No	72 (18)	38 (14)	32 (25)	142 (18)		56 (15)	47 (19)	12 (10)	115 (16)	
Initial Prevalence	< 25	93 (23)	38 (14)	47 (36)	178 (22)	≤0,001 (27.73)	123 (33)	47 (19)	78 (64)	248 (33)	≤0,001 (75.42)
	25-35	128 (32)	95 (34)	36 (28)	259 (32)		154 (41)	127 (51)	23 (19)	304 (41)	
	>35	179 (45)	145 (52)	46 (36)	370 (46)		97 (26)	73 (30)	21 (17)	191 (26)	
Proportion of Heifers	< 33%	131 (33)	55 (20)	83 (64)	269 (33)	≤0,001 (78.84)	156 (42)	66 (27)	81 (66)	303 (41)	≤0,001 (53.49)
	> 33%	269 (67)	223 (80)	46 (36)	538 (67)		218 (58)	181 (73)	41 (34)	440 (59)	
Herd Size[a]	< 100 LC	144 (36)	49 (18)	92 (71)	285 (35)	≤0,001 (111.35)	90 (24)	24 (10)	62 (51)	176 (24)	≤0,001 (76.38)
	> 100 LC	256 (64)	229 (82)	37 (28)	522 (65)		284 (76)	223 (90)	60 (49)	567 (76)	
Farm Feet hygiene	Good to Fair N < 2	181 (45)	127 (46)	46 (36)	354 (44)	0,38 (1.90)	151 (40)	132 (53)	21 (17)	304 (41)	≤0,001 (44.42)
	Poor ≥2	219 (54)	151 (54)	83 (64)	453 (56)		223 (60)	115 (47)	101 (83)	439 (59)	

[a]LC=Lactating Cows

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B. Description of the dynamics of the skin microbiota in feet affected by bovine digital dermatitis, before and after the implementation of a footbath disinfectant

In collaboration with Dr. Dörte Döpfer and her team from the School of Veterinary Medicine of the University of Wisconsin.

Description of the dynamics of the skin microbiota in feet affected by bovine digital dermatitis, before and after the implementation of a footbath disinfectant

INTRODUCTION

The increased prevalence of lameness and its impact on the productivity and animal welfare reflects one the main issue facing modern dairy farming. The first cause of infectious lameness is bovine digital dermatitis (bDD) (Laven and Lawrence, 2006), a disease spread across the world and characterized for ulcerative and painful lesions located in the inter-digital cleft.

Digital dermatitis is considered as a multifactorial disease. The presence of specific *Treponema* species on feet suffering from cutaneous maceration is recognized as the major components involved in disease development. Nevertheless, to induce the disease in controlled studies, macerates of bDD lesions were importantly more effective than the bDD *Treponemas* alone (Gomez et al., 2012). These findings support the recent highlights from metagenomics studies on the subject which revealed that even if treponemes are the most representative bacteria in bDD lesions, other different families of bacteria might be involved and interacting with them to promote the disease development such as mycoplasma for instance (Chapter 1, Section 4.1). However, most of those others pathogens frequently involved in bDD are ubiquitous in the farm environment. Therefore, the putative incrimination of a specific bacteria for their simply presence seem to overestimate their role in the disease. Across the different studies investigating the microbial structure of the bDD, the importance of *Treponema spp.* remains undisputable (Brandt et al., 2011; Zinicola et al., 2015). Besides, the connection between bDD infection and some other bacterial phylum, genera, and species across the studies have been pointed, such as a broad range of *Firmicutes* (Santos et al., 2012), specifically *Mycoplasma* (Krull et al., 2014; Nielsen et al., 2016); some *Bacteroides* (Yano et al., 2010), specifically *Porphyromonas levii* (Berry et al., 2010); different *Proteobacteria*, such as *Campylobacter* (Döpfer et al., 1997), and *Dichelobacter nodosus* (Rasmussen et al., 2012; Knappe-Poindecker et al., 2013); and finally *Fusobacterium necrophorum* (Moe et al., 2010b). All these facts reinforce the conception of bDD as a poly-microbial and poly-treponemal disorder and thereby suggest that particular bacterial communities (microbiota) may drive the lesion environment and affect the clinical evolution of such lesions over the time. These insights represent (i) a new factor to explore the

pathophysiology of the disease and (ii) a putative outcome of interest to look at when assessing the effectiveness of collective or individual treatments for the healing of bDD lesions, not only from a clinical point of view but from a bacteriological perspective.

Despite several advances in understanding bDD, the current control measures implemented in dairy farms have demonstrated variable effectiveness. Control strategies are focused on the reduction of the main risk factors for the disease, such as the wet and unhygienic conditions of the barns or the presence of reservoir cows in the herds. Therefore, among the bDD control strategies, the individual treatment of ulcerative lesions and the administration of collective treatments to the entire herd are strongly advised to limit the spread of the disease (Relun et al., 2012, Solano et al., 2017). Currently, the collective treatments are mainly administered by footbaths and encompass multiple types of disinfectants. However, the disinfectants most commonly used represent a hazard either for the environment or for the farmers. Even more, there is a lack of evidence about the effectiveness and the conditions in which these collective treatments could improve the healing or prevention of the bDD lesions (Ariza et al., 2017). Therefore, it seems that the development of alternative and safe disinfectants of confirmed effectiveness might benefit the dairy farming industry. Theoretically, effective treatments for the bDD control must regulate and drive the microbiota of foot with clinical lesions to those of a normal healthy skin by preventing or decreasing the pathogens proliferation. Nevertheless, as the clinical lesions may evolve in a dynamic way driven by multiple factors, it seems crucial to investigate in a longitudinal follow-up setting if there are specific microbial profiles or dynamic patterns in the skin microbiota which may explain the dynamics of the disease.

Therefore, in this study, the microbiotas of the foot skin of 10 cows, from 5 different bDD infected dairy farms, were gathered to investigate and explore their dynamics before and after the implementation of a footbath regimen using the Pink-Step™ solution, and thereby evaluate the potential impact of footbathing on those microbiotas across the time.

MATERIAL AND 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, France (CERVO, France) (registered number: CERVO-2016-12-V).

Study population

The study was conducted on 5 dairy farms located in western France. These farms were known to have experienced bDD for over at least the last two years. The herds were composed of Holstein cows to limit the putative breed effect, and in average 90 cows were milked twice a day. Cows were housed in cubicles and have no access to pastures during this study to avoid the putative cleaning impact of grazing practices over the feet hygiene. Table 1 presents the main characteristics of the 5 farms participating in the study.

Table 1. Main farms characteristics before side randomization

Farm ID	Lactating Cows	Heifers	Initial bDD prevalence (%)[a]	Housing system	Global Feet Hygiene[b]	Grazing Practices	Milking system	Preventive Hoof Trimming Practices	Allocation of the feet sampled
1	99	37	20	Cubicles	2.03	Yes	Conventional	No	Control
2	85	33	40	Cubicles	2.31	Yes	Conventional	Yes	Control
3	123	38	31	Cubicles	2.02	Yes	Rotary	Yes	Footbath/ Control
4	145	49	59	Cubicles	2.18	Yes	Rotary	Yes	Footbath
5	105	35	30	Cubicles	2.11	Yes	Conventional	Yes	Footbath
Total	562	198	36 %[d]		2.13 [d]				

[a]Prevalence of active lesions at the pre-study visit
[b]Average feet hygiene score (1-3) among the animals recorded at the pre-study visit
[c] Total count and mean proportion in parenthesis, unless otherwise specified.
[d]Total average

The enrolled farms from this study participate simultaneously in a controlled clinical trial which evaluates the effectiveness of a footbath solution for the healing of bDD lesions (Chapter 4.1). 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™). During the trial each farm implemented a split-footbath which allowed the administration of the biocide to one side of the cows, the other side being used as an empty control bath. 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 bDD by using 2 applications

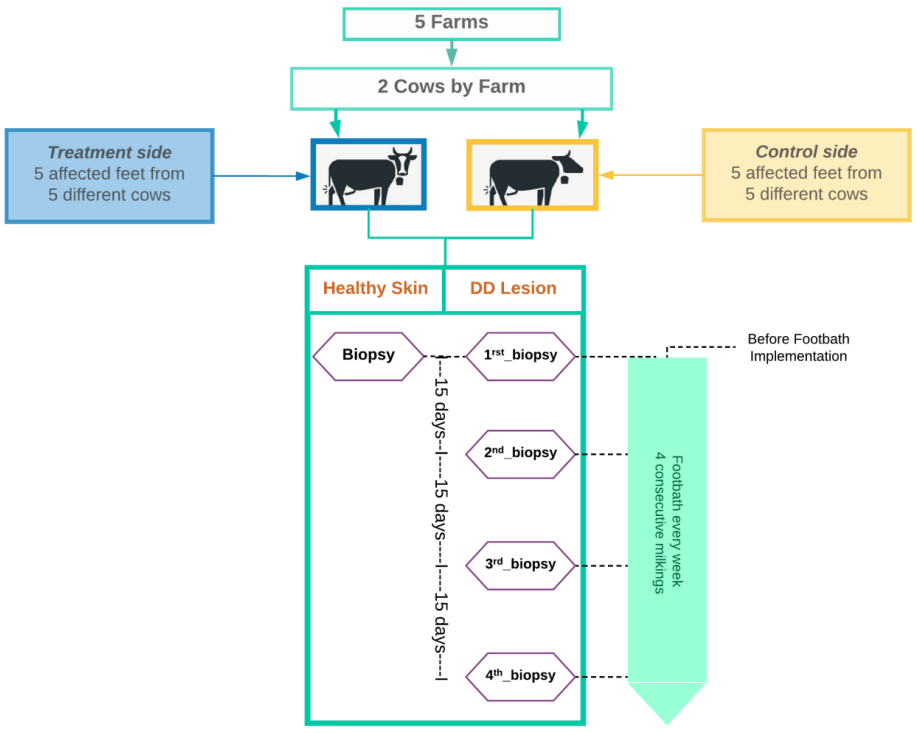
of oxytetracycline (30 mg/ml) (Oxytetrin™, MSD) 2 days apart. For the current investigation, from each farm, two animals suffering from a bDD lesion were chosen by their owners.

Therefore, for ethical and welfare concerns, the study population consisted of 10 different affected feet from 10 different cows. To compare the dynamics of bDD lesion according to the footbath treatment, from the 10 effected 5 belonged to the control side and the remaining 5 to the footbath side.

Follow-Up and Data Collection

Farms were visited by 2 investigators trained by practical lessons to practice the biopsies. To perform the biopsy sampling every cow was carefully restrained in a trimming chute. For each foot, the skin 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 a sample with a sterile biopsy punch (6 mm). Then, the incisional samples were washed with the PBS solution and stored in sterile 2.0 ml microcentrifuge tubes at -20°C until analysis. Finally, an aerosol bandage of aluminum was sprayed directly over the incisional biopsy wound of each cow. In case of pain detected by famers, NSAIDS were provided after the biopsy sampling.

Figure1. Sampling protocol scheme.



The biopsy sampling began immediately before the start of the footbaths. For the first sampling and for this unique occasion each cow was sampled twice in the same foot, allowing recovering a healthy skin sample and a bDD lesion sample. Afterwards, during the following 3 visits, single lesion biopsies were sampled at intervals of 15 days as a compromise for allowing the partial skin recovery while investigating the dynamics. The location of each biopsy was approximately closer to the site where the precedent sample was taken (Figure 1). In total each animal was sampled 5 times over a period of 45 days. Thus, at the end of the study, a total of 50 biopsies were recovered, 25 from effected feet allocated to the control group (Empty footbaths) and 25 from affected feet allocated to the footbath treatment (Pink-Step™ footbaths). All lesions sampled were photographed and scored using the M5 score system (Berry et al., 2010). 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.

DNA Extraction, PCR amplification, illumina MiSeq sequencing.

All procedures were performed in collaboration with the School of Veterinary Medicine of the University of Wisconsin and the Team of Dörte Döpfer.

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 V4 region of the bacteria 16S rRNA gene was amplified by PCR, using primers ### (####) and (###), 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 the 3 PCR assays. The amplicons were extracted and purified from the gel using the Zymo gel extraction kit according to the manufacter’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 (### cycles) on the MiSeq platform (Illumina, Inc., CA, USA).

Sequencing data processing

The generated 16S rRNA gene sequences were processed through the open source MG-RAST pipeline (Glass and Meyer, 2011). All the sequences were screened for quality control. Additionally, other artifacts were removed from the dataset, such as reads that matched to bovine genomes. Finally, the retained reads were clustered into Operational Taxonomic Units (OTU) based on 97% identity and taxonomy was assigned using the SILVA database (Glöckner et al., 2017). Therefore, for every sample, their respective OTU counts represented the number of similar sequences assigned to a specific taxon.

Data analyses

The statistical analyses were performed with R (R Core Team, 2017). For the analyses, 4 different categorical covariates were created from the records encompassing potential factors supposed to impact the dynamics of skin microbiota. The covariates include: the sampling time points (Initial healthy skin, initial bDD lesion, 2nd lesion sample, 3rd lesion sample and 4th lesion sample), the allocation groups (control vs. footbath), the lesion aspect (proliferative vs. nonproliferative), and finally, the time since the last administration of an antibiotic to the foot (TLA) (no antibiotic vs 10-20 days vs > 20 days).

The analyses were conducted in 3 steps. First, using only the information from the first sampling time (Healthy skin and Initial bDD lesion), the baseline bacterial diversity within the sample (alpha diversity) was compared using ANOVA and Kruskal-Wallis tests. Similarly, in order to determine which taxonomic groups at baseline were different between the healthy and diseased samples, a negative binomial GLM model and the respective Wald test were used accounting for paired data (McMurdie and Holmes, 2014).
Second, the alpha diversity across the time was compared against the covariates of interest by the calculation of the Shannon diversity and Chao richness indexes and using linear mixed-effects models for test accounting for paired data (Hill, 1973; Chao and Chiu, 2014).

Third, to allow between samples comparisons, the OTU counts were normalized by variance-stabilization transformations using the package *Deseq2* (McMurdie and Holmes, 2014). Thereafter, relative abundances were calculated and pie charts were created using Krona for each animal sampled and for the samples group in the control and footbath group, respectively (Ondov et al., 2011). In order to calculate whether the overall microbial community (beta diversity) differed for the covariates studied, OTU were grouped into genera, and NMDS ordination on Bray-Curtis distance was constructed with *phyloseq* (McMurdie and Holmes, 2013). Thereafter, CCA (or PCA) were run from the OTU counts using the vegan package and tridimensional plots were designed for display samples across the time, the OTUs, and additional centroids representing the contribution of the covariates studied. Beta diversity differences were tested using adonis PERMANOVA.

RESULTS

In this study was explored the microbiota diversity from the skin of 10 cows affected by bDD across 4 subsequent samples over 45 days. Using 16S rRNA sequencing 1631292, high-quality sequences were obtained, with an average of 32625 (SE 18190) sequences per sample.

Table 2. Baseline alpha diversity at first sampling within each group of biopsy samples and within each allocation group.

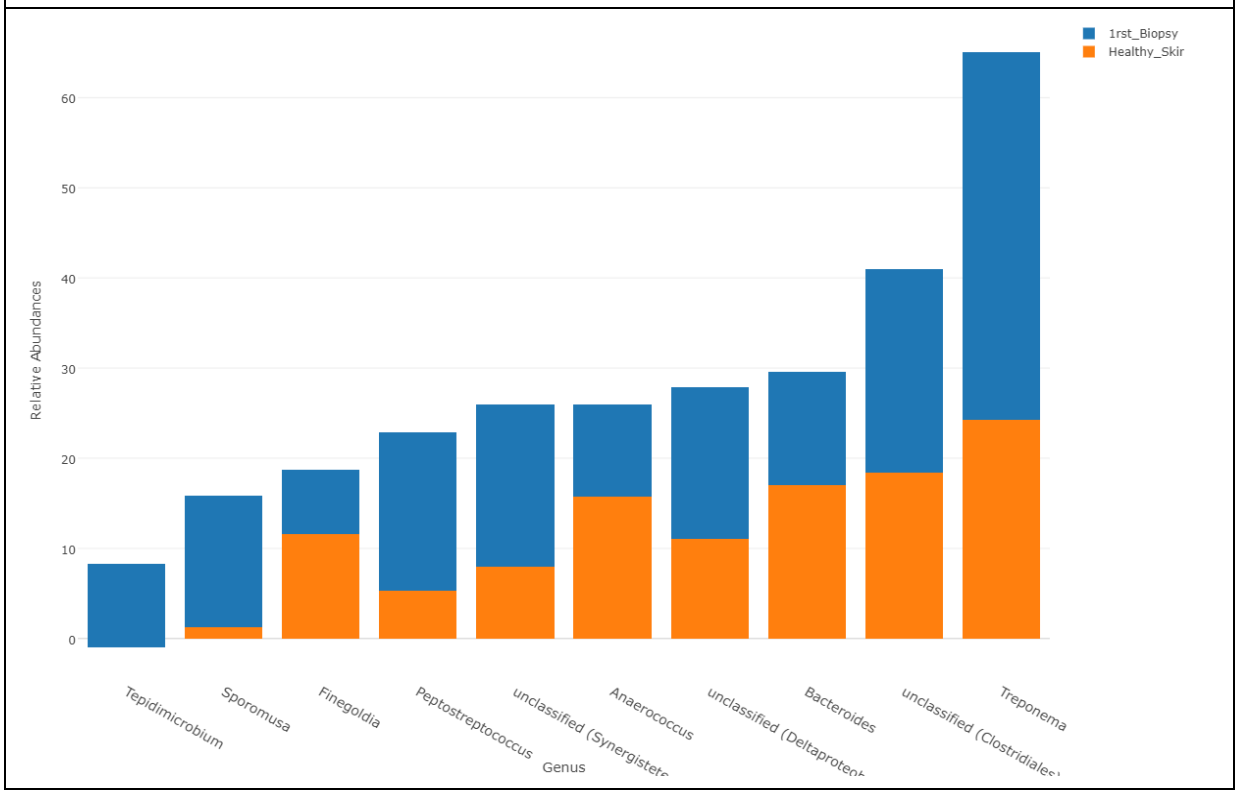
Biopsy samples	Alpha – diversity (Shannon index)			p-Value		
	Overall	Control	Footbath	Overall	Control Vs. Footbath	Between Farms
Healthy Skin	2.88 (0.78)	2.88 (0.68)	2.88 (0.95)	0.43	0.60	0.24
bDD Lesion	1.10 (0.60)	1.11 (0.62)	1.09 (0.66)	0.43	0.91	0.67

Baseline skin microbiota from healthy and bDD samples

At the first sampling time, no difference in the alpha diversity (Shannon index) was detected between the healthy samples and the bDD lesions samples neither according to their allocation group (footbath vs control) nor between the farms included (Table 2). The significant baseline differences between diseased and healthy samples were related to the following top 10 OTU from the phylum (genera) *Spirochaetes* (*Treponema*), *Firmicutes*

(*Anaerococcus, Finegoldia, Peptostreptococcus, Sporomusa, Tepidimicrobium*, and unclassified derived from *Clostridiales*), *Proteobacteria* (*Deltaproteobacteria*), *Bacteroidetes, Synergistetes*. The treponemes were the most different compared to healthy samples (Figure 2).

Figure 2. Principal baseline taxonomic differences between bDD lesion and healthy skin samples.



Differences in the microbiota diversity and richness between the samples

Within sample diversity metrics (Shannon and Chao indexes) indicated no difference between the samples from foot within footbath and control groups ($P>0.49$), the TLA categories ($P>0.74$) or between farms ($P<0.09$). Among the samples obtained from the footbath group, the alpha diversity of bDD samples estimated across the different time points was not significantly different from the initial healthy samples. Contrarily, among the samples obtained from the control group, compared to the initial healthy samples, the alpha diversity of bDD samples remained significantly different across time points until the last sampling (Table 3). Otherwise, a significant difference was evidenced between the alpha diversity of the different sampling time points studied ($P<0.001$), and between proliferative and non-

proliferative lesions (P<0.05). Differences in the richness index were only detectable between farms (P<0.05) (Figure S1). Similarly, the OTU numbers observed in every sample were not different across the time neither between the allocation groups nor comparing in others covariates.

Differences in the microbiota composition between the samples

Through the visual assessments of the pie chart, no differences in the relative abundances between the footbath and control groups were detected over time (Figure 3 and 3.1). Otherwise, high variability in the relative bacterial abundances was evidenced within cows (Figure S3). After the visual inspection of the tridimensional plots, differences between the OTU grouped according to the sampling time points (Figure 4) or to the lesion aspect (Figure 4) seemed evident and contrary to the similarities appreciated between the allocation groups over the time (Figure 5), or for the TLA (Figure 6). Visual differences in the beta diversity of the samples were as well perceived between farms (Figure 8). All these findings were confirmed statistically. Therefore, the overall microbial community differed significantly over time, in terms of its composition, between the footbath and control groups, the farms and the lesion aspect. The figures generated display individually the categories studied for every covariate (Figures 4, 5, 6, 7, and 8).

Table 3. Alpha diversity metrics of bacterial communities of bDD lesions across the time and according to the allocation group[1].

Covariates	Control Samples			Footbath Samples		
	OTUs	**Richness (Chao index)**	**Diversity (Shannon index)**	**OTUs**	**Richness (Chao index)**	**Diversity (Shannon index)**
Healthy Samples	114.4	156.10^{a}	2.88^{a}	121.4	175.06^{a}	2.89^{a}
1^{rst} bDD samples	87.8	132.03^{a}	1.12^{b}	74.8	99.59^{b}	1.09^{b}
2nd bDD samples	71	104.82^{a}	0.93^{bc}	107.2	147.53^{b}	1.88^{a}
3rd bDD samples	78.6	108.12^{a}	1.50^{bcd}	100.8	143.62^{b}	1.85^{a}
4th bDD samples	103	129.48^{a}	2.26^{ad}	107.4	164.36^{b}	1.91^{a}
SEM	17.71	20.75	0.81	17.12	28.90	0.63
P value		<0.001	<0.001		<0.001	<0.001

[1]Means within the same column with different subscripts are significantly different from one another.

DISCUSSION

The present investigation described over time the dynamics of the skin microbiota in feet affected by bDD taking into account putative factors such as treatment. For the first time, the microbiotas of bDD lesions were studied over time within the same animal. Whereas in other animals, the microbiotas were compared between treated or not treated or healthy and affected samples coming from separate animals From the findings of this investigation, no difference was evidenced between the skin microbiota diversity or richness within and among the samples of feet receiving the Pink-Step footbaths compared to the control feet. The skin microbiota in affected feet differed across the time and independent of the footbath or individual treatment usage. Similarly, the microbial diversity of non-proliferative lesion was statistically different from proliferative lesions. Between farm, differences were detected indicating that particularities in unmeasured factors at farm level may affect the microbial structure of the foot skin.

Our findings support the concept of multi-microbial disease previously highlighted in other studies by metagenomics approaches (Krull et al., 2014; Zinicola et al., 2015; Nielsen et al., 2016). Similarly, treponemes were the microorganism most related to lesions when compared to the healthy skin. Besides, the microbiota profiles from healthy and bDD affected skin described in this study resembles partially to previous profiles reported (Santos et al., 2012; Zinicola et al., 2015). Compared to the findings of previous studies, coincidences in the main bacteria linked to bDD lesions were found for *Treponema, Fusobacterium* (Krull et al., 2014; Zinicola et al., 2015; Nielsen et al., 2016), *Peptostreptococcus* (Berry et al., 2010; Santos et al., 2012), *Mycoplasma* (Krull et al., 2014), *Synergistetes* (Santos et al., 2012), *Tissierella* (Krull et al., 2014), *Proteobacteria* (Yano et al., 2010), *Prevotella* (Berry et al., 2010), and *Corynebacterium* (Nielsen et al., 2016). Contrarily, from the bacteria consistently associated in previous studies with bDD, such as *Dichelobacter nodosus* (Rasmussen et al., 2012; Knappe-Poindecker et al., 2013) in this study was not related to the lesions or to play a major role in the disease dynamics. Furthermore, in the present study some particular genera, not previously involved with bDD, were as well detected at important levels. Indeed, the phylum *Firmicutes* was overrepresented by the genera *Sporomusa*, bacteria already identified in endodontic infections in humans (Rolph et al., 2001), *Finegoldia* an inhabitant of human mucocutaneous tissues (Raz-Pasteur, 2014), *Anaerococcus* an aerobic cocci opportunistic

pathogen in different human infections (Smith et al., 2016), and finally *Tepidimicrobium* an anaerobic bacteria generally increased during decomposition (Marchandin et al., 2003). However, the associations of these specific anaerobic bacteria have been already described in foot ulcers of humans (Murphy and Frick, 2013), and therefore probably they represent as well opportunistic flora of bDD lesions. Another explanation for these particular findings might be linked to regional particularities of the farms sampled. Indeed, being the first profiles studied in France no comparison can be done. Additionally, the fact that previous studies have not linked these bacteria to bDD might be explained by the original statistical procedures implemented in the analyses of the 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 after the rough normalization which may entail the loss of valuable information (McMurdie and Holmes, 2014). In other words, due to the method implemented for the data analyses, samples with a scarce number of sequences, as the retrieved in healthy samples, were included for the analyses as their low numbers of sequences reflected the true clinical nature of these samples. Contrary to other studies in which such samples with scare numbers of sequences are excluded in the quality screening.

One of the main limitations of this study was related to the nature and the environment of bDD lesions, which is characterized by the close contact with the ground and thereby with dirtiness. Indeed, the distinction of pathogens results in a challenge in such conditions and additionally the follow-up of the clinical evolution of the skin after an incisional biopsy in a contaminated environment was another challenge in the design of the present study. Therefore, from our clinical but subjective perception, all the sampled lesions followed a progressive recovery during the trial, which let us infer that these observations coincide with the dynamics evidenced in the skin microbiota over the time. 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 bDD. In this study, the changes evidenced in the microbiota allowed the clear distinction between diseased and healthy states. However, the potential usefulness of this tool for measuring the effectiveness of treatment strategies seemed inferior or at least different to standard observational technics, in terms of practicability, precision and more importantly, invasive technics are limited by ethical concerns. Moreover, in this study, the effect of footbathing practices and individual antibiotics treatments might be highly reduced or masked by the exacerbated anti-inflammatory response

of the animals after every biopsy. Lastly, the washing and scrubbing procedures entailed by the biopsy procedures might have modified the skin microbiota.

The covariates studied revealed important differences between the farms included. As several risk factors at farm level are involved with the disease, such as hygienic conditions, differences in the microbial profile according to the farms were expected. In our findings, these differences were visually obvious. However, the small sample size (5 farms) and the number of observations within farm restrain the magnitude of our results. Nevertheless, for example, in the Farm 3 was evident the important abundance of *Fusobacterium* genera (Figure S3.3), and in Farm 2 the abundances of *Mycoplasma* were as well easily recognized when compared to the other samples (Figure S3.2). Therefore, the number of farms, animals, and time points sampled must be increased in further studies aiming to explore the impact of different risk factors linked to the feet hygiene on the skin microbiota. Similarly, using larger samples, particular microbial profiles might be associated with high virulent forms of bDD or to outbreaks episodes.

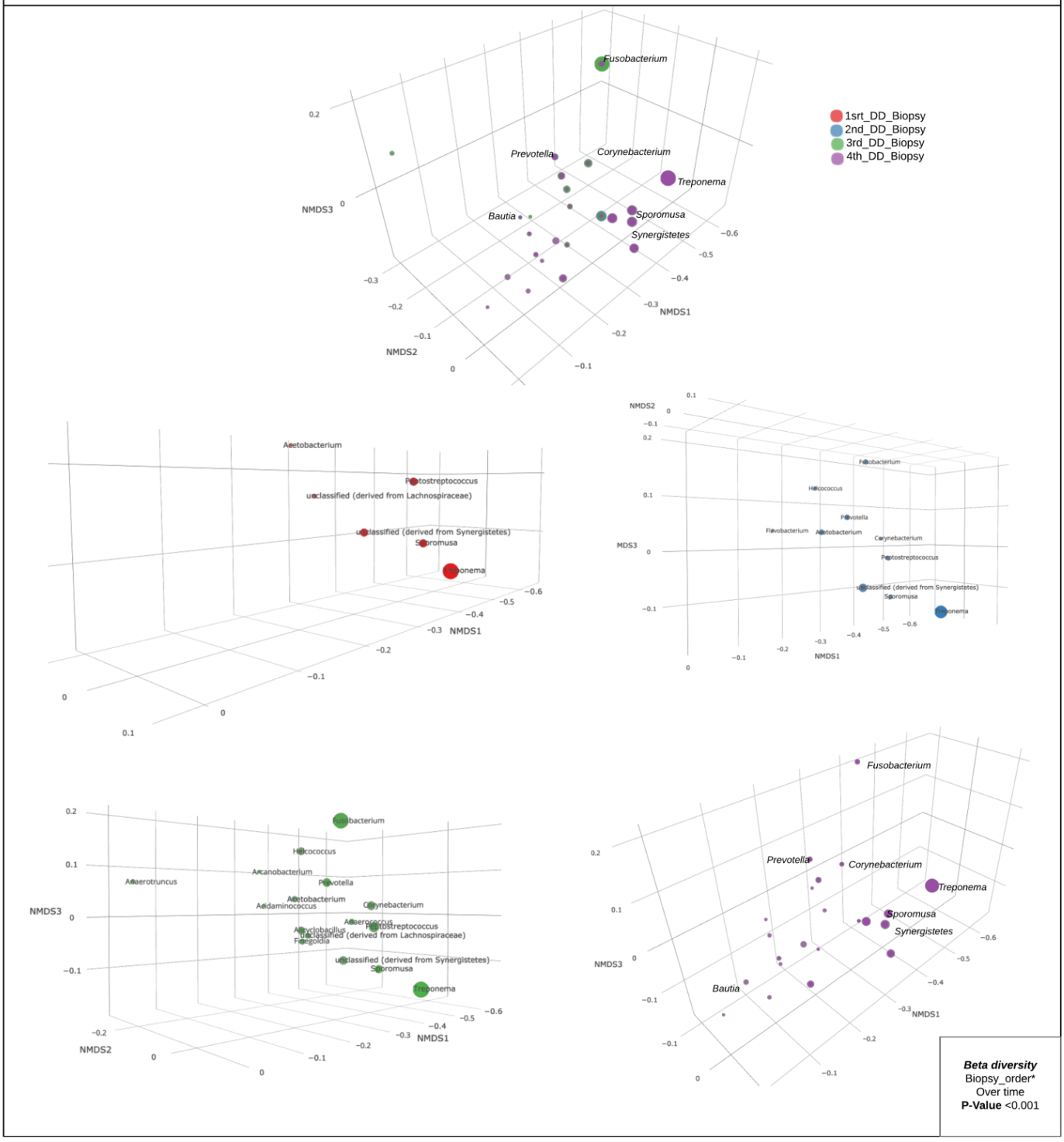
The absence of difference in the microbiota diversity related to individual antibiotic treatments could be explained because their effect on the microbial structure is short and not persistent over time. Otherwise, this study supports previous findings indicating that microbiotas of nonproliferative lesions (inactive lesions) were closer to the healthy skin microbiota (Zinicola et al., 2015). This raises the question about how to consider inactive lesion in the process of bDD pathogenesis and therefore in the monitoring of the prevention or the treatment of bDD lesions.

The technologies implemented in the analyses of this study did not allow to precise any inference at the species level. 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. Therefore, future studies may be approached by shotgun sequencing tools; enhancing the sensibility in the recognition of specific communities and allowing the identification of pathogenicity mechanism. Beyond the overall similarities between the dynamics in which the skin microbiota of bDD lesions evolved over time until recovering the same diversity of the healthy skin, the microbiotas studied were heterogeneous between farms indicating that other factors affecting the microbiota dynamics differ between farms. Therefore further investigations linking the skin microbiota to different herd management practices might enhance the current understanding of the disease.

CONCLUSION

This investigation described the dynamics of the skin microbiota in feet affected with bDD. The composition and diversity of the bacterial communities present in each sample did not varied over time according to the usage of Pink-Step™ in footbaths or the individual treatment of the bDD lesions. The microbiota diversity of bDD lesions evolve over 45 days until recovering the same diversity metrics of healthy skin microbiotas. Differences in the microbiota diversity over time were as well detected between the nonproliferative and proliferative lesions and between the included farms. Therefore, the evaluation of the skin microbiota over time may distinguish the healthy and affected status of a foot, but the usefulness of this tool for measuring the effectiveness of treatment strategies results questionable. Finally, the differences detected between the included farms highlight the probability that specific farm conditions may impact the structure of the skin microbiota and therefore determine the clinical evolution of the affected animals.

Figure 4. Diversity comparison of the spatial distribution of microbiota from DD lesions according to 4 time points over 45 days.



Beta diversity
Biopsy_order*
Over time
P-Value <0.001

Figure 5. Diversity comparison of the spatial distribution of microbiota from DD lesions according to their lesion aspect (Non_proliferative or proliferative) over 45 days.

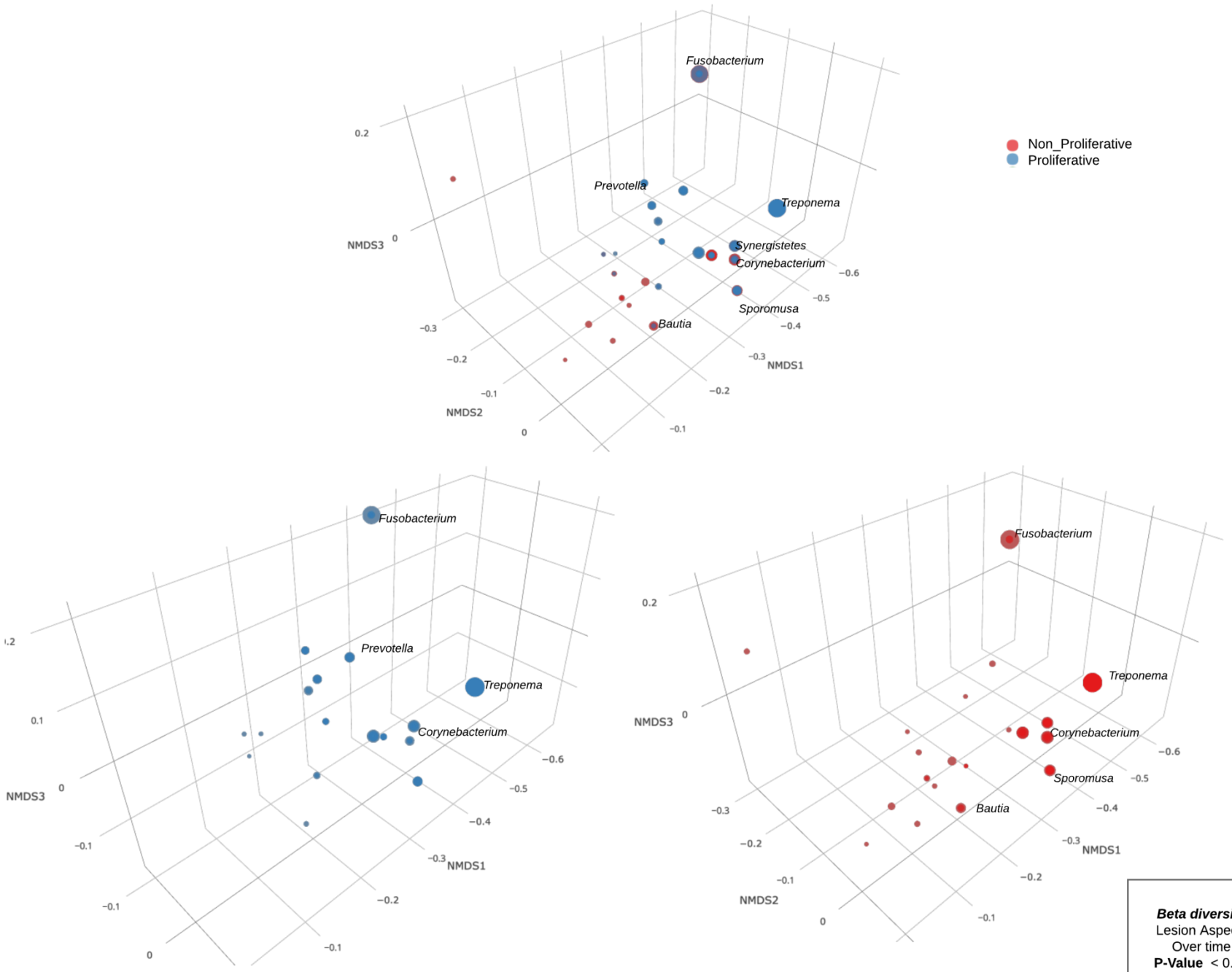


Figure 6. Diversity comparison of the spatial distribution of microbiota from DD lesions according to the allocation group (Control or Footbath) over 45 days.

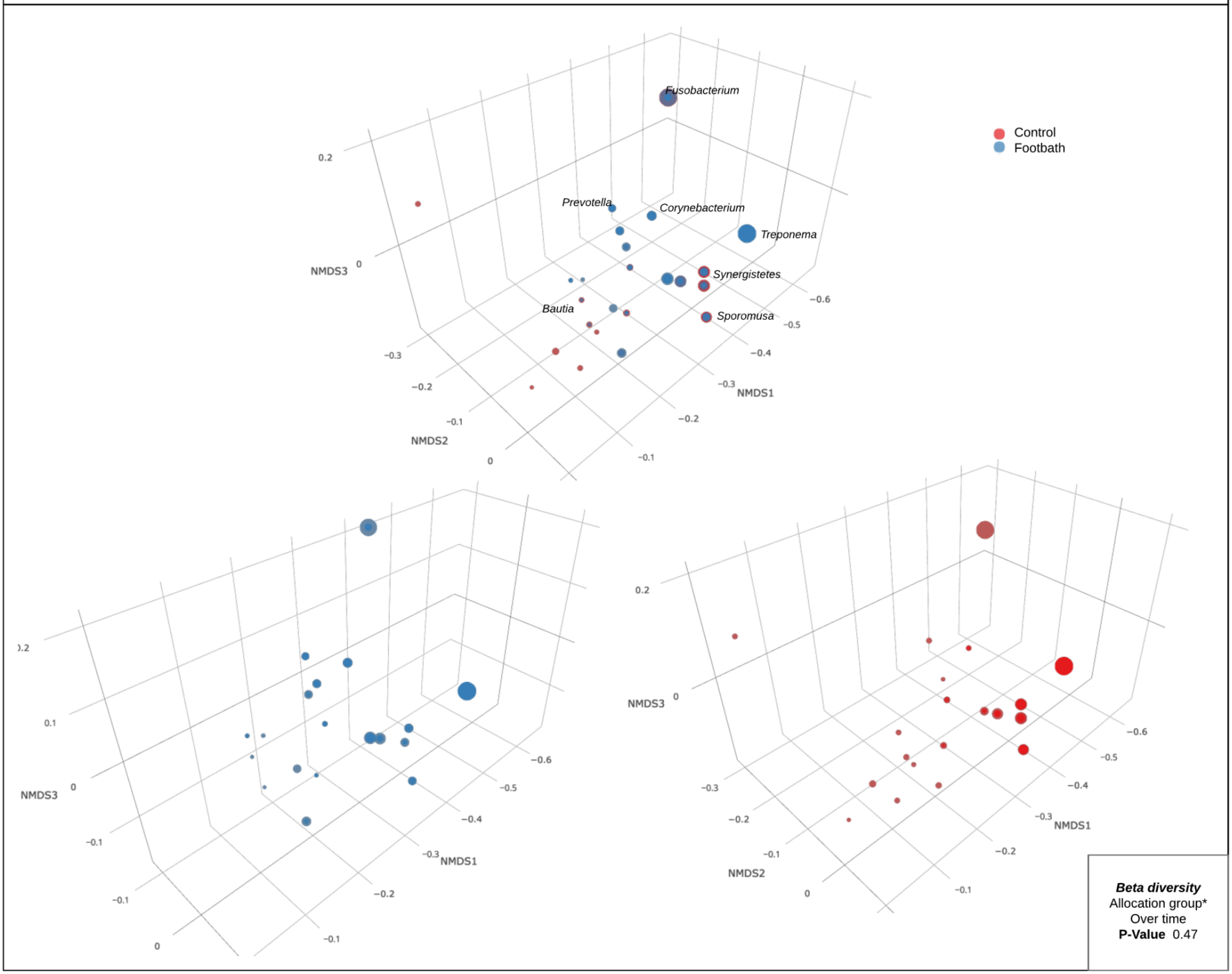


Figure 7. Diversity comparison of the spatial distribution of microbiota from DD lesions according to the time since the last administation of an antibiotic over 45 days.

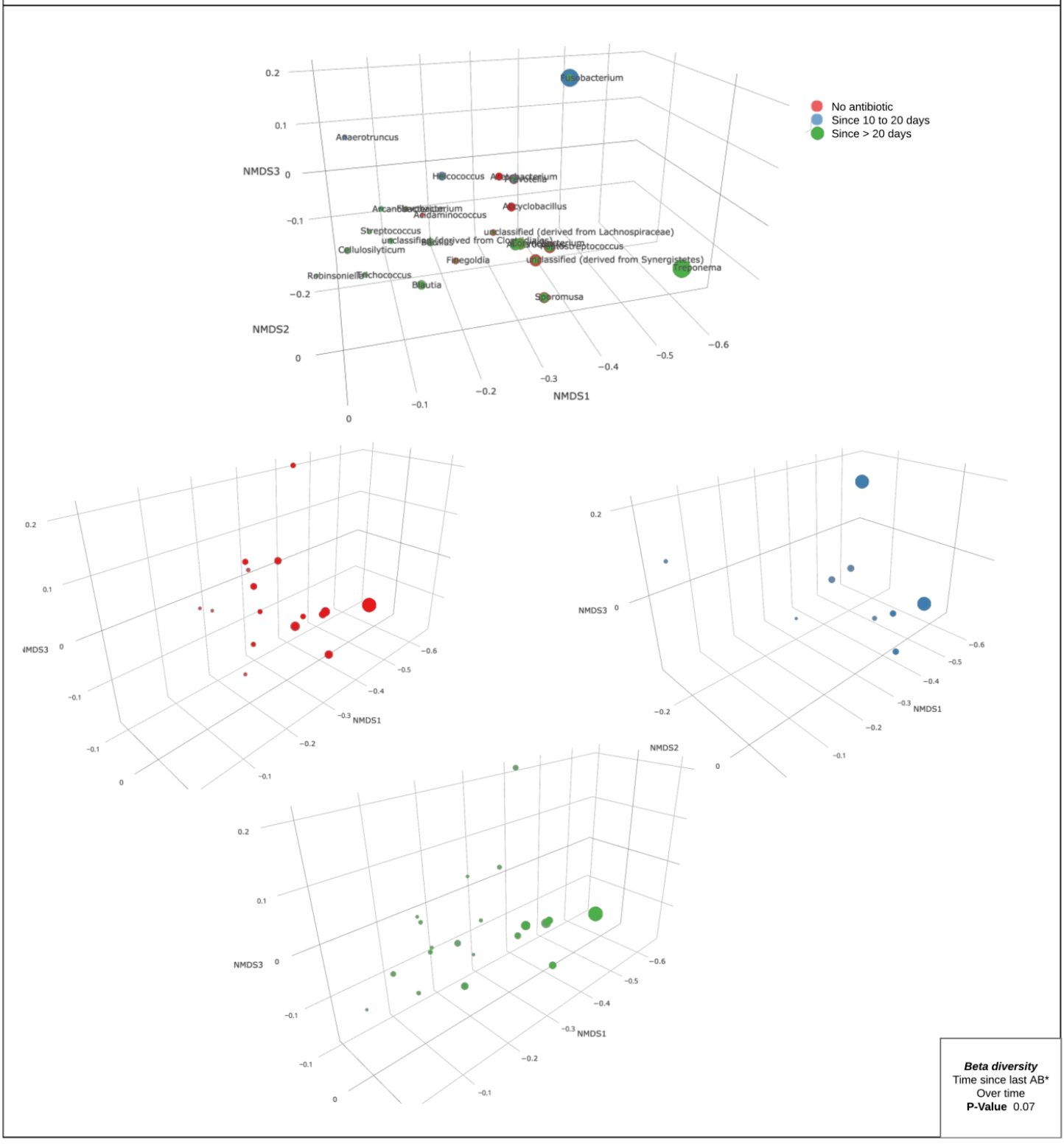
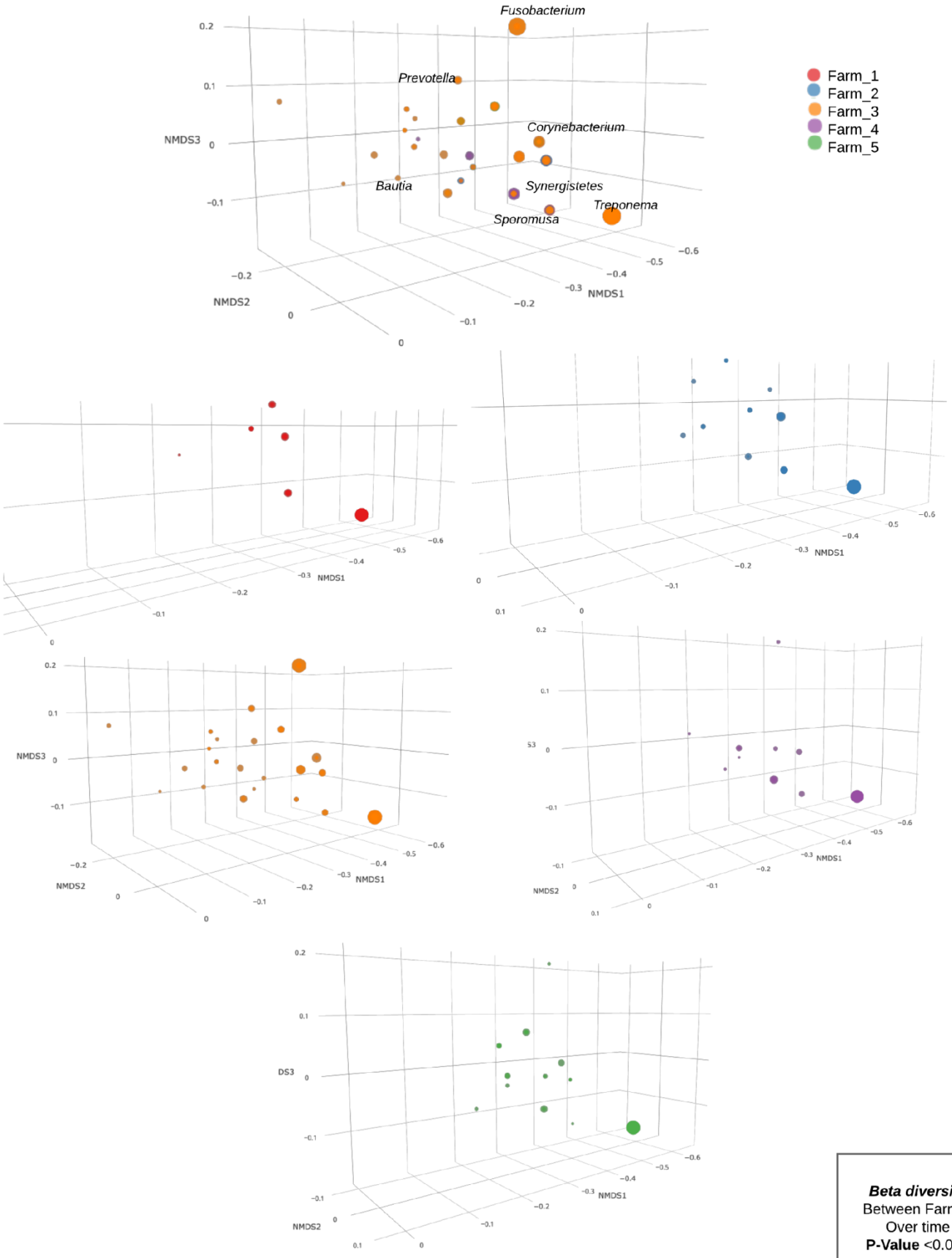
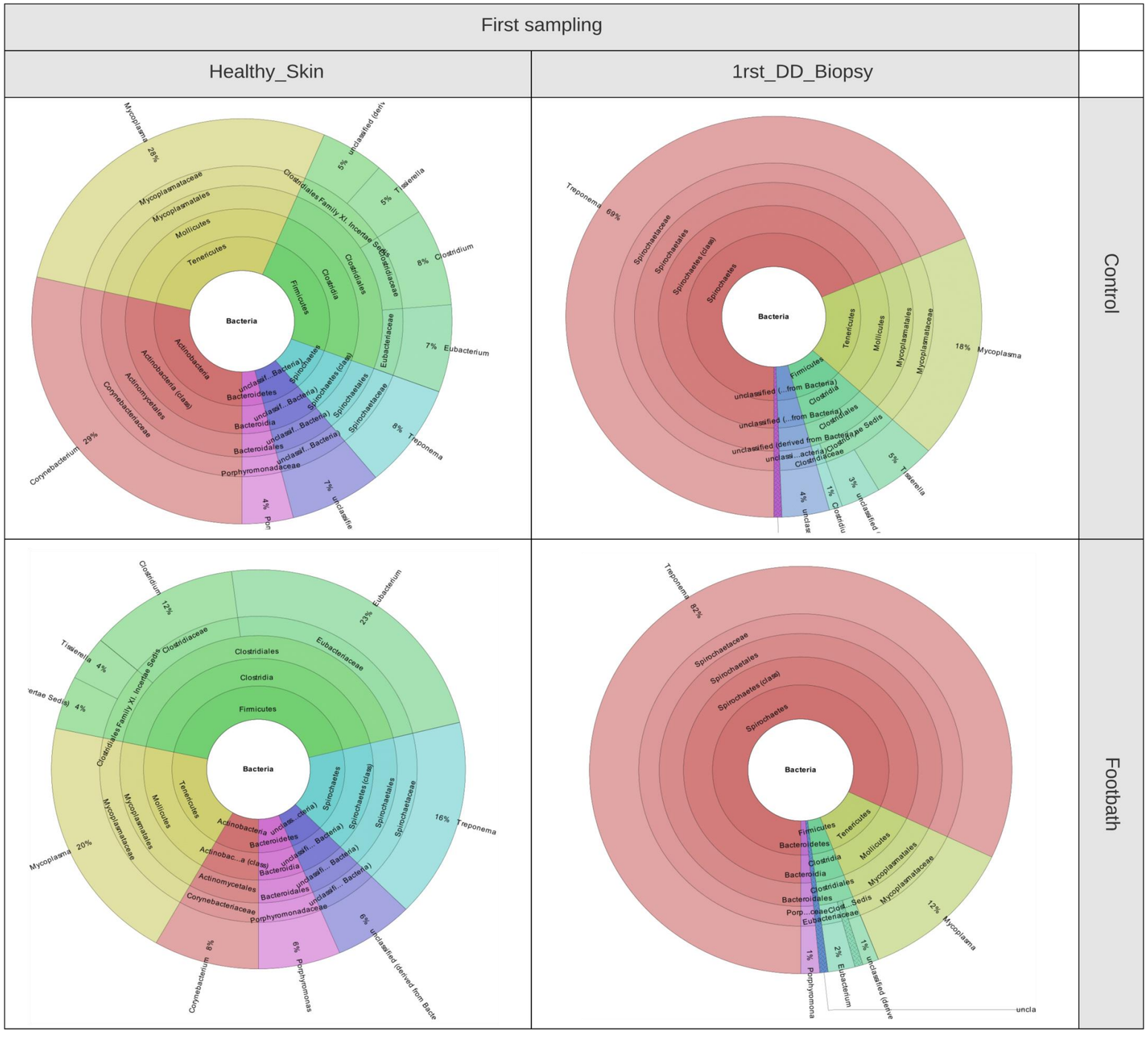


Figure 8. Diversity comparison of the spatial distribution of microbiota from DD lesions according to each farm over 45 days.



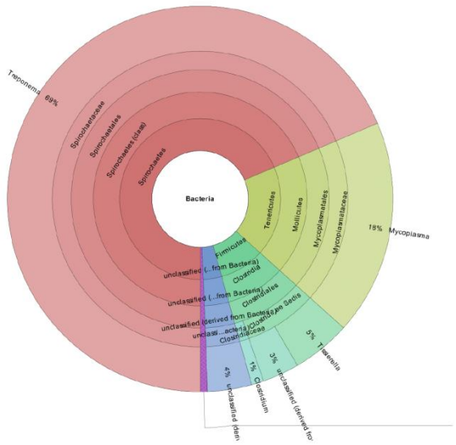
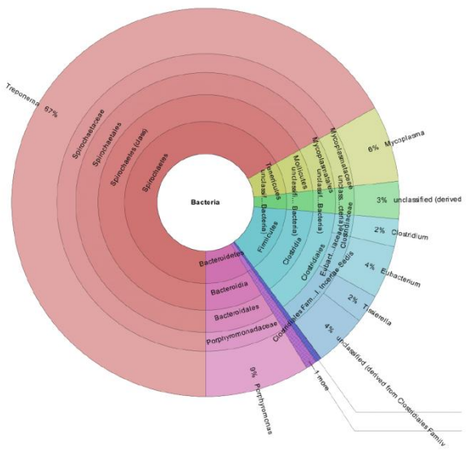
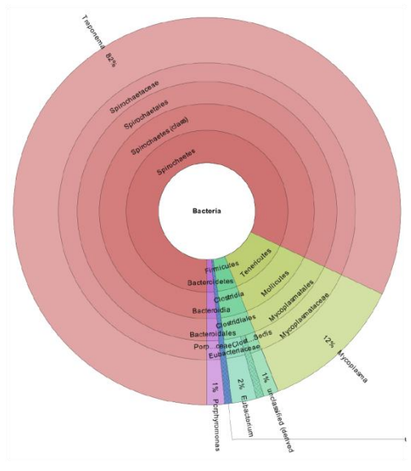
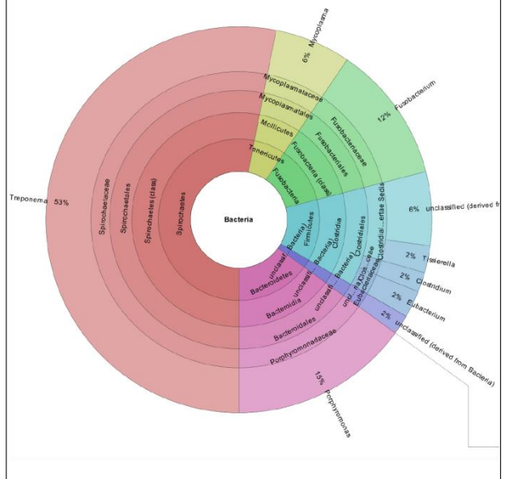
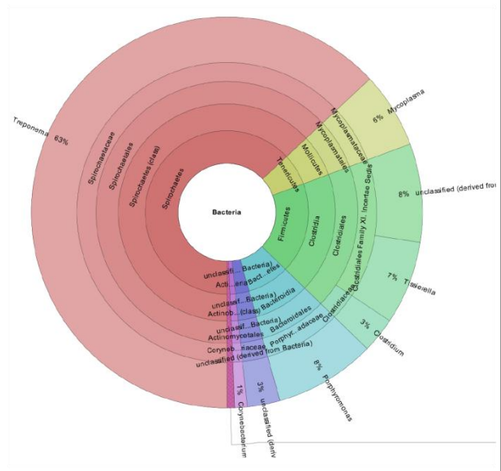
Beta diversity
Between Farms*
Over time
P-Value <0.001

Figure 3. *Average relative abundances of microbial communities from bDD lesions according to the allocation group at the first sampling*[1].



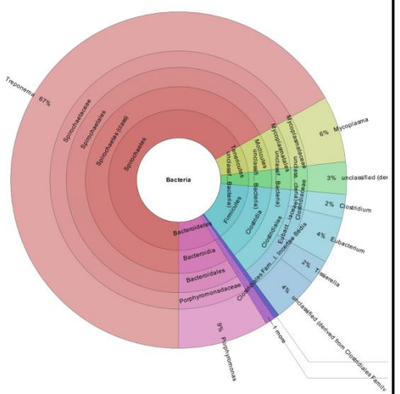
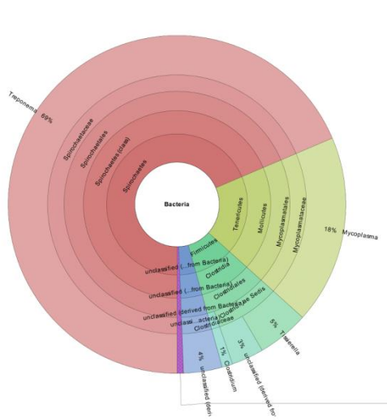
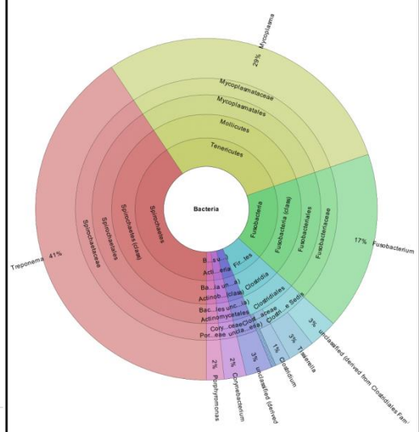
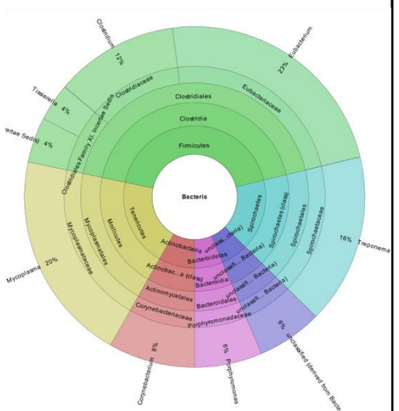
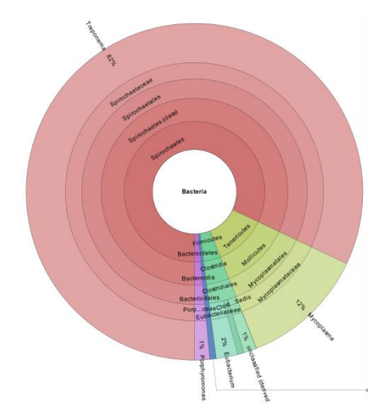
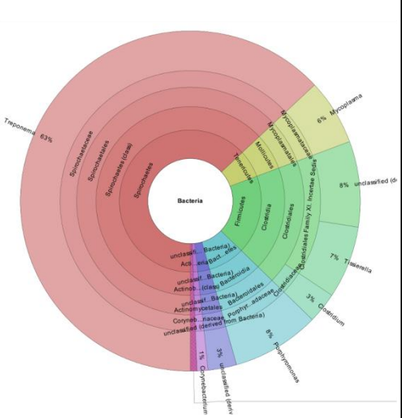
[1]Colors changes proportionally to the taxonomical abundances and diversities, being red colors the most abundant and green colors the most diverse.

Figure 3.1 Average relative abundances of microbial communities from bDD lesions according to the allocation group over 45 days[1].

Consecutive DD sampling				
1rst_DD_Biopsy	2nd_DD_Biopsy	3rd_DD_Biopsy	4th_DD_Biopsy	
				Control
				Footbath

[1]Colors changes proportionally to the taxonomical abundances and diversities, being red colors the most abundant and green colors the most diverse.

Figure 3.1.2 Average relative abundances of microbial communities from initial healthy samples and bDD lesions according to the allocation group over 45 days[1].

First sampling		Consecutive DD sampling			
Healthy_Skin	1rst_DD_Biopsy	2nd_DD_Biopsy	3rd_DD_Biopsy	4th_DD_Biopsy	
					Control
					Footbath

[1]Colors changes proportionally to the taxonomical abundances and diversities, being red colors the most abundant and green colors the most diverse.

Figure S1. Relative abundances of microbial communities from bDD lesions according to the farm, and group allocations.

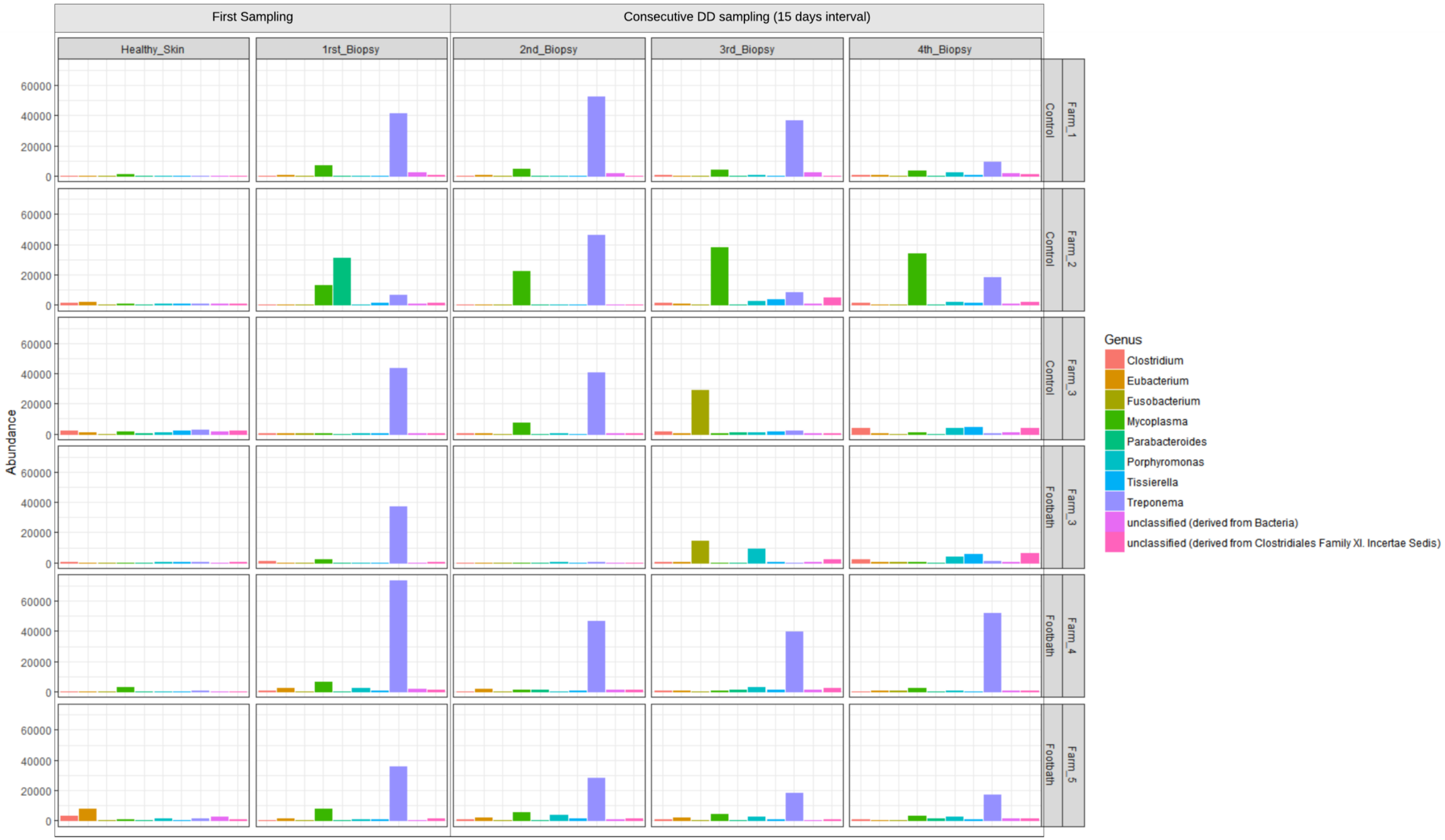


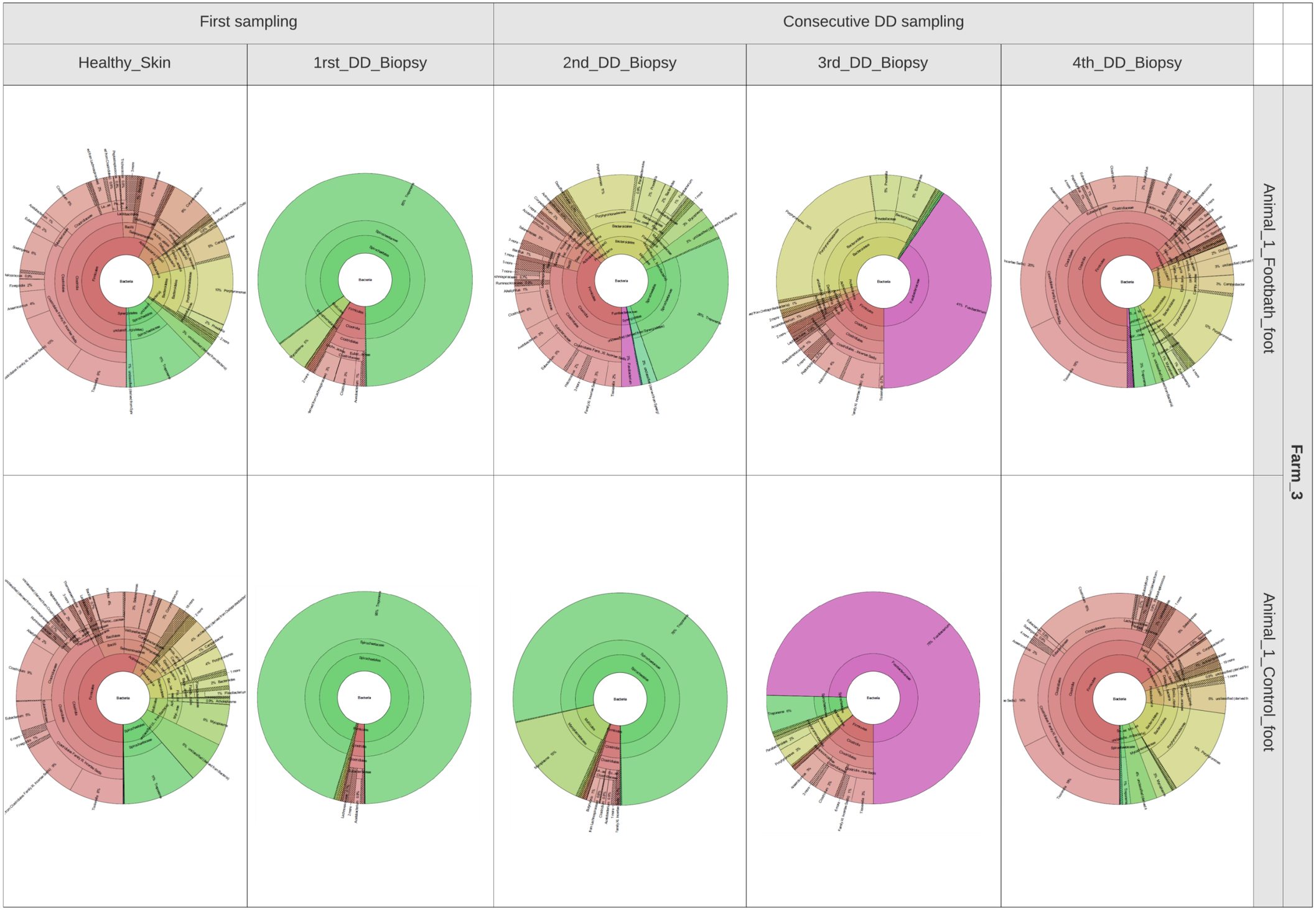
Figure S3.1. Average relative abundances of microbial communities from bDD lesions according to the Farm_1 [1].

[1]Colors changes proportionally to the taxonomical abundances and diversities, being red colors the most abundant and green colors the most diverse.

Figure S3.2. Average relative abundances of microbial communities from bDD lesions according to the Farm_2 [1].

[1]Colors changes proportionally to the taxonomical abundances and diversities, being red colors the most abundant and green colors the most diverse.

Figure S3.3. Average relative abundances of microbial communities from bDD lesions according to the Farm_3 [1].



[1]Colors changes proportionally to the taxonomical abundances and diversities, being red colors the most abundant and green colors the most diverse.

Figure S3.4. Average relative abundances of microbial communities from bDD lesions according to the Farm_4 [1].

[1]Colors changes proportionally to the taxonomical abundances and diversities, being red colors the most abundant and green colors the most diverse.

Figure S3.5. Average relative abundances of microbial communities from bDD lesions according to the Farm_5 [1].

[1]Colors changes proportionally to the taxonomical abundances and diversities, being red colors the most abundant and green colors the most diverse.

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C. PROTOCOL: Evaluation of a new disinfectant solution in the collective prevention and treatment of digital dermatitis in dairy cows. A clinical trial

Titre du projet de recherche clinique et/ou épidémiologique vétérinaire :

Evaluation of a new disinfectant solution in the collective prevention and treatment of digital dermatitis in dairy cows. A clinical trial

Investigateur principal : Raphaël GUATTEO

Durée estimée du projet : 8 -10 Months

Date souhaitée de début du projet : October 2016

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RESUME NON TECHNIQUE DU PROJET

Digital dermatitis (DD) is currently the principal infectious cause of lameness in dairy cows. The disease is associated with economic and animal welfare concerns, such as lameness, reduced milk production, impaired reproductive performance, and increased risk of culling. Digital dermatitis is considered a multifactorial disease, where the presence of specific *Treponema* species on feet suffering from cutaneous maceration, are recognized as the major components involved in disease development. Recent studies have been demonstrated differences in the microbial diversity of healthy, active and non-active DD lesions. The concept of "polymicrobial etiology" adds a new variable on the pathophysiology of the disease, suggesting the possibility that particular microbiotas drive the lesion environment affecting their outcome.

Despite several advances in understanding the disease, the current control strategies evidenced variable efficacy in practice. Control measures are focused on the reduction of the main risk factors of the disease, such moist and unhygienic conditions. Additionally, individual and collective treatments are strongly advised to limit the spread of the disease. Nevertheless, there is lack of knowledge about the effectiveness of treatments in the prevention and treatment of DD lesions.

These facts reflect the importance to develop new control strategies adapted to the current scenario of the disease. Hypothetically, effective treatments must regulate and drive the microbiota diversity of foot with clinical lesions to those of a normal healthy foot. The multifactorial and poly-microbial nature of DD, represents a challenge for the evaluation of treatments strategies, and the possible influence of the different recognized risk factor on the treatment efficacy must be concomitantly accounted. This approach could allow the extrapolation of the results to different field conditions. Therefore, the development of a standardized protocol and high-quality clinical trials are urgently needed to investigate the effectiveness of DD control strategies.

The main objective of this study is to evaluate the effectiveness of a new biocide solution in the collective prevention and treatment of DD in comparison with a negative control, at two different regimens of application frequency. Additionally, for the first time in France, and contrary to the few existent studies in the subject, one part of this clinical trial aims to explore and characterize the microbiotas of clinical lesions across their evolution after the administration of a collective treatment. The expected sample size is greater than previous studies to explore between farms variations and the repeated samples over time may allow us to assess for the first time, the putative evolution of the microbiota, not only across lesions stages but also within-cows over time-lesions.

Abstract

Main Objective: To evaluate the effectiveness of two regimens of a new biocide solution for the collective prevention and treatment of DD, in comparison with the absence of disinfectant footbaths.

Secondary Objective: To explore and characterize the microbiotas of clinical lesions across their evolution after the administration of disinfectant footbaths.

Design: A prospective randomized controlled clinical trial.

Population: Dairy Holstein farms from Brittany region, France.

Subjects and methods: A split footbath will be placed in the milking parlor exit of 10 farms (528 lactating cows). One side of the cow will be treated and the other side will be used as a negative control. The side of the cows to be treated will be selected by systematic random sampling, thereby ensuring that half of the farms within each footbath regime will be treated on the left side only and the other half of farms will be treated on the right side only. To minimize possible imbalances between treatments farms, only farms with DD prevalences ranging from 15 to 30% will be included. The DD prevalences will be determined by the proportion of hind feet detected with active DD lesions during the pre-study visit by visual inspection in the milking parlor.

Administration regimens: The moderate regimen (MR) consists in the footbath usage weekly for 1 month, then every fortnight for 1 month, and then once a month for 2 months (regime frequently used by the farmer). The intensive regimen (IR) consists in the footbath usage weekly for 2 months, and then every fortnight for 2 months.

Main outcomes measure: Based on the M5 stage classification (Döpfer et al. 1997). Healing failures of active (M1,2 and 4.1) and/or chronic (M4) DD lesions in feet. Preventing failures in the occurrence of active (M1,2 and 4.1) and/or chronic (M4) DD lesions in feet. Farms will be visited at least once a month for lesion scoring.

Secondary outcomes measures: Changes in the microbiota load and diversity of DD lesions during the treatment period of the IR group. The follow-up evaluations consist of fortnight skin biopsies during the first two months of the trial on 2 selected animals of each farm (Total= 10 cows).

Study power: (α=0.05 and Power=80%).

Statistical methods: Survival analysis of the main outcome observations (Cox proportional hazard model).

Key words: bovine digital dermatitis, microbiota, disinfectant, prevention, healing.

Experimental protocol:

1. Duration of the study

Length of the project: The project length will depend on the recruitment process times. We planned to expend between 8 to 10 months since the start of the trial in the first farm until the end of the trial in the last farm recruited.

Length of the trial: For each farm, the clinical trial will last 4 months (±133 days), time since the first until the last evaluation of the main outcomes.

2. Pre-study

Baseline records on the prevalence and other covariables of the potential participant farms will be taken one month before the footbaths regime started.

3. Interventions arms and co-interventions.

Co-interventions
For ethical and welfare reasons, farmers will be expected to individually treat all ulcerative-active DD lesions detected during the month before the start of the trial period by 2 applications of oxytetracycline (30 mg/ml) 2 days apart, regardless of the group or treatment regimens assigned for the trial.

Control Group

I. Negative control Group
Untreated feet. In each farm the footbath side selected as a control will be empty, avoiding possible interpretation bias due to interactions of placebo treatments in DD lesions.

Intervention Groups
A new biocide solution of recognized *in-vitro* efficacy will be administered by footbaths in each one of the included farms. The cows will walk through the footbaths after 4 consecutive milkings at two different frequencies as follows:

I. Moderate regimen (MR) Group
Footbath usage weekly for 1 month, then every fortnight for 1 month, and then once a month for 2 months.

II. Intensive regimen (IR) Group
Footbath usage weekly for 2 months, and then every fortnight for 2 months.

4. Outcomes Evaluations: Main and secondary outcomes

Outcomes measures	Main Outcome	
	Curative	Preventive
Outcome Definition	Healing failures of lesions.	Preventing failures in the occurrence of lesions
Case Definition	Feet lesions scored at M 1-2-4-4.1 stages.	Feet considered at M 0 - 3 stages
Success Definition	Evolution to stages M 0-3 during at least 2 consecutive visits.	Feet resting at M 0 – 3 stages during at least 2 consecutive visits.
Measure	Time to heal (days until the first day without any active lesion	Time until lesion occurrence (days until the first day with any lesion).

Outcomes measures	Secondary Outcome*	
	Curative	Preventive
Outcome Definition	Changes in the Microbiota diversity of DD lesions.	
Case Definition	Increase in the bacterial diversity of lesions evolving from M1-2-4-4.1 to M0–3	Regulation of the bacterial diversity of feet considered at M0 – 3 stages.
Measure	**Diversity index.**	
Analyses	Differences between the bacterial diversity of lesions followed for 45 days.	
Comparisons	○ Within-cow ○ Between-cows ○ Between-farms.	
* **Secondary outcomes:** will be only measured in the Intensive regimen (IR) group (5 farms).		

Lesion recording system

Digital dermatitis lesion scoring system modified from Döpfer et al. (1997) and Berry et al. (2012). M1 is an early-stage ulcerative lesion (0–2 cm diameter); M2 is an ulcerative painful lesion with a diameter>2 cm; M3 is the healing stage with a lesion covered by a scab; M4 is the chronic stage characterized by dyskeratosis or surface proliferation; and M4.1 consists of a chronic lesion with a small area of ulceration. Stage M0 corresponds to healthy foots without DD lesions. Stages M1 - 2- 4.1 are considered as active lesions. Stage M4 is considered as a chronic lesion (non-active). Stage M3 is considered as healing stage (non-active).

5. Complementary Evaluations

Questionary covariables

During each farm visit, assessors in the company of farmers will fill the follow-up questionnaire that includes all the co-variables accounted.

Covariates to adjust (Confounding factors)

- Farm-level:

1. Using of individual treatments.
2. DD initial prevalence.
3. Poor foot cleanness.
4. Microbial pattern.

-Cow level:

5. Cow parity.
6. Stage if lactation.
7. Grazing.

-Feet level:

8. Individual treatment.
9. Initial lesion stage.
10. Hoof-trimming.
11. Active DD on contralateral foot.

6. Experimental procedures: Data collection Frequency and method.

I. Main outcomes: Severity= Mild ***(Légère)***

Observation frequencies for the main outcomes: Using the method to score lesions in the miking parlour described by Relun (2011). A first scoring will be performed immediately before the footbaths regimes start. Consecutive feet scoring at intervals of maximum 30 days will be performed during the trial period (minimum 7 days, minimal reported time for lesion evolution). Ulcerative lesions identified and treated by the owner will be reported and included in the analyses.

Note= The observational diagnostic procedures represent a low risk for the animals.

Training: Assessors will be trained for the lesion scoring by practical lessons.

Possible Complications

- **Adverse events:** the possible iatrogenic risks of secondary reactions caused by the usage of the biocide product will be previously explained to owners and assessors. During the experimental period, owners and trained assessors will assure the follow-up of possible complications linked to the biocide usage. Any adverse effect reported, will be registered and their clinical implications will be measured in regard to welfare concerns.
- **Ingestion of the disinfectant:** possible risk of ingestion will be decreased by the repulsive nature of the disinfectant solution and by the usage of a split footbath that limits the cow access to drink.

Decision rules for terminating the study

In farms in which the overall clinical status increases drastically or over the 20% of the baseline status of active lesions (M1 - 2 -4.1) of the herd, the experiment will be considered finished. In case of any adverse reaction evidenced the experiment as well will be considered finished.

Note: The M5 scoring system is not a completely linear scoring, the M1 - 2 – M4.1 represent the ulcerative and painful states. The experiment will be considered finished in farms experiencing a drastic increase over the 20% of their M1 -2 - M4.1 baseline status (determined in the pre-study visit). **(i.e: Farm X "Baseline status" = 20% of animals with lesions M 1-2 –M4.1, Farm X "status during trial" = ↑↑40% of animals with lesions M2-M4.1)**

II. Secondary outcomes: Severity= Mild ***(Légère)***

Observation and Biopsy frequencies for the secondary outcomes: 4 consecutive biopsies at 15 days of the interval after the start of footbaths regimen. Biopsies will be performed in only two animals by farm (10 animals) during the treatment period of the IR group. The biopsies will be performed in cows presenting active lesions (ulcerative lesions M1, M2, and M4.1) and chronic lesions (non-active lesions M4). The animals will be selected based on their baseline status. For each farm, one active lesion will be sampled and one chronic lesion (2 animals), assuring that half of the biopsies will be performed in feet receiving the biocide footbath disinfection, and the other half of the biopsies will be performed in feet under the control side footbath.

Biopsies Protocol: The animals should be adequately restrained in a trimming chute. The foot surface selected will be cleaned by brushing and washing with tap water, and then gently dried with a paper towel. Local anesthesia of the selected area will be provided before the beginning of the procedure by the multipoint subcutaneous application of 3 – 5 mL of 2% Procamidor (Procaine hydrochloride, withdrawal period of zero hours for milk, and meat)
A 4 - 6 mm sterile biopsy punch will be used to penetrate the skin layers. The punch will be inserted up to its full length and then, after 6 -8 rotations, carefully withdrawn. The biopsy tissue is removed from the punch, divided into two pieces and fixed immediately in 10% neutral phosphate-buffered formalin or placed in a sterile 2.0 ml microcentrifuge tube and transported on ice to the laboratory and then stored at -20°C until analysis. The hole should be gently washed with an iodine solution and slightly cover with sterile gauze to promote second-intention healing. After the biopsy procedure, animals evidencing pain, lameness or severe local discomfort (assessed by the farmer) due to the biopsy will be treated with an NSAID's. Only NSAID's with zero-day withdrawal periods for milk and meat will be administered, according to the convenience of the owner and with the agreement of him referring veterinarian (Tolfenamic Acid 4% (2mg/Kg), or Ketoprofen 10% (3mg/kg) or Carprofen 5% (1.4mg/kg)).

Biopsies sampling: All biopsies will be performed by Dr. Juan Manuel ARIZA (Docteur Véterinaire).

Possible Complications
- **Iatrogenic infection:** the risk will be decreased by the strict hygienic conditions of the procedure.
- **Minor hemorrhage:** a special follow up period of 24 hr will be established after the biopsies procedure to ensure the hemostasis of the lesion, any bleeding lesions will be cleaned and cover.

7. Sample sizes

Main Outcomes: Based in a previous study of similar approach (Relun et al., 2013), we use the preventive outcome for the sample size calculations because the detectable differences in the rates of failures between the treatment and control group are lower for the preventive outcome (10%) in comparison with the curative outcome (20%). Therefore, a larger number of animals are needed to achieve statistical power for the preventive outcome when compared to the curative outcome. To account the within cow control for the sample size calculation, we use a mean cluster size (m) of **2 (right and left feet, treatment, and control).** The number of farms is calculated from our inclusion criteria and the median size of the herds in France (minimal farm size= 50 lactating cows). The table describes two calculations using two different intra-cluster correlation coefficients (ρ= 0.05 -0.1) advised in the related scientific literature.

$$e = (Z_{1-\alpha/2} + Z_{1-\beta})^2 \frac{(1+rHR)^2}{r(1-HR)^2} \rightarrow e_c = e(1+(m-1)\rho) \rightarrow n = \frac{(1+r)e_c}{m((1-P_{Tx})+r(1-P_{Control}))}$$

A	β	ρ	m	r	P_{Tx}	$P_{Control}$	HR	e	N Total	N x group	N Adjusted (Loss 30 %)	# Farms
0.05	80%	0,05	2	1	0.90	0.80	0.47	65	214	107	252	5
0.05	80%	0,1	2	1	0.90	0.80	0.47	68	224	112	264	5

α= Type 1 error, **β=** Type II error, **ρ=** intra-cluster correlation coefficient, **m=** mean cluster size, **r=** ratio of subjects allocated to control and treatment groups, $\mathbf{P_{Tx}}$**=** rate of healing or preventing failures expected in treatment group, $\mathbf{P_{Control}}$**=** rate of healing or preventing failures expected in control group, **HR=** Hazard ratio calculated, **e=** number of events, **n=** number of cows (or feet) by comparison group.

Secondary Outcomes: The absence of previous studies evaluating longitudinally the changes in the microbiotas of DD lesions under an active treatment, does not allow us to argue the exact number of animals necessary to statically test any hypothesis. Nevertheless, we are requesting 10 animals (Total= 50 biopsies procedures) for this second and exploratory aim of our study. Based on previous publications approaching the DD pathogenesis by metagenomic methods and some personal communications of different researchers specialized in the subject, the metagenomic sequencing data obtained from these biopsies procedures will represent a considerable material for the statistical analysis and interpretation of the bacterial evolution of DD lesions in terms of diversity and load.

8. Data analyses

Survival analysis

A clustered within cow, and within farm using a frailty Cox proportional hazard model, including different covariates (specified below), will be employed to investigate the differences in time to healing and time for the first occurrence of DD lesions between the groups (Control, IR, MR). Feet included in the curative outcome analysis will be those considered to have active lesions (M1, M2, and M4.1) or chronic lesions (M4) at the first observation of the trial, and not being treated individually a least for two weeks before the first observation. In the preventive outcome analysis feet included will be those considered to have no active lesions or no chronic lesions (M 0 – 3) at the first observation of the trial. The animals experiencing during the trial time a healing or occurrence lesion event will be censored in the analysis. Nevertheless, their follow-up observations will continue until the end of the trial time. The survival analyses take into account the dynamic pattern of some putative risk or protective factors, providing an additional precision to the calculated cumulative incidence. All analyses will be performed in Survival kit® v6.0. Hazard ratios will be estimated for each exposure (footbath treatments and control) and for each covariate.

Metagenomic analysis

In collaboration with the veterinary faculty of the Wisconsin University (USA), 16S rRNA metagenomic sequencing analyses will be performed to investigate the microbial diversity across different stages of DD. Biopsy samples will be analyzed to obtain 16S rRNA bacterial gene sequences, the diversity of the present bacterias for each sample will be evaluated and compared by the Chao1 index methodology. Additionally, the abundance of microbial taxon types will be measured from the binned Operational Taxonomic Units (OTUs) obtained from the 16S rRNA analyses. Comparisons analyses between control and treatment arms will be performed between farms and between cows. However, intra-cows comparisons will not be performed due to ethical concerns regarding the unfeasibility to practice biopsies on both hind feet of the same cow.

Chapter 5. Discussion

Chapter 5. Discussion

DISCUSSION

The overall goal of this thesis was to generate knowledge about the effectiveness of a new footbath biocide solution for the control of Bovine Digital Dermatitis in dairy herds, and consequently investigate deeply the conditions which may determine the success or failure of such a control strategy. In this order, the thesis formulated the next objectives, briefly:

Objective 1. Systematic assessment of the evidence about the effectiveness of collective treatments and evaluate the strengths and limitations of the different study designs to avoid such problems in future clinical trials.
Objective 2. Determine the parameters to adapt the renewal frequencies of a new biocide solution for the bDD control, according to best conditions for their implementation in field conditions.
Objective 3. Evaluate the effectiveness of a new footbath solution in the control of bDD under field conditions through a clinical trial taking into account other risk factors and assessing the skin microbiota of the affected feet.

The main results of our thesis according to the objectives formulated were:

Objective 1. The evidence about the effectiveness of collective treatment for bDD is scarce and highly heterogeneous, therefore their effectiveness remains uncertain. Otherwise, the main drawbacks and strengths of the design for the conduction of high-quality trials were deducted from the review process.
Objective 2. After pre-clinical investigations integrating the field conditions, the renewal frequencies for a new footbath biocide were established according to the levels of contamination (100 cow passages).
Objective 3. From the findings of a clinical trial evaluating the new footbath biocide. The healing effectiveness of the product used in a moderate frequency was evidenced. However, the preventive effectiveness of the product was not evidenced. The overall results reinforced the crucial role of hygiene in the bDD control. Otherwise, from the findings related to the skin microbiota of bDD lesions, a description of the bDD microbiota dynamics over time was achieved. Finally, according to the design of our study, the assessments of the skin microbiota revealed to be not adapted for the effectiveness evaluation of control strategies.

These objectives were set out to accomplish our main goal and their main results will be discussed according to their contribution to (A) the understanding of the pathogenesis and etiology of bDD, (B) the elaboration of strategies of control for bDD, and finally to (C) the establishment of guidelines for the evaluation of control strategies for bDD.

A. Contribution of our findings for understanding the pathogenesis and etiology of bDD

Despite the recent years of research towards the complete understanding of bDD, its precise pathogenesis and etiology remains unclear and masked under the complexity of its nature. Although several investigations have succeeded to reproduce bDD through controlled studies, the Koch's postulates remain partially fulfilled, especially for treponemes as single responsible for bDD. The treponemes were early recognized in the early nineties, and are still consistently incriminated as a major etiological component of bDD. First, the second Koch's postulate, indicating that the incriminate pathogen is not found in healthy subjects, is hardly approachable under the current circumstances where treponemes are everywhere, as inhabitants of the foot skin, the rumen, the saliva and the gastrointestinal tract of ruminants (Klitgaard et al., 2014; Zinicola et al., 2015; Nascimento et al., 2015). Moreover, once inoculated into the skin of feet previously damaged, the disease is reproduced inconsistently. Consequently, the third Koch's postulate, regarding the inoculation of the incriminate pathogen to induce disease, is still unaccomplished using only treponemes alone. Therefore it seems like the scientific rigor imposed by these postulates fails to accomplish the complexity of bDD. There is a need to incorporate the complexity of bDD into a rigorous modern guideline for evaluating disease causation. Our findings have confirmed the multi-factorial and poly-microbial origin of the disease. Indeed, a diversity of bacteria was associated with bDD lesions and led us to question whether these microbiotas induce or not the disease and how to address the role of particular microbiotas in the disease pathogenesis?

From the epidemiological point of view, other criteria for causality than those formulated by Koch, stipulated by Hill's, can be used to evaluate the relationships between exposure and disease outcome (Bradford-Hill, 1965). These criteria are flexible without losing the scientific rigor look for outline the mechanisms that lead to the disease. To approach the presumed causality of bDD microbiota advocated by our findings and supported by previous studies, the main Hill's criteria were theoretically approached in this discussion by formulating the respective questions which address these criteria in the bDD context (Table 1). For this

purpose, the main taxonomical phylum identified in bDD lesions in our findings were gathered with the shared findings of previous studies using next-generation sequencing methodologies (Krull et al., 2014; Zinicola et al., 2015; Nielsen et al., 2016) (Figure 3, Chapter 1). In this order, the average structure of the proposed "bDD microbiota" would be composed (hypothetically) in relative abundances by: *Spirochaetes* (35%), *Firmicutes* (20%), *Tenericutes* (15%), *Bacteroidetes* (15%), *Fusobacterium* (10%), and other bacteria (5%).

Table 1. Hill's criteria causality applied for the association between "bDD microbiota" and bovine Digital Dermatitis (bDD).

Hill's Criteria	**Digital Dermatitis Context**	**Criteria Answer from scientific literature and our findings.**
Biological plausibility and Coherence	Does bDD microbiota known to cause the characteristic bDD ulcerative lesions according to the current state of knowledge?	Yes. To successfully induce the disease, lesion macerates containing the bDD microbiota must be used.
Consistency	Do other studies found similar bDD microbiota when comparing to healthy skin microbiota?	Yes, particular microbiotas dominated notably by treponemes have been consistently identified in bDD lesions across the studies.
Specificity	Is bDD microbiota associated with diseases other than bDD?	Unknown, different diseases have been associated with some of the presumed pathogens present in the bDD microbiota. However, the description of the microbiota of such diseases remains unexplored.
Temporality	Does bDD microbiota precede disease or lesion development?	Unknown, the skin microbiota has not been studied before the occurrence of a lesion in longitudinal follow-up.
Experiment	Does bDD microbiota change their structure or composition after a treatment?	Not consistent. Form our findings, bDD microbiota did not change according to individual or collective treatments. However, multiple factors related to the study setting conditioned these results. Besides, across the literature evidence support that topical treatment successfully reduces the disease and change the microbiota until approaching healthy skin.
Dose-response	Less diversified bDD microbiota is associated with bDD?	Yes. Form our findings bDD microbiota was less diverse than healthy skin
Strength of the association	What is the association between the identification of bDD microbiota and the risk of bDD?	Small, larger samples are necessaries to measure the risk of bDD associated with the bDD microbiota.

From this exercise the current concepts of bDD were challenged by the putative microbiota identified in recent findings, revealing that there are still many questions to solve regarding the pathogenesis and the etiology of the disease and that multiple disciplines and new approaches must be integrated to enhance the current understanding of bDD. Therefore, our "bDD microbiota" remains a potential candidate and further investigations are necessaries to attribute causality. However, from a practical perspective, the standards measuring the efficacy of biocides for the feet disinfection should at least add the main bacteria present in the bDD microbiota to the challenging standard bacteria.

Otherwise, when comparing the "Koch's postulates" and "Hill's criteria", both concepts share the temporality as a common principle for determining causality. The fact that bDD lesion appears subsequently to the skin damage and maceration imposes the crucial paradigm about the temporality of bDD. Therefore, results decisively to determine: (i) if there is a particular microbiota that drives or favor the maceration and damage of the skin for its posterior colonization or the proliferation of a specific pathogen and then inducing disease. (ii) Else, the mechanical maceration and damage to the skin product of wet environments allow the colonization by specific microbiotas that induce the disease. However, the design of our investigation that includes only affected animals restricted the possibility to assess this main aspect and thereby determine causality. Consequently, further investigations using longitudinal designs are needed to evaluate the association between the microbiotas of healthy feet and the occurrence of bDD lesion. Furthermore, microbiota from lesions located in areas which are closer to the ground might be exposed more importantly to the main risk factors of bDD (wet and dirty environments). Thus, in the study protocol and in the interpretation of the microbiota, another factor to take into account is the sample location. The contralateral feet, if healthy, could have been an alternative but it was not done for ethical reasons (invasive sample). In this perspective, future studies must explore if, by less invasive sampling technics, such as lesion swabs, it is possible to achieve representative microbial material to be analyzed. Indeed, the immune response and the incisional lesion generated by the biopsy sampling may represent an important confounding factor impacting the occurrence and persistence of bDD lesions. This bias can be avoided by less invasive technics easier to implement under field conditions and with reduced ethical concerns. Similarly, less invasive samples may allow more easily the evaluation of the clinical outcome of bDD lesions and the contamination by opportunistic pathogens could be avoided. Another limitation related to next-generation sequencing technologies, as the one used in our study to identify microbiotas, is concerning their specificity. Indeed, these technologies fail to distinguish between death

and live bacteria simply because they were not designed for this purpose. Consequently, to conserve comparability, studies using metagenomics approaches should take into account the analyses the potential confounding factors, and besides, always include within animal or within feet controls. Once again, and due to this limitation, longitudinal designs and a larger number of samples might improve the precision of the findings.

Other questions unsolved by our works were related to the fact that within a herd some cows under the exposure to the same risk factors did not develop the disease implies that individual factors determine in some degree the bDD infection. Besides the genetic component linked to the disease and the overall clinical status of a subject (diseased, debilitated or immunosuppressed subjects), it have been demonstrated that skin microbiota can defend the host against pathogenic bacteria either by directly inhibiting the pathogen or by enhancing the host immunity (Rosenthal et al., 2011), a phenomena known as "colonization resistance", which is sometimes claimed by some dry footbath based on bacteria supposed to colonize the skin. In our investigation, the skin microbiota of bDD lesions evolved over time until approaching the healthy microbiota. However, the methodologies applied did not allow to investigate the immune response of the host or the bacterial mechanism for inhibiting pathogens. Therefore, further studies should explore the association between immune response and skin microbiota, and the bacterial mechanism associated with bDD. Otherwise, the dynamics evidenced over time in the bacteria communities that compose the bDD microbiota of our findings, added to the ubiquitous nature of these bacteria, matches the deterministic concept of microbiology discipline. This concept entails the precept that microbiota is guided by selective forces exerted by the farming environment contrary to a random principle of ubiquity. Therefore modeling the dynamics of microbiotas over time using dynamics Bayesian models may approach the reality of this complex system. By this approach the dynamic dependence of an specific bacterial family in a time point may be modeled according to the measures stated in the previous time point and are calculated as a function of its own cyclic presence, the co-occurrence of other bacterial families, and the skin environmental changes, such as the usage of an individual or collective treatment, the trimming foot or the access to pastures of the cow. This novel approach entails the evolution of the network across the time and therefore represents a challenge for the analyses and graphical representation. In the recent year's important advances are accounted for the dynamics Bayesian models, however, the related scientific literature still limited. Therefore, the perspectives offered by this model represent an asset to implement in further studies about bDD.

Among the perspectives generated from our findings, the association of the skin microbiota and the histological description of bDD lesions remain unexplored. The histological evaluation of bDD sample may highlight the potential mechanism of disease linked to the microorganism detected and thereby facilitate the attributability. Additionally, spirochetes can be stained and therefore differentiated in the tissues studied to confirm the sequencing findings. From our findings, we have hypothesized that every farm may determine a specific bDD microbiota pattern according to the factors affecting the hygiene and environment of the feet. However, to evaluate this association, a larger number of farms and observations are necessary. Additionally, the fact that multiple organisms were identified among this putative bDD microbiota may increase the specificity in the microorganisms targeted by novel treatment measures thereby enhancing their performance. Similarly, standards for determining the efficacy of collective solutions for bDD could be amended to include the microorganism related to the bDD microbiota. New footbaths products composed of drying agents or active micro-organism might take advantage of metagenomics approaches to support their claimed efficacy and as well effectiveness. Finally, the description of this particular bDD microbiota may enlarge the perspectives through the development of effective vaccines for bDD.

B. Contribution of our findings for the elaboration of strategies of control for bDD

About the renewal rates for footbaths

Throughout this manuscript was pointed that multiple factors affect the effectiveness of footbaths in field conditions. Although the impact of some factors is strictly associated with the feet outcome, other factors impact directly the footbath substance reducing its presumed efficacy, the contamination being the main limiting for footbaths solutions. However, to our knowledge, there was not scientific literature reporting how and at which levels footbaths are contaminated and needed to be renewed. Then, the number of passages for the renewal of a footbath solution became a questionable number determined probably by empirical observations or in the worst scenario by commercial motivations. This missing information in the footbaths within the bDD context led us to recognize a new point of disagreement between the laboratory and the practice. Therefore, a field investigation was designed expecting to bring into the laboratory from a field experience, the missing information about the conditions in which footbaths are challenged (Chapter 3.1). From this experience, we recovered and reported valuable information about how footbaths are contaminated; being the

most astonishing finding the larger losses of solution across the increasing number of passages. Although the plastic baths used during this study are amply commercialized and are often implemented in French farms, their designs are contrary to the scientific dimensions advised (Cook et al., 2012) and after our findings, many doubts about their utility were raised leading to highlight the importance of further investigations on the subject. It can be noticed at this stage that most footbath commercialized failed to reach the recommendations of at least 3 to 3.5 meters length. Otherwise, concerning the levels of contamination, the results of our study revealed surprisingly that the historical renewal rates (200 passages) partially matched the levels of contamination in which biocides for footbaths are tested in-vitro. Indeed, biocides are tested under restricted levels of contamination according to controlled conditions of time, temperature, and concentration. European standards for biocides used in the veterinary area (PT3), are tested against specific bacteria strains and challenged during 30 minutes at 10°C by 20g/L of organic matter (EN 1956). However, the circumstances corresponding to 20g/L of contamination are mainly determined by the number of passages. Therefore, the missing information in the literature was supplied by the findings of a field experience to thereafter evaluate in-vitro again, yet with more precision, the bactericidal efficacy of a footbath solution according to the contamination levels related to a specific number of passages. From this in-vitro experiment, the guidelines for the usage of a footbath solution could be established including a renewal frequency close to the field conditions. Thereafter, to finalize the experimental cycle of this control measure for bDD, a clinical trial was designed and conducted. This research structure which has integrated the field and laboratory experiences in a coherent manner has fulfilled the lack of preclinical evidence supporting a new footbath solution. By this experience, it can be proposed that novel measures of control for bDD must support their claimed efficacy in robust preclinical investigations approaching field conditions. Furthermore, due to the high impact of organic matter over biocides, footbaths solutions or other collective solutions indicated for bDD should specify the optimal conditions for their usage supported by scientific evidence. Consequently, this thesis project aimed to develop a product which supports their potential in scientific evidence and not only on the administrative requirements for the market. Furthermore, the scope of developing an efficient alternative with regimens supported by evidence to replace the current harmful biocides represented one of the main motivations for our project. Finally, as footbaths are implemented in other scenarios in dairy farming, new perspectives should concern the investigation about the optimal conditions for the usage of footbaths or collective treatments in farms using milking robots, where the access to footbaths

is often unrestricted. Therefore alternative measures to determine the renewal of the solutions should be explored. In addition, for farms with milking robots, it is promising to explore automatized methodologies for the lesion recognition, to substitute to observation in the milking parlor, and its subsequent treatment by alternatives types of administration, such as spraying when milked. Similarly, for dry products administered through footbaths, such as drying agents or dry biocides based on disinfectants or in active micro-organisms, the contamination rates of these products, and the losses of the product after the cow passages is unknown. Further studies in the area of "dry" footbaths are needed, especially because the regular washing of the feet necessary for classical footbath could be there detrimental to the colonization of the skin by the flora for instance. Then, the precise conditions in which such new products should be assessed remains to be elaborated. Lastly, the amount of contaminants present on feet might determine the effectiveness of a biocide applied by collective spraying. For this reason, measuring the mean quantities of contaminants that challenge the sprayed solution might highlight the optimal conditions for its implementation.

About the prevention of bDD lesions

The preventive effectiveness of collective treatments was still not evidenced across the literature (Chapter 2). In these previous studies, multiple reasons have been proposed to explain the lack or small effect of these strategies in the control of bDD. Nevertheless, it seems incoherent to expect to achieve protection through the disinfection of healthy feet which are biologically already aseptic. Otherwise, if the pathogens were in important numbers in the skin, such in the lesion stages, the effective measure results in the healing, and therefore the reduction of infected animals results in the prevention of the bDD occurrence. Besides, footbathing practices are not related by any means to limit the maceration of the skin and thereby protect the feet against the bDD occurrence (except maybe drying agents but this has to be evidenced). Although all these arguments contradict the plausibility of a preventive effect in collective treatments, footbaths are nevertheless considered in practice as preventive measures. An explanation could be that effective footbathing allow the early healing of M1 lesion, small and then not detected, leading to consider the absence of occurrence of M2 lesion (more frequently associated with lameness and thereby easily detected) as prevention n(from M0 to M2) while it could be a fast cure avoiding the transition from M1 to M2. Similarly, the term "treatment" seems punished to make reference to disinfectants as like healing enhancers. In the commercial and political context of veterinary labeling, disinfectants are only contemplated as prophylactic measures. Consequently, from our perspective, there is

a major concern regarding the preventive potential of footbaths strategies when measuring the incidence of bDD infection. Therefore, in the perspective of establishing guidelines for the usage and implementation of strategies of control for bDD, it seems crucial to define the indication of footbaths measures.

About the healing of bDD lesions

The results of our clinical trial indicate that the risk of healing of bDD lesion was increased significantly by the implementation of footbaths using the Pink-Step™ solution (HR 1.51 CI 1.07-2.11) at a moderate frequency. Comparing these results against the findings of our systematic review (Chapter 2), it is likely that by integrating our clinical trial into the meta-analyses, the overall sample reduces its heterogeneity and thereby increasing the precision on the previously combined effect estimate (OR 1.22 CI 0.73-2.02). Therefore, the overall effectiveness of collective measures for the healing of bDD lesions might be confirmed in a future systematic review. In addition, using the results of this meta-analysis, in order to roughly estimate the magnitude of the healing effect of Pink-Step™ when compared to the overall estimated effect of collective treatments, its effectiveness may double the mean effect expected by those collective treatments.

In our clinical trial, other factors were found as well at cow level affecting negatively the risk of healing, such as the lactation stage, the trimming and the presence of contralateral lesions. Thus, those factors inherent to the cow characteristic in a precise moment of the trial remains crucial to be systematically measured in order to avoid the over or underestimation of the true effect of collective treatments. Otherwise, after our investigation, the healing properties beyond the bactericidal effect of the biocide solution remained unexplored. The components of biocide solutions may promote the healing of bDD lesion by another mechanism than the formal disinfection. Biocides often support their efficacy in their bactericidal efficacy, and therefore effective biocides might be differentiated from others bactericidal by an additional cosmetic or healing property. In the case of Pink-Step™, there is some evidence in human medicine supporting the effect of glycolic acid in the healing of damaged skin (Green et al., 2009). However, these additional claimed properties of treatment measures must be investigated trough pertinent models. Otherwise, footbath products often lead to acids solutions rounding a pH from 2 to 4 (Cook, 2017), whether the normal skin pH round 7.13 (Meyer and Neurand, 1991). Thus, even if probably the skin pH in dairy cows is affected by the farming environment, the frequent usage of footbaths at acids concentration could alter the physiology of the skin. Therefore, the potential caustic effect of footbath solutions and their

relation to the occurrence and persistence of chronic lesions must be explored. It means that safety of the product should be assessed in parallel to efficacy.

From our results, the skin microbiotas of inactive lesions were closer to the ones of the healthy skin, hence supporting similar findings of previous studies (Zinicola et al., 2015). This fact represents a paradigm in the interpretation and the measure of efficacy. Indeed, if when measuring the healing or preventive efficacy of a treatment measure the inactive lesions are considered, according to their microbiota, as healthy stages of the disease, the estimated effect could vary considerably. Nevertheless, because the small sample size and the invasive technics used during the sampling of our study, the clinical evolution could not be appraised, therefore, enabling the evaluation of the relation between microbiotas and clinical improvements. Consequently, evaluate and measure clinically and microbiologically the differences between inactive and healthy stages seem essential to complete the clinical understanding of bDD. The importance of inactive lesions could be considered as negligible by welfare reasons due to its painless nature. Besides, it is important to remark that from the clinical perspective chronic lesions represent reservoirs of the disease associated with encysted forms of treponemes. Therefore, future studies should consider the implications of considering inactive lesions as like healthy stage when measuring the effectiveness of treatment measures according to their real benefit at short and long term.

Hygiene is the key

When measuring the risks associated with the occurrence of bDD lesions, the results of our clinical trial pointed the crucial role of farm and feet hygiene. From all the risk factors linked to bDD, the hygiene is probably the convergence point between the different studies across more than 20 years of research on the subject. Consequently, all improvements in the farm and feet hygiene remained the best measure for preventing bDD. Nevertheless, the mechanisms in which dirty and wet conditions favor the skin maceration have been scarcely studied. Furthermore, the role of the presumed pathogens in this process still unknown (Chapter 4.2). Hence, further studies concerning the feet hygiene are needed to highlight potential mechanisms to avoid the skin maceration. Besides, investigate the impact of slatted floors on the animal comfort, the feet hygiene, and the occurrence of claw lesions, remains a thematic largely interesting to explore because we can somehow expect contradictory result in terms of hygiene (increased with slatted floors) and lameness (decreased with slatted floors) (Ménard et al., 2016). Mixed floors combining concrete and soft material should be studied (Ménard et al., 2016). The fact that the feet hygiene plays the main role in the pathogenesis of

the disease reveals the importance of focus the hygiene assessments in the feet as a reflection of the multiple factors that may or not affect the overall farm hygiene (Guatteo et al., 2013).

Finally, the need for a multidisciplinary approach

The long persistence of the disease inside a herd once it is affected and its high between herd prevalence, open the perspectives to conduct large retrospective studies to approach potential temporal or regional factors linked to environmental conditions, nutritional management or massive trade of animals. On the other hand, mathematical models may provide the means to generate evidence-based information on bDD control at a reduced cost, exploring a range of diversity of possible strategies and play an important role in understanding its dynamics at long-term. For example, using the information generated in our clinical trial about the dynamics in the transition between the different bDD stages, it is possible to model how the exposure to different factors could affect these transitions in a long term. Therefore, revealing potential benefits of strategies of control at long-term. Besides, the findings of multiple previous studies can be integrated into the model to measure the impact of factors of interest at long-term, such as the use of dietary supplements (Gomez et al., 2014b), or programs of genetic selection (Scholey et al., 2012). Furthermore, through mathematical modeling artificial scenarios might be reproduced to simulate the singularities of a specific farm, and thereby determine the best strategy of control according to each farm scenario. These complex approaches open the perspectives to multidisciplinary collaborations integrating biological sense, informatics skills, and mathematical reasoning to converge in plausible models with factual scopes.

C. Contribution to the establishment of guidelines for the evaluation of control strategies of bDD

The methodology and the results from this Ph.D. led us to consider that there is a need for a standardized frame or approach to conducting the evaluation of a control strategy for bDD. Below, we would like to discuss, from our point of view, what could be the different steps in this process.

1. Evidence concerning a specific control strategy for bDD

The strategies of control for bDD might include single or multiple measures targeting the reduction of the disease by the healing of infected animals or/and by reducing the occurrence of infections within the herd. First, before beginning the evaluation of a control strategy for bDD, the assessment of the current scientific literature on the subject should be performed, to determine if there is already evidence supporting the implementation of the control strategy expected to be tested.

In the scope of evidence-based veterinary medicine, a systematic review of the control strategy in question will provide a comprehensive and transparent summary of the evidence on the subject (O'Connor et al., 2014). Nevertheless, the number of systematic reviews in veterinary medicine is limited, mainly because the feasibility for the conduction of clinical trials is restricted by ethical and economic concerns (O'Connor and Sargeant, 2014). This phenomenon is easy to perceive in the context of bDD. Indeed, to our knowledge, only 2 systematic reviews related to the bDD control are reported in the scientific literature (Thomsen, 2015; Ariza et al., 2017). Therefore, in veterinary medicine, in some cases, the evidence is mostly represented by epidemiological and non-randomized controlled trials. Thus, when evaluating the evidence about a control strategy for bDD, and in the case that systematic reviews are absent, the current evidence should be evaluated in same manner than systematic reviews to determine if it is necessary to conduct a complete evaluative process for the control strategy or if the evidence is already strong enough to support their implementation.

As pointed in Chapter 1, we reported that although the number of publications related to bDD has continuously increased since its first description (Chapter 1, Figure 1), the systematic assessment of this evidence remains unaccomplished. From this research process as well and beyond the evidence synthesis and the statistical summary, the main difficulties, drawbacks, and strengths on the design of the studies were highlighted, and these findings represent one the most important results of the review process conducted. This was for us the first step

before the elaboration of our own clinical trial trying thus to avoid the bias mostly encountered during the review. Moreover, through the review process, the benefit of a control strategy could be quantified. However, these statistical assessments are often difficult to conduct in the context of bDD. For instance, in the case of collective treatments for the bDD control, multiple substances are used through different systems of administration across the literature. Although the comparison of these measures may represent a goal for the review, the comparability of these different measures is restricted by the heterogeneity of the included studies. In other words, the fact that different studies comparing different measures of control limit their comparability when they do not share the same point of comparison, as for example when every study uses a different control intervention to compare a collective treatment. In these scenarios, network meta-analyses may be useful. Network meta-analyses allow indirect comparisons adjusting the effect estimates according to the sample size of the trials and other baseline parameters. These approaches, even if complex, could represent a perspective and a further step for evaluating qualitatively the evidence about strategies of control for bDD.

2. How strong is the preclinical evidence supporting the control strategy?

In the second place, the preclinical evidence that supports the questioned strategy must be assessed, to determine which evidence must be produced or reinforced before conducting the final trial. Although the quality of pre-clinical evidence might be high, its applicability in field conditions is sometimes importantly limited for instance by the design of these types of investigations. Thus, from our findings, it was demonstrated that the design of pre-clinical trials must fit with the field conditions (level of contamination by organic matter for instance), and for this purpose, pre-clinical must explore and include field measures. Consequently, the strength of the evidence from preclinical studies should be evaluated according to their coherence with the field condition in which the strategy will be implemented.

- Particular case of the evaluation of biocides

The optimal conditions for the implementation of a control strategy might be determined in an important degree in preclinical studies. Indeed, biocides should be tested and challenged in conditions approaching those encountered in the field. Beyond the levels of contamination that can affect the bactericidal efficacy of biocides, it could be primordial to enlarge the variety of bacteria that are standardly tested. Indeed, from our findings, different families and species of bacteria were associated with the bDD lesions. Even if the standard tests were

conceived to cover a large spectrum of bacteria by the 4 species used to follow the UE guideline (Chapter 1, Section 4.1), target bacteria of bDD should be tested to confirm the specificity of the biocide against the bDD associated-pathogens. Therefore, according to our results, biocides for the control of bDD must be tested against representative species of the phylum: *Spirochaetes* (*Treponema spp.*), *Firmicutes*, *Tenericutes*, *Bacteroidetes*, and *Fusobacterium.*

Otherwise, another aspect about biocides important to evaluate in pre-clinical studies is the safety of their usage. Indeed, the potential adverse effects of these substances in terms of tolerance to the product by the animals and farmers and their environmental impact should be evaluated approaching the dairy farming environment. Lastly, the acceptability of the footbath or administration route (such as spraying for instance) should be investigated to ensure the fact that realistic solutions would be assessed (Relun et al., 2013).

- How to proceed with the evaluation of the administration methods for collective treatments

The method implemented for the administration of collective treatments should as well be evaluated to guarantee the optimal usage of the products administered. For the specific case of footbaths, we determined the levels of contamination at which they are confronted in field conditions. We evidenced that the dimensions of the baths affect not only the content of organic matter but also the residual volume of solution after passages, and thereby determining the renewal frequency of the solution. Therefore, the evaluation of these baths seems crucial because even if the solution used to support high levels of contamination, the residual volume is not enough to cover the feet of the animals and provide their claimed effect.

In conclusion, pre-clinical evidence confers confidence to the guidelines protocols of a strategy of control. Therefore, strong pre-clinical studies should be a prerequisite to enhance the probabilities of success of a control strategy in field conditions.

4. Control strategies for bDD: How to define Success or Effectiveness?

- About statistical unit and risk factors

In the complex scenario of bDD where multiple factors can impact the estimation of the effectiveness of the strategy in question, the design of the trial protocol is fundamental. As multiple strategies for the data analyses might be implemented, it is mandatory to account for all the risk factors which can impact the measures of effectiveness. In our trial, survival analyses were implemented because considered as the most valuable based on the dynamic nature of the disease. These strategies of analyses allow accounting for unobserved covariates and therefore are suitable when evaluating bDD. Otherwise, the statistical unit used for the measure of the outcome and in the analyses is another factor that must be carefully chosen according to the biology of the disease and the scope of the results expected. Indeed, as in herds several animals are affected, and within animal multiple feet can also be affected, the inferences made it at cow and herd levels must be carefully approached. The intercorrelation between feet is another criterion for which we provide new insight and may allow more precisely determining the sample size when feet are the statistical unit.

- Which outcome can/shall we chose?

The outcomes definition is another important criterion to evaluate control strategies and also to communicate the results to farmers and veterinarians. The importance of outcomes is that they measure the success of a control strategy. Therefore no ambiguities are allowed in their definition, and, in a large perspective, success should be defined in an international consensus to allow comparability between trials. The outcomes related to the successful control of bDD might encompass among others, the healing of lesion, the prevention of the occurrence of lesions, the recurrence of lesions, or the reduction of the prevalence. Besides, outcomes might target specific stages or types of lesions. The ROI (return on investment) is another criteria which could be considered once the technical of the impact is well known in different herd context (prevalence, housing, herd size).

- Once the outcome is defined, how to measure it?

In the perspective of establishing criteria to evaluate a strategy of control for bDD, it is crucial to homogenize the methodologies implemented for measure the disease in field conditions. To remark, for the evaluation of strategies of control for bDD, outcomes should be measured always by objective and reproducible methods. Two different standardized methodologies are currently available for these mean (M scores – Iowa scoring). Nevertheless, these methodologies, as observational tools, are prone to subjective interpretations, and therefore their precision still controversial (Cramer et al., 2017). The gold standard remains the

trimming chute, but their usage is limited to small studies. Otherwise, more invasive methodologies have emerged, such the histological or metagenomic analyses of biopsy samples, and the ELISA test to detect titers of anti-bodies against bDD-treponemes in serum and milk samples. Nevertheless, the feasibility, the precision and the benefit of these methodologies must be evaluated. For instance, from our results, the metagenomic approach does not seem yet adapted to measure effectiveness. In conclusion, the homogenization of the observation methodologies is the main concern to guaranty comparability between studies and the perspectives for less invasive methodologies remain largely promising.

- About Efficiency?

Finally, the success of a control strategy might be perceived from different points of view. Indeed, clinical effectiveness may not be related overall efficiency. Therefore, cost-benefit indicators must be evaluated for the implementation of control strategies. Additionally, control strategies can be evaluated in a long-term perspective, through surveys approaching the perception and motivations of farmers for the continuity or interruption of the measure. By these means, the entire process of evaluation might be assessed, and improvement to the protocols might be performed. Mathematical modeling as suggested in the previous section could help to identify and assess ex-ante the opportunity of different strategies especially at a regional scale taking into account the potential impact of cattle trade or genetic selection on the herd status of bDD.

5. How to communicate the results obtained from strategies of control trials?

The results of trials might be reported in different metrics, being the most used the relative measures. Nevertheless, the way in which these relative measures are communicated may be complex for farmers and farms advisers. Therefore, the benefits of strategies of control for bDD can be expressed and homogenized through numbers needed to treat (NNT) (Cook and Sackett, 1995). The NNT provides useful insights for the decision-making process by including the notion of the effort required to achieve a successful objective in a specific context. By these means, positive numbers, are obtained when the beneficial effect of the strategy is superior to the benefits achieved by the comparison group, and can be interpreted as the "numbers of animals needed to treat (or to be exposed to the control strategy) for evidence an additional beneficial outcome or to prevent a fewer negative outcome". Otherwise, when the differences between the effect of the strategy studied and the comparison

group not statistically significant (P>0.05), the NTT 95% CI includes the infinity (∞), entailing the possibility of incertitude between the benefit or not of the strategy, exactly as other relative measures. The value obtained is relevant for the specific context of the study on which is it calculated (study period and the baseline spontaneous healing rate or the baseline incidence rate of bDD). As pointed before the NNT calculations may help in the decision-making process for strategies of control for bDD which often are measures applied to a large number of animals. The representation of efforts necessary to achieve an additional success by the strategy in comparison with a comparison group can guide the decision to implement or not the strategy. When NNT are low, a minor effort is necessary to achieve success by the implementation of the strategy, compared with when the NNT are high. Other relatives measures of treatment effect such as OR or "relative risk" are difficult to represent on the practical context because the benefit depends on the baseline risk. However, even if the NNT can only be compared within the same trial because it represents the effect of the compared interventions under the specific study conditions, they reflect easily the baseline incidence rates (for preventive outcomes) and the spontaneous healing rates (for healing outcomes).

To conclude, we considered that the most important perspective encompasses the establishment of international guidelines for the evaluation of strategies of control for bDD from the review of the literature to the clinical trial through a consensus statement including the experience, the evidence and the skills of the different research teams of every country.

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Chapter 6. Conclusion

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CONCLUSION

The focus of this thesis was to gain insights about the effectiveness of a new footbath biocide solution for the control of Bovine Digital Dermatitis in dairy herds and consequently investigate deeply the conditions which may determine the success or failure of such a control strategy. The facts that the current disinfectant solutions for the bDD control represented an environmental hazard or were unsafe for farmers motivate our research for the development of an effective alternative for the control of a highly prevalent, costly and painful disease.
This collaboration between a veterinary pharmaceutical laboratory, Qalian (Neovia group), and higher education institutions, Oniris-INRA, made it possible to carry out a very original epidemiological study on the evaluation of the effectiveness of a footbath solution for the control of bDD from the lab to the farm. The presumed efficacy of a novel footbath biocide solution (Pink-Step™) was assessed integrally and the healing effectiveness of the product was confirmed through a clinical trial of high quality.

The first part of this thesis which consisted in a systematic review and meta-analyses of the existing literature about the effectiveness of collective treatments on the healing and prevention of bDD lesions revealed the lack of evidence supporting collective treatments and allowed to highlight the drawbacks to avoid in future clinical trials.
During the second part of the project, before to the clinical trial, the impact of organic matter and slurry on the efficacy of a new biocide was assessed in vitro according to records obtained in field conditions. This step, rarely conducted for other products, led to determine the most appropriate renewal rate for the footbath under field conditions.
A third and last part consisted in a clinical trial conducted to implement the footbath solution under its optimal conditions of usage. The healing and preventive effect of different regimes of the solution were compared to a placebo group, using a split footbath allowing to treat one side of the cow, this latter being, therefore, its own control. The effectiveness was assessed through (i) the evolution over time of bDD lesions using survival analysis and (ii) through the description over time of the microbiota found in feet skin biopsies performed before and after treatment using 16s rRNA analysis. The findings of this clinical trial indicate that the collective disinfection of feet using Pink-step™ footbaths improved significantly the healing of bDD lesions. Nevertheless, the preventive effectiveness of the solution was not evidenced. The healing rates of bDD were also affected importantly in feet with active lesions, in

trimmed feet, in cows in late lactation and without contralateral lesions, and especially in larger herds (>100 cows). Besides, the occurrence of bDD lesions was mainly affected by the feet cleanliness at cow and herd level. Otherwise, the microbiota diversity of bDD lesions evolves over 45 days until recovering the same diversity metrics of healthy skin microbiotas. Differences in the microbiota diversity over time were as well detected between the nonproliferative and proliferative lesions and between the included farms. No differences between the microbiota diversity of feet receiving footbath and control feet were detected.

The overall results obtained from this work revealed insights about the preclinical methodologies to implement in the development of control strategies for bDD. Besides, through a clinical trial the effectiveness a new footbath solution was evaluated, evidencing its healing effectiveness and confirming the need for a global approach including other measures of control such as hygiene improvements and the concomitant usage of individual treatments over ulcerative lesions to control this multifactorial disease.

New perspectives of research have been identified from this work for the control of bDD. Thus, further studies should evaluate in a long-term if the improvements in the overall hygiene of farms and the implementation of footbaths using better biocides in optimal conditions, might lead to control the disease. Besides, the potential protective effect of footbath solutions through their healing effects must be assessed in a long term. Finally, further studies about the bDD microbiotas and their dynamics might elucidate the pathogenesis and the true etiology of the disease, and thereby gain insights for the bDD control.

UNIVERSITE BRETAGNE LOIRE

Oniris
ÉCOLE NATIONALE VÉTÉRINAIRE, AGROALIMENTAIRE ET DE L'ALIMENTATION
SANTÉ ET ALIMENTATION AU CŒUR DE LA VIE

Thèse de Doctorat

Juan Manuel ARIZA

Évaluation de l'efficacité et conditions optimales d'utilisation d'une solution de désinfection collective pour la maîtrise de la dermatite digitée en troupeaux bovins laitiers

Assessment of the effectiveness of a new footbath biocide and the best management practices for the prevention and treatment of the bovine digital dermatitis in dairy cows

Résumé

L'objectif de cette thèse était d'évaluer l'efficacité d'une nouvelle solution de désinfection collective en jugée prometteuse pour diminuer durablement la prévalence de la dermatite digitée (DD) en élevage bovin laitier. La solution devait être acceptable non toxique pour l'Homme et l'Environnement et permettre de raisonner le recours aux antibactériens. Une première partie a consisté en une méta-analyse de la littérature existante sur l'efficacité des solutions de désinfection collective. Cette méta-analyse a souligné le peu d'études robustes correctement menées et a permis d'identifier les erreurs à éviter dans notre essai clinique ultérieur. Une deuxième partie, préalable de l'essai clinique, a permis d'évaluer à la fois *in vitro* et *in vivo* l'interaction entre la matière organique apportée par les animaux et l'efficacité du produit testé afin d'identifier les préconisations de renouvellement de la solution en élevage, étape souvent négligée dans les essais cliniques. Une troisième et dernière partie a consisté en un essai clinique original, sous bonnes pratiques cliniques, dans 10 fermes sur plus de 2000 pieds visant à évaluer l'efficacité à la fois curative et préventive de la nouvelle solution désinfectante en comparaison à un placebo, à l'aide d'un bi-pédiluve permettant de ne traiter qu'un seul côté de l'animal qui était alors son propre témoin. L'efficacité a été évaluée (i) à travers l'évolution de la dynamique des lésions au sein du troupeau (par analyse de survie) et (ii) également via une partie originale d'analyse du microbiote présent sur des biopsies prélevées avant et après traitement permettant d'explorer la dynamique microbienne. Les résultats rapportent un effet principalement curatif de la solution et confirment la nécessité d'une approche globale incluant hygiène et le traitement individuel sélective pour maitriser cette maladie multifactorielle.

Mots clés : vache laitière, dermatite digitée, épidémiologie, méta-analyse, essai-clinique, microbiota.

Abstract

The aim of the PhD thesis was to assess the effectiveness of a promising original disinfectant product to decrease the prevalence of digital dermatitis (DD) in dairy herds. This product should be acceptable for farmers, not toxic both for human and the environment and finally allow a rational use of antibiotics. A first part consisted in a meta-analysis of the existing literature about the effectiveness of collective treatments on the healing and prevention of DD lesions. This meta-analysis, besides revealing weak evidence for supporting collective treatment, allowed us to highlight the drawbacks to avoid for our further clinical trial. In a second step, prior to the clinical trial, we assessed both in vitro and in vivo the impact of organic matter and slurry on the efficacy of the disinfectant. This step, rarely conducted for other products, led to determine the most appropriate renewal rate for the footbath under field conditions. A third and last part was devoted to a clinical trial, under good clinical practices, to assess both healing and preventive effect of different regimes of the new disinfectant in comparison to a placebo group, using a split footbath allowing to treat one side of the cow, this latter being, therefore, its own control. The effectiveness was assessed through (i) the evolution over time of DD lesions using survival analysis and (ii) through the description over time of the microbiota found in feet skin biopsies performed before and after treatment using 16s rRNA analysis. The results reported mainly a healing effect of the disinfectant and confirmed the need for a global approach including other risk factors such as hygiene and concomitant individual treatments, to control this multifactorial disease.

Key Words: dairy cow, digital dermatitis, epidemiology, meta-analysis, clinical trial, microbiota.

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