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EPIDEMIOLOGIC SURVEY OF HOOF HEALTH IN GRAZING CATTLE UNDER TROPICAL CONDITION AND ETIOLOGY OF BOVINE DIGITAL DERMATITIS

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Thesis presented as partial requirement for the degree of Doctor of Philosophy in Veterinary Science at the Veterinary School, Universidade Federal de Minas Gerais.

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"Só se vê bem com o coração, o essencial é invisível aos olhos."

"It is only with the heart that one can see rightly; what is essential is invisible to the eye."

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ABREVIATION LIST

HHE Heel horn erosion

WLF White line fissure

SH Sole hemorrhage

SC Scissor claws

DS Double sole

TS Thin sole

SU Sole ulcer

ID Interdigital dermatitis

DD Digital dermatitis

TP *Tunga* spp.

IP Interdigital phlegmon

IH Interdigital hyperplasia

OG/TRA Overgrown claws/ trauma

OTH Others

ABSTRACT

Hoof lesions and the resulting lameness are considered one of the three most common occurrences in dairy cattle and despite the advancement of knowledge his prevalence is increasing over the years. The objective of this study was to determine the prevalence of foot lesions and lameness in grazing herds in Minas Gerais state and identify the main risk factors. Furthermore, this work aims to investigate the core pathogens related with digital dermatitis in grazing cattle under tropical conditions. A total of 48 farms divided equally in four production groups were visited, 2267 animals were mobility scored and 392 animals had all hoofs inspected. A questionnaire and a checklist were applied to the owner or stockholder to identify risk factors. The prevalence of hoof lesions, lameness and severe lameness were calculated and a multivariable linear regression model were built to identify risk factors. Digital dermatitis lesions were sampled for further analysis by histopathology, fluorescent *in situ* hybridization (FISH) and gene sequencing. Among the evaluated cows, 16.0% were scored as lame and 6.8% as severely lame. Nearly all cows presented at least one type of hoof lesion, of which heel horn erosion (HHE; 90.1%), white line fissure (WLF; 50.4%), and digital dermatitis (DD; 32.7%) were the most frequent. DD was present in all but two farms. Sole ulcer was observed in only one animal. HHE and DD presented the highest proportion of severe cases, while the majority of WLF were mild. DD was correlated to an increase chance of 2.5 times in mobility score. Track features was the most significant factor increasing more than threefold the odds for HHE, WLF and sole hemorrhage (SH). Several factors related to unhygienic conditions such as frequency of corral cleaning, condition of corral exit, access to pile of manure and keep animals in paddocks during the dry period were identify as risk factors for hoof lesions. Poor human-animal relation was related with an increase odds for SH while patience of the farmer handling the cows on the track decrease in more than half the odds for interdigital hyperplasia. The microbiological analyses of DD samples revealed *Treponema* spp. as the most abundant bacteria and eleven different *Treponema* strains belonging to the six major phylotypes were identified. Furthermore, *D. nodosus* was also identified in a high proportion of samples in both FISH and sequencing. It was present in areas with mild epithelial damage and together with *Treponema*. Collectively, our results demonstrate that digital dermatitis is the main concern and the biggest cause of lameness in grazing cattle under tropical condition and it is related with unhygienic environment. The present data support the hypothesis that *Treponema* constitutes the main pathogens in DD and it further suggests *D. nodosus* as another potentially important pathogen.

Key words: Digital dermatitis, heel horn erosion, hoof lesions, lameness, welfare, fluorescent in situ hybridization, next generation sequencing, 16S rRNA, *Treponema*.

RESUMO

Afecções podais e a resultante claudicação são considerados uma das três ocorrências mais comuns no gado leiteiro e, apesar do avanço do conhecimento, sua prevalência continua a aumentar ao longo dos anos. O objetivo deste estudo foi determinar a prevalência de lesões podais e a claudicação em rebanhos a pasto em Minas Gerais e identificar os principais fatores de risco. Além disso, este trabalho objetivou investigar os patógenos relacionados à dermatite digital em bovino a pasto sob condições tropicais. Para atingir o objetivo, foram visitadas um total de 48 fazendas divididas igualmente em quatro grupos de produção, 2267 animais foram avaliados quanto a escore de claudicação e 392 animais tiveram os cascos inspecionados. Um questionário e uma lista de verificação foram usados para identificar fatores riscos. Lesões de dermatite digital foram amostradas para posterior análise por histopatologia, hibridização fluorescente in situ e sequenciamento genético. Entre os animais avaliados, 16,0% estavam claudicantes e 6,8% severamente claudicantes. Quase todas as vacas apresentaram pelo menos um tipo de lesão, do qual erosão de talão (ET; 90,1%), fissura de linha branca (FLB; 50,4%) e dermatite digital (DD; 32,7%) foram as mais frequentes. DD foi observada em 46 de 48 fazendas. A úlcera de sola foi encontrada em apenas um animal. ET e DD apresentaram a maior proporção de casos graves, enquanto a maioria das FLB eram leves. Além disso, a DD foi correlacionada com um aumento de 2,5 vezes no escore de claudicação. A condição das trilhas foi o fator mais impactante, aumentando mais do que três vezes a chance de ET, FLB e hemorragia de sola. Vários fatores relacionados as condições de higiene, como frequência de curral, limpeza, condição de saída do curral, acesso a pilha de estrume e manter animais em piquetes durante o período seco foram identificados como fatores de risco. A relação homem-animal, quando era inadequada, foi relacionada com um aumento das chances para hemorragia de sola, enquanto a paciência do agricultor ao tocar as vacas na pista diminui em mais da metade as chances de hiperplasia interdigital. As análises microbiológicas das amostras de DD revelaram *Treponema* spp. como as bactérias mais abundantes e foram identificadas onze diferentes cepas de *Treponema* pertencentes aos seis principais filotipos. Além disso, *Dichelobacter nodosus* também foi identificado em uma alta proporção de amostras tanto no FISH como no sequenciamento, estando presente em áreas com dano epitelial discreto e juntamente com *Treponema*. Coletivamente, nossos resultados demonstram que a dermatite digital é a principal preocupação e a maior causa de claudicação em bovinos leiteiros a pasto sob condições tropicais e está relacionada com as condições higiênicas do ambiente. Os presentes dados suportam a hipótese de que o *Treponema* constitui os principais patógenos em DD e sugere o *D. nodosus* como outro potencialmente importante patógeno.

Palavras-chave: Dermatite Digital, erosão de talão, lesões de cascos, claudicação, bem-estar, hibridização fluorescente in situ, sequenciamento de próxima geração, 16S rRNA, Treponema.

1. GENERAL INTRODUCTION

Lameness is considered one of the three most common occurrences in dairy cattle (Hernandez et al., 2002; Bruijnis et al, 2010), and, in some cases, reaches incidence above 100% (Ferreira, 2003). It causes a great economic loss by impairing milk production, reproduction and premature culling (Souza, 2002; Booth et al. 2004; Bruijnis et al., 2010). Besides that, lameness is one of the greatest welfare concerns in dairy industry. Along with its high prevalence worldwide, lameness itself is an indicator of pain and affect animal behavior (Whay et al. 2003; Bruijnis et al., 2010; 2012).

Despite advances in knowledge of the control practices and treatment of lameness, their frequency has not decreased over time, and though unlikely, it is apparently increasing and today reaches unacceptable prevalence (Leach et al, 2010; Potterton et al, 2012; Casagrande, 2013). Studies executed in Brazil show that this situation also happens in our country. Ferreira (2003) described an incidence of lameness of 122% in a free-stall farm while Moreira et al. (2012) observed hoof lesions in 100% of the examined animals in a farm in Minas Gerais state.

Various diseases or conditions can cause lameness, but in most cases is a consequence of hoof lesions, most of whom are present in subclinical form in the herd (Murray et al., 1996). The cause of hoof lesions are multifactorial and therefore a holistic approach encompassing all the characters involved throughout the dairy industry and identifying the risk factors in both individual and herd level must be understood and taken into account in order to reach success in reducing these diseases (Leach et al., 2010).

This research is part of the “Cascos Saudáveis” (Healthy Hooves) project, which was established in co-operation between COOPRATA and the Escola de Veterinária from the Universidade Federal de Minas Gerais (Veterinary School of Federal University of Minas Gerais). The project counted with the collaboration of farmers, veterinarians, technicians and hoof trimmers to achieve the objective to access and further improve the hoof health in the region. Specifically, the project aimed to identify the prevalence of lameness and hoof lesion, its causes and associated risks, what can be considered the first step in a control program. Furthermore, this study also investigated the pathogens related to DD in grazing cattle in Brazil.

The project began after some years of collaboration between COOPRATA and the Escola de Veterinária (Veterinary School) from UFMG. The first collaboration happened when occurred an outbreak of trypanosomiases in the region. The university helped the farmers and the local cooperative by diagnosing and teaching the local technicians about the disease. After some visits in the regions, it was very common farmers complaining about the number of lame cows.

It was interesting to follow the history of the regions and, because of that, it was possible to connect all the facts. We believe that the trypanosomiases outbreak occurred in consequence of the culling of lame cows and the necessity of buying new ones for replacement. Trying to respond to the farmers demand, and understanding that the lameness was one of the factors that helped the trypanosomiases outbreak to occur, the two organizations established a cooperation and the project “Cascos Saudáveis” begun.

There are two main approaches for reduction of foot diseases which should be made together to achieve best results. The first is based in actions aimed at early diagnosis and treatment of diseases preventing further damage to the animal and financial loss for the owner. The second is to establish control measures and prevention to reduce the number of new cases (Potterton et al., 2012). However, both approaches usually suffer the resistance of producers due to reluctance to make changes in the management (Leach et al., 2010). In order to make any changes to reduce hoof lesions everyone involved should be motivated and the obstacles that may impair the progress should be identify and swiftly reduced (Leach et al., 2010). Main et al. (2012) described a decrease prevalence of hoof lesions with increasing the awareness and providing a better understanding of the subject by the farmers. Thereafter, in “Cascos Saudáveis” project we tried to encompass all the three main characters involved in dairy industry (farmers, veterinarians and technical advisors) aiming to bring a long and lasting benefit for the region.

Most of the studies regarding lameness and hoof lesions were performed in the north hemisphere, with confined and high yielding dairy cows (Barker et al., 2010; Capion et al., 2009; Foditsch et al., 2016). Consequently, little is known about hoof lesions and lameness prevalence in grazing cattle under tropical condition. In this context, a study that clarify the management and husbandry practices that encompass risk factor for both lameness and hoof lesion under Brazilian conditions is warranted.

Brazil has 212.340.000 heads of cattle (IBGE, 2012), the second largest herd in the world, just behind India. It is also the fourth largest milk producer (FAO, 2012) with a production of 35.17 billion liters in 2014 (IBGE, 2012). However, the average productivity of milk in Brazil is very low as 1525 liters/cow/year, or 4.17 l/cow/day. Minas Gerais has 23,707,042 heads of cattle, and is the first milk producer in the country with 9.37 billion liters in 2014, representing 77.0% of all production in the Southeast and 26.6% of the total national production (IBGE, 2012). The majority of milking cows are crossbreed with the predominance of zebu breeds. The dairy farms in Brazil are predominantly of the semi-intensive systems, with average milk yield of up to 20L milk/day/cow. In this kind of system, despite the fact that the cows spend most part of the day in pastures, lameness is a great concern, and represents an expressive loss (Souza, 2002; Moreira et al., 2012).

The municipality of Prata has the largest cattle herd in the state of Minas Gerais with 356,491 heads and is the second largest milk producer in the "Triangle Region" with 78,900 cows milked (IBGE, 2012). It has a total area of 4847.544 square kilometers and an estimated population of 27,293 habitants in 2014 (IBGE, 2014). The city is also known as "Milk Capital", which demonstrates the importance of this activity for that region and, consequently, the relevance of a work to assist the development of this activity. In addition, a work that has the intention to educate all three characters involved with the activity in the region (farmers, veterinary and agricultural technicians) on lameness has great potential to change the local scenario.

The great territorial extension and large diversity of the dairy farms presented in Prata make possible to extend the finding to a larger area of Brazil, especially for the southeast region, with is characterized by very similar conditions. Thus, this study represent a groundwork to elucidate the lameness and hoof health situation in Brazilian farms.

Previous studies show that the primary cause of lameness in grazing cattle in Brazil is infectious diseases and particularly bovine digital dermatitis (DD), with in some cases reach prevalence above 40% (Mouchle, 2001; Souza, 2002; Moreira et al., 2012; Casagrande, 2013). However, few studies have try to determine the prevalence of hoof lesions in a regional perspective, rather than in individual farms. Digital dermatitis is a growing concern worldwide since its first description in 1974 (Refaai et al., 2013). Despite the growing interest, the etiology of this disease continue to be an unsolved challenge (Evans et al., 2016). Similar to what happen with lameness research, the works to clarify DD etiology has focused in free-stall cattle, despite its importance for grazing cattle too.

The partial results from the epidemiological study carried out in the "Cascos Saudáveis" project confirmed the great importance and huge impact of DD for the hoof health in the studied region. Therefore, we decided to dedicate further attention to this disease and try to elucidate its related pathogens.

The etiology of bovine DD appears to be polymicrobial by the wide variety of bacteria that have been isolated (Marcatili et al., 2016; Nielsen et al., 2016). However, evidences indicate an important role of spirochetes, especially from the genus *Treponema* (Klitgaard et al., 2008; Yano et al., 2009). This is the most abundant group of bacteria in DD lesions, and they are found deep in the lesions suggesting an active hole in disease development (Klitgaard et al., 2008; Rasmussen et al., 2012; Klitgaard et al., 2013). To date, at least 20 different phylotypes of *Treponema* have been identified from DD biopsy specimens in different parts of the world (Klitgaard et al., 2013).

Because *Treponema* species are extremely challenging to culture, culture-independent methods have been used to identify pathogens in DD lesions, mostly using comparative 16S rRNA gene sequences analysis and fluorescence *in situ* hybridization (FISH) (Klitgaard et al., 2008). FISH has been largely used to detect treponemes in DD lesions. This method also provides information on the localization of individual microorganisms at the infection site (Klitgaard et al., 2008; Rasmussen et al., 2012; Klitgaard et al., 2013).

2. OBJECTIVES

The aim of this thesis is to provide an epidemiological background for future development of programs for a better hoof health in all year round grazing cattle under tropical condition. For this, the first part aims to determine the prevalence of hoof lesions and lameness in a representative region of the Minas Gerais state, while the second part aims to identify the main risk factors present. Furthermore, as the research was developing, digital dermatitis was identified as the biggest challenge in the visited farms. Therefore, a second objective was proposed, to identify the main pathogens present in digital dermatitis lesions in grazed cows and to elucidate the role of each identified bacteria.

3. LITERATURE REVIEW

3.1. Lameness, a growing issue

Lameness is considered one of the three biggest burden in dairy industry. It has been highlighted as a major welfare problem in dairy cattle and represent an expressive economic loss (Bruijnis et al., 2012, 2010). Furthermore, reports from different parts of the world indicate an increase in incidence of lameness despite the efforts to control and reduce it. Nevertheless, the research in the area is scarce compared to mastitis and others infectious diseases (Bicalho and Oikonomou, 2013).

3.1.1.Cow wealfare

Lameness is usually caused by discomfort and pain, normally associated with hoof injuries (Whay et al., 1998, 1997). The signs of pain in lame animals are very clear and well recognize. From both animal welfare and public relations perspectives, cattle lameness is an issue of growing attention for the industry and scientific community (Von Keyserlingk et al., 2012). Lame animals change their behavior, spending more time lying and less time feeding (Ito et al., 2010; Thomsen et al., 2012). Besides, lame cows are less likely to start social interactions with other cows, although they are as likely to be subjected to aggressive behavior by other animals as sound cows (Galindo and Broom, 2010).

Dairy operation has more often been placed under scrutiny by the consumers and animal well-being was indicated as the most important concern for nonfarming respondents to a recent US survey (Boogaard et al., 2011; Cardoso et al., 2016). When the question "What do you consider to be an ideal dairy farm and why are these characteristics important to you?" was asked to US citizens, answers included the following: "They [the cows] should be treated humanely because it's the right and moral thing to do" and "This is a humane issue and people should treat animals with respect. It is just common decency". In this scenario lameness seems to be an important factor once the signs of pain in lame cows are easy noticed (O'Callaghan et al., 2003).

3.1.2.Lameness cost

Lameness has a great economic impact because it can lead to numerous consequences. The most recognizable by the farmers is milk production losses, but it also causes reproductive impairment, increases the risk of culling and also of other diseases such as mastitis and milk fever, increases expenses with hoof trimming, treatment and labor required (Bruijnis et al., 2010; Cramer et al., 2009a; Raboisson et al., 2014; Somers et al., 2015). It also changes animal behavior decreasing the time spent feeding (Galindo and Broom, 2000).

The average cost per case (US$) of sole ulcer, digital dermatitis and foot rot was estimated at 216, 133 and 121 (Cha et al., 2010). In Brazil, the annual cost produced by lameness was calculated to be US$ 125.36 for each cow in a herd (Souza et al., 2006). The estimated cost only for treating each lameness case was US$ 44.68 (Ferreira et al., 2004). However, most of the hoof lesions occur in subclinical form (Manske et al., 2002). According to Bruijnis et al. (2010), a clinical foot disorder costs, on average, US$ 95, and a subclinical foot disorder US$ 18. In a hypothetical situation, Bruijnis et al. (2010) found that 32% of the total cost is due to subclinical cases.

3.1.3.Increasing incidence

Despite the increasing knowledge regarding foot health of dairy cattle, the annual incidence of lameness in dairy herds worldwide is apparently increasing, rising from 5% at the beginning of the ' 80s (Prentice and Neal, 1972; Eddy and Scott, 1980) to currently exceed 100% in some cases (Ferreira, 2003). Although, this conclusion can be considered precipitated, through the analysis of Table 1, it is easy to observe incidence rates of less than 10% in works conducted in the '70s and '80s, while later works do not report prevalence below 30%. It is important to consider the methodology used to calculate the incidence of lameness. The studies carried out in the decades of 70 and 80 were normally done by calculating the cases attended by veterinarians, which represents only about 30% of the animals treated, while farmers or other professionals treat the other 70% (Whitaker et al., 1983). These researchers already believed that the actual incidence of lameness in the United Kingdom, was already close to 30% and up to 60% in some cases (Whitaker et al., 1983; Esslemont and Spincer, 1993; Clarkson et al, 1996), but still lower than incidence rates reported nowadays.

Table 1. Overview of lameness incidence around the word in the last decades.

Prevalence/incidence	**Country**	**Author and year**	**Observations**
7% (0-50%)*	Australia	Harris et al. (1988)	
2,7%*	Australia	McLennan (1988)	Treted by the vet.
14%	New Zealand	Dewes (1978)	
20.7% (2-38%)	New Zealand	Tranter and Morris (1991)	
3,88%	United Kingdom	Leech et al. (1960)	
30%*	United Kingdom	Prentice and Neal (1972)	
4,7% (0-32%)*	United Kingdom	Eddy and Scott (1980)	Treated by the vet.
5,5% (1,8-11,8%)	United Kingdom	Russel et al. (1982)	Treated by the vet.
25%*	United Kingdom	Whitaker et al. (1983)	
6,3%*	United Kingdom	Whitaker et al. (1983)	Treated by the vet.
36% (1-94%)	United Kingdom	Esslemont and Spincer (1993)	
55% (11-170%)	United Kingdom	Clarkson et al. (1996)	
38% (4-69%)	United Kingdom	Kossaibati and Esslemont (1999)	
69% (32-112%)	United Kingdom	Hedges et al. (2001)	
36%	United Kingdom	Barker et al. (2010)	
4,4	US	Barlett et al. (1986)	
5,1	US	Kaneene and Hurd (1990)	
46% (40-52%)*	US	Warnick et al. (2001)	
31%*	US	Hernandez et al. (2002)	
90%	Denmark	Capion et al. (2009)	
30,3%	Brazil (Minas Gerais)	Molina (1999)	Prevalence of hoof lesions
55%*	Brazil (Minas Gerais)	Souza (2006)	
50,2%	Brazil (Rio Grande do Sul)	Cruz et al. (2001)	
29,67%	Brazil (Goiás)	Silva et al. (2001)	Prevalence of hoof lesions
122%*	Brazil (Minas Gerais)	Ferreira (2003)	One Free-stall herd
4,8%	Brazil (Goiás)	Romani et al. (2004)	Partly housed

*Incidence

3.2. Lameness assessment

In dairy cows, the most recognized signs of lameness are head nodding, slow and hesitant walk, asymmetric gait, non-rhythmic timing of footfall, shortened strides in one or more feet, spine-arching and abnormal limb motion or limping (O'Callaghan et al., 2003; Van Nuffel et al., 2015). The cow performs this alteration in posture and weight barring to preserve the affected limb. The "arched spine" posture helps to distribute more weight away from the affected feet (Van Nuffel et al., 2015).

Visual assessments of locomotion remain the usual approach despite the growing options for automatic monitoring. Visual assessments are done using subjective methods that can be taught to lay people to detect gait impairment. This way, visual assessments can be performed by the farmer, employee, veterinarian or agricultural consultant and constitutes an inexpensive and quick to apply method (Van Nuffel et al., 2015). However, many scoring system (more than 25) have been developed based on different signs. The most used is the one proposed by Sprecher et al. (1997). This score system is based in the back posture during walking and standing and it is divided in 5 scales. Another very used score system is the one described by Barker et al., (2010), which does not focus in only one sign of pain, but rather use a more broad characteristics to diagnose lameness. This two methods are described in Table 2.

Table 2. Comparison of lameness score proposed by Sprecher et al. (1997) and Barker et al. (2010).

Author	Score	Description
Sprecher et al. (1997)	1	Cow stands and walks with a level back posture. Her gait is normal walking. Gait is normal
	2	Stands with level back but arched back when walking. Gait is norma
	3	Arch back while standing and walking. Gait is affected. Shortened strides with one or more limb
	4	Arched back posture always evident. Gait best described as one deliberate step at a time
	5	The cow demonstrates an inability or extreme reluctance to bear weight on one or more limb
Barker et al. (2010)	0	Sound. Walks confidently, with even weight on all 4 feet; tracks up (hind feet in prints of fore feet); no swinging of legs inward or outward.
	1	Imperfect locomotion. May walk cautiously, possibly because of tenderness, or does not track up, or legs swing out or in, but no obvious limp.
	2	Lame. Definite limp (foot fall uneven, dew claws on affected limb do not drop as far) or arched spine. A favored limb will move more quickly than the lame limb. Speed of walk not noticeably affected.
	3	Severely lame. Cannot walk at a brisk human pace. Animal shows obvious signs of limb pain (e.g., reluctance to bear weight, very obvious shifts in body posture).

Adapted from Sprecher et al. (1997) and Barker (2010)

Barker et al. (2010) found the percentage of agreement between the paired observers using the 4-point scale to range from 61.3 to 83.3%. When comparing lame versus non-lame cows the agreement ranged from 83.9 to 96.8%. Kappa values between the paired observers ranged from 0.67 (moderate) to 0.93 (good) when comparing lame to non-lame cows.

3.3. Bovine claw

By international agreement (in identifying the location of a lesion), the claw capsule has been broken up into different zones. Those zones has different names and functions. In Figure 1 is possible to see how the bovine claw zones are named, while Figure 2 shows the internal structure of the bovine hoof.

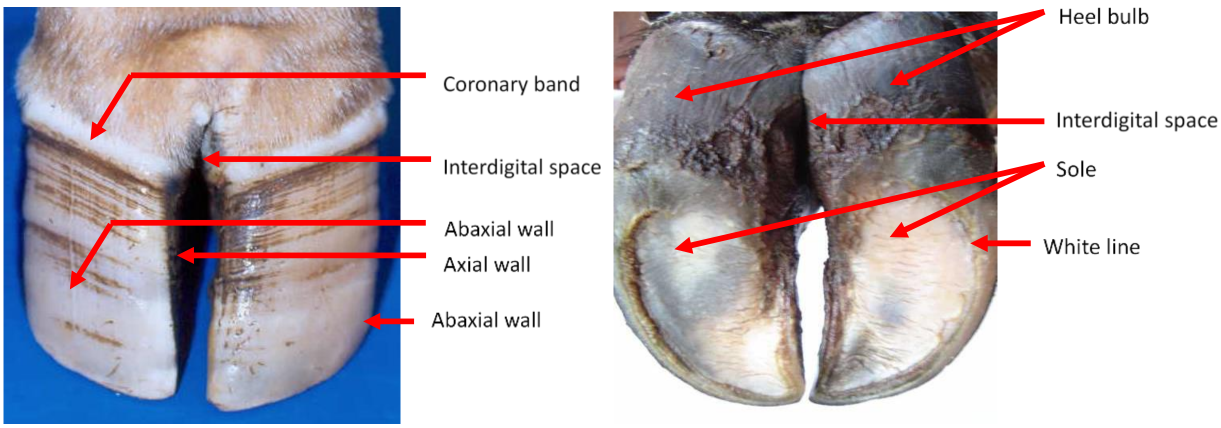

Figure 1. Picture of the palmar (left) and plantar (right) view of a bovine hoof showing the name of different zones.

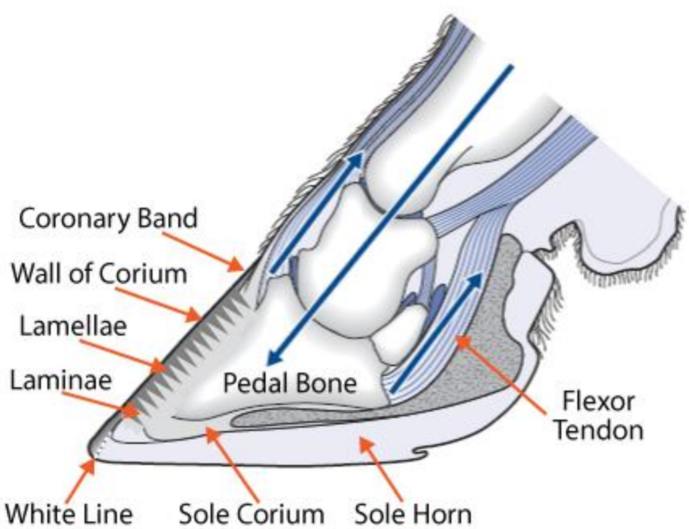

Figure 2. Representation of the internal structure of the bovine hoof. The pedal bone is suspended by the interdigitation of the lamellae in the corium wall with the dermal laminae attached to the bone. Figure from Greenough (2007).

The claw or hull capsule acts as a barrier that protects the internal tissues and transmits the burden between the ground and the skeleton (Hoblet and Weiss, 2001). The hull is formed by a layer of living cells in its deepest part and a layer of keratin in its surface (Tomlinson et al., 2004). The monthly growth rate of healthy cattle hooves is around 0.4 to 0.5 cm, but this can vary depending on the nutrition, physiological status, age, environment and season of the year (Tranter and Morris, 1991; Vermunt and Greenough, 1995).

The keratinization is the key process for the production of a claw with good quality and appropriate characteristics. This process is performed by the keratinocytes, highly specialized cells in proteins production (Tomlinson et al., 2004). Keratin is formed by the combination of various proteins with a high content of cysteine and other sulfur-rich amino acids. This is important for the structure of keratin, due to the disulfide bonds formed from the sulfhydryl groups. As the process of keratinization progresses, the formation of these bridges is accelerated and the keratin filaments interlink providing the hardness and chemical stability to the hull (Tomlinson et al., 2004).

An important structure on the hull is the digital cushion, which is formed mainly of adipose tissue and has the function to absorb the impact between the ground and the third phalanx (Räber et al., 2004; Bicalho and Okimonu, 2013). Its composition suffers the influence of age, and the adipose tissue is gradually replaced.

3.4. Hoof lesions and hoof inspection

Lameness can be caused by pain and several others neurological or muscle-skeleton disturbances that impairs the function of the locomotor system. However, the most common lameness cause is found in the hoof or in adjacent skin (Murray et al., 1996; Ferreira, 2003). Of the cases affecting the feet of dairy cattle from 80 to 95% are in the hind feet and 65% of these are in the lateral claw (Murray et al., 1996; Ferreira, 2003; Solano et al., 2016). As the main cause of lameness, hoof record may provide a better understanding of hoof health than only lameness records. Thus, direct measures of hoof diseases or a combination of direct measures of hoof diseases and lameness are preferred if data are available (Buch et al., 2011).

The list of conditions that can affect the hoof and skin of bovines is extensive. For simplicity, these conditions can be grouped according to whether they affect claw horn and corium, skin or deeper tissues or it can be categorized according to aetiopathogenesis. Using this last approach, almost all hoof lesions can be grouped in two categories: infectious lesions diseases such as digital dermatitis, interdigital dermatitis, phlegmon, and heel horn erosion; and non-infectious lesions or claw horn disruption lesions (CHDL) such as sole ulcers, sole hemorrhage and white line disease (Greenough and Weaver, 1997; Manske et al., 2002)

Most hoof lesion can be present in subclinical form, without being associated with clinical lameness (Manske et al., 2002). The relationship between lameness and the early stages of foot lesion development may be complex for a variety of reasons (O'Callaghan et al., 2003). Under normal conditions of hoof growth and wear, hoof lesions that are consequence of laminitis or corium tissue damage only reach the bearing surface and become visible 8 to 10 weeks after the injury has occurred (Bergsten and Frank, 1996; Lischer and Ossent, 2000). Thereafter, hoof lesions can reflect damage inflicted weeks or months before the examination and thus fail to offer accurate insight to the present status of the hoof. However, in the case of faster developing conditions of the distal limb, such as digital and inter-digital dermatitis, hoof inspection can give an accurate insight into possible causes of lameness.

3.5. Infectious lesions

3.5.1.Heel horn erosion

Heel horn erosion (HHE) is characterized as an erosion of the bulbs that can present a typically V-shaped in severe cases, possibly extending to the corium (ICAR 2015). The loss of horn tissue can be caused by a structural breakdown induced by manure and urine or by proteases produced by bacteria such as *Dichelobater nodosus* (Mülling and Budras, 1998). The lesion is related with a prolonged exposure of the foot to wet and unhygienic environments. A horn of poor quality enhance the effects of the environment such as in cases of laminitis and nutritional deficiency (Greenough and Vermunt, 1991, Shearer et al., 1999).

The HHE is often observed as the most prevalent foot lesion, both in grazing and non-grazing cattle. A survey from 2009 stated that HHE was found in Danish dairy heifers with a prevalence greater than 90% in freestall herds (Capion et al., 2009). Similar results was found by Moreira et al. (2012) in a semi intensive farm in Minas Gerais, Brazil.

3.5.2.Interdigital hyperplasia

Interdigital hyperplasia (IH), also known as corns, fibroma or tyloma, is caused by a chonic irritation of the skin and the development of a proliferative reaction in the interdigital cleft made of fibrous connective tissue. The affected animals is not often lame. The predispose conditions are poor claw conformation, incorrect claw trimming, interdigital phlegmon, dermatitis, lesions and slippery floor (Hoblet and Weiss, 2001; Manske et al., 2002). IH is correlated with other infectious conditions as DD.

3.5.3.Interdigital phlegmon

Interdigital phlegmon (IP) was also described as interdigital necrobacillosis and foul in the foot. It is an acute inflammation of the subcutaneous tissues of the interdigital space and adjacent coronary band caused by the penetration of bacteria through open lesions (Greenough and Weaver, 1997). Bacteria related with IP are *Fusobacterium necrophorum*, *Porphyromonas levii* and other opportunistic pathogens. The affected limb became hyperemic, swollen and painful, what makes the animal to become lame (Shearer et al., 1999). If not treated promptly arthritis may follow.

3.5.4.Digital dermatitis

Digital Dermatitis (DD) is a relatively new disease and was first reported by Cheli and Mortellaro (1974), in Italy. There are some reports and photographs of similar injuries before the 70´s, but the disease certainly didn´t have the same distribution and epidemic situation as at the present time. Blowey (2012) describes that his first experience with DD was in the late 80´s. When visiting a farm in England he noticed injuries and open wounds in the region between the heels of cows. According to him, diagnose was difficult because few people knew at that time about DD. Nowadays, it is virtually impossible that a veterinarian practitioner has never treated or at least seen an animal with DD.

DD, also called hairy heel wart, strawberry heel, or raspberry warts, is spread all over the world and is considered the first cause of lameness in dairy cattle causing great economic loss (Mouclhe, 2001; Logue et al., 2005, 2011; Laven e Logue, 2006; Logue e Bergsten, 2007; Casagrande 2013). Data suggests that 10%–40% of all lameness cases can be attributed to DD depending of the region (Refaai et al., 2013). Surveys report that DD is present in 70-95% of dairy herds in the United States and Denmark (Capion et al., 2008; Cramer et al., 2008), with a prevalence of average 20-30% (Capion et al., 2008; Cramer et al., 2008; Barker et al, 2009; Moreira, 2012; Casagrande 2013).

The DD lesion can present diverse aspects as show in Figure 3. The typical lesion is characterized by proliferative and/or ulcerative dermatitis that is typically located on the plantar aspect of the hoof between the heels (Laven and Logue, 2006; Krull et al., 2016). Pain upon palpation and lameness is common, but not always present. The disease can present a diverse aspect and it can be classified according to Döpfer et al. (1997) in four different stages. The score M1 corresponds to an initial lesion presenting a small focal active state (<2 cm across). M2 is the classical aspect of DD, characterized by an ulcerative active lesion with more than 2 cm across, extensively mottled red– grey. M3 stage correspond a healing lesion where the ulcerated surface is covered by a dry brown and firm rubbery scab and normally it is not painful. M4 is the chronic lesion with a proliferative hyperkeratotic growth that vary from papilliform to mass-like projections, usually brown or black, rubbery and irregular surface. Barry et al. (2012) introduced a fifth classification, the score M4.1 where the

Figure 3. Different aspects of digital dermatitis. From left to right a typical active lesion presenting a circumscribed moist ulcerative erosive mass, a larger lesion with an erosive aspect and a chronic case with erythematous masses with papilliform projections.

chronic stage dominates the lesion but a small active painful M1 focus is present.

Although most epidemiologic studies regarding DD are limited to the north hemisphere and focus in confined high yield dairy cows, studies show that DD is the primary cause of lameness in semi-intensive systems, the predominant type in Brazil, reaching over 40% of prevalence (Mouchle, 2001; Souza, 2002; Moreira et al., 2012; Casagrande, 2013). Souza (2002) found an average prevalence of DD of almost 60% in 63 farms in Minas Gerais state and Moreira et al. (2012) found 40% of prevalence in one semi-intensive farm.

Despite the great importance and the increase knowledge of the dynamics of infection, causative agents and forms of control, DD continues to be an endemic disease with a prevalence that often exceed 50%, representing a grave concern (Somers et al., 2005; Nielsen et al., 2012; Speijers et al., 2013). Because of that, LeBlanc et al. (2006) consider DD an example of a disease that is still a challenge nowadays.

3.5.5.Digital dermatitis etiology

The precise etiology of DD remains to be determined. The etiology of DD is predominantly bacterial since lesions regress after antibiotic treatment and virus and fungus participation have been discarded by several studies (Demirkan et al., 1999; Brandt et al., 2011; Berry et al. 2012; Cutler et al., 2013). DD is polymicrobial disease by the wide variety of bacteria that have been isolated, however, the role of different species is not well known. The isolated and identified bacteria from DD varying from *Fusobacterium necrophorus*, *Guggenheimella*, *Prevotella*, *Camplyobacter*, *Clostridium*, *Mycoplasma* and *Dichelobacter nodosus* to various spirochaetes (Demirkan et al., 2000; Schroeder et al., 2003; Klitgaard et al., 2008, 2013; Yano et al, 2009; Yano et al, 2010; Rasmussen et al., 2012; Nielsen et al., 2016).

Evidence indicates an important role of the spirochetes, especially of the genus *Treponema* in the etiology of DD. They would represent the most abundant bacteria group, mainly in the deepest parts of the lesions, suggesting that they are invasive pathogens and not simply colonizers of the infected tissue (Klitgaard et al., 2013; Nielsen et al 2016). *Treponema* spp. are the only bacteria that have been consistent identified in DD lesions (Evans et al., 2008; Apley, 2015). To date at least 20 different phylotypes of *Treponema* have been identified from DD biopsy specimens (Wilson-Welder et al., 2015a).

The most common phylotypes were *T. vincentii/T. medium*-like, *T. phagedenis*-like, *T. denticola*/*T. putidum*-like, *T. refringens*/*T. calligyrum*-like, *T. pedis*-like and *T. denticola*/ *T. putidum*-like, but other researches have expanded this list incliding *T. brennaborense*, *T. maltophilum*-like and *Spirocheta zuelzerae* (Evans et al., 2008, 2009; Klitgaard et al., 2013; Yano et al., 2010). Other identified by 16S rDNA but yet uncultured phylotypes defined as clusters of treponemes in which the 16S rDNA sequence differs by ~2% from known species and which are ≥99% similar to other members of their cluster were identified by the PT prefix (Klitgaard et al., 2013). These studies have been carried out in different countries of Europe, in the USA and Japan, and present a variance of the treponemes identified by 16S rDNA what may indicates a regional/geographical variance in DD-associated treponemes.

Attempts to induce the disease with pure cultures of these bacteria by inoculation on healthy skin were largely unsuccessful (Wilson-Welder et al., 2015b). However, Gomez et al. (2012) and (Krull et al., 2016a) successfully reproduced the disease by different methods. After diverse tests, the methodology with higher success rate (95% of success) was to inoculate in healthy skin a macerate of DD lesion, wrap to produce an anaerobic environment and keep it moistened. After a period of 12 to 25 days in these conditions, the authors successfully induced DD lesions and detected the presence of *Treponema*.

In the last few years, DD has been considered a politreponemal disease rather than a polimicrobial disease. However, some evidences points toward other bacteria acting synergistically and have an active involvement in lesion development or progression, with some indication that this bacteria belongs to the phylum Bacteroidetes (Marcatili et al., 2016). In studies trying to induced DD, pure *Treponema* cultures as well as *D. nodosus* cultures were capable to reproduce an incipient DD lesion, but better results were achieved when a homogenate material from a naturally occurring DD lesion were used (Gomez et al., 2012; Knappe-Poindecker et al., 2015).

Treponema genus are not associated only with DD, they are also responsible for periodontal diseases in humans and animals. Another example of the involvement of these bacteria are the skin ulcers found in swine and equine (Karlsson et al., 2014; Svartstrom et al, 2013) and syphilis in humans. The National monitoring system of Animal health of the United States (USDA) showed that more than 50% of cases of lameness in dairy cows and heifers have as main cause the DD and has estimated a yearly loss of $190 million dollars for this disease. Determining the cost per case of DD is $95 and when milk production losses and decreased reproductive performance were incorporated into the calculation, the losses were estimated at $126 per clinical case (USDA, 2009).

3.6. Laminitis and Claw Horn Disruption Lesions (CHDL)

Laminitis or aseptic diffuse pododermatitis is a diffuse not infectious inflammation in the corium (Greenough and Vermunt, 1991; Vermunt, 1994; Ossent et al., 1997). The laminitis results in an imperfect keratinization process, with consequent production of corneal tissue of poorer quality and less resistance, making the hoof horn more susceptible to diseases (Lean et al., 2013).

Laminitis can develop in three distinct forms: acute laminitis, subclinical laminitis and chronic laminitis. The first is the rarest and causes a lot of pain and the animal tends to get into abnormal positions. Inflammation of the corium is evident with dilation of veins and arteries and local hyperthermia (Ossent et al., 1997; Lean et al., 2013). The subclinical laminitis is a chronic condition that only becomes apparent over time. It is characterized by low quality and softer corneal tissue. Its consequences are sole and white line hemorrhages, double sole, white line disease and sole ulcers (Ossent et al., 1997; Lean et al., 2013). This group of conditions is referred to as claw horn disruption lesions (CHDL) (Machado et al., 2010). The chronic laminitis occurs because of successive cases of clinical or subclinical laminitis. It occurs mainly in older animals, showing deformed, elongated, wide hooves, and plans with horizontal striations (Lean et al., 2013). This represents one of the main causes of culling (Ossent et al., 1997).

The etiology of laminitis is complex and multifactorial and its pathogenesis is still a challenge for researchers (Osset et al., 1997; Danscher et al., 2010). Many studies show the relation between nutrition and the occurrence of laminitis; particularly rapidly fermentable carbohydrates content, capable of inducing subclinical acidosis (Nocek, 1997). However, more recent research points to an interrelationship between ruminal acidosis, trauma, physiological alterations around parturition and the type of flooring (Leacher and Ossent, 2002; Cook et al., 2004; Lean et al., 2013; Bicalho and Oikonomou, 2013).

The pathogenesis of laminitis initiates with metabolic disorders with subsequent mechanical degradation (Nocek, 1997). Several studies have demonstrated the potential of vasoactive substances, which are produced in rumen acidosis conditions, metritis and mastitis to change the vascular perfusion of the corium. Histamine, lactic acid, endotoxin and serotonin receptors are the main substances studied (Nocek, 1997; Leacher e Ossent, 2002; Cook et al., 2004; Lean et al., 2013). The hemodinamic alteration promoted by this substances can increase the pressure in blood vessels causing internal bleeding and edema (Nocek, 1997). However, evidences that laminitis can occur even without the expected vascular changes; strengthen the hypothesis that it is associated with a degradation of bone support apparatus in the foot. This theory proposes that the vasoactive substances activate the Metalloproteinases matrix (MMPs) which causes degradation of collagen fibers and a weakening of the support apparatus (Leacher and Ossent, 2002; Tarlton et al., 2002). Therefore, you may experience the offset of the third phalanx, increased pressure exerted by the third phalanx over the corium, mainly in the area of the tuberosity of the flexor tendon, causing ulcers and sole hemorrage **(**Leacher e Ossent, 2002; Tarlton et al., 2002; Danscher et al, 2010). This processes is demonstrated in the Fig. 5.

The bovine hooves are constantly influenced by the animal metabolism and at the same time by the external environmental factors. In this way, the interaction between the pathophysiological processes that occur inside the hoof with the external factors can be the determining factor for the occurrence of laminitis. Bergsten and Frank (1996) demonstrate that neither a diet richer in grains (6 kg) nor a died less rich in grains (0.4 Kg) nor rubber or concrete free stall floors influenced the prevalence of sole hemorrhage. However, by combining the diet rich in grains with the concrete floor, heifers of the study presented higher scores of sole hemorrhage. In this way, more attention has been given to animal comfort, housing facilities and especially floor type (Mülling e Greenough, 2006; Vermunt, 2007; Bicalho and Oikonomou, 2013).

Body condition score (BCS) has been demonstrated to have a strong association with CHDL pathology. BCS reflects the amount of adipose tissue and influence the thickness of the digital cushion (Bicalho et al., 2009; Green et al., 2014). The digital cushion of cattle serves to mitigate the impact between the ground and the distal phalange, protecting the corium of trauma (Räber et al., 2004). The movement of the distal phalanx at the joint capsule and the pressure exerted by this on the corium are factors that determine the occurrence of CHDL (Machado et al., 2010; Oikonomou et al., 2014). This movement can be facilitated in cases in which the digital cushion can not exert its function properly, because it is thinner than it should be, or when environmental challenges such as long time standing on hard floors (Räber et al., 2004; Bicalho et al., 2009; Machado et al., 2011; Bicalho e Oikonomou, 2013).

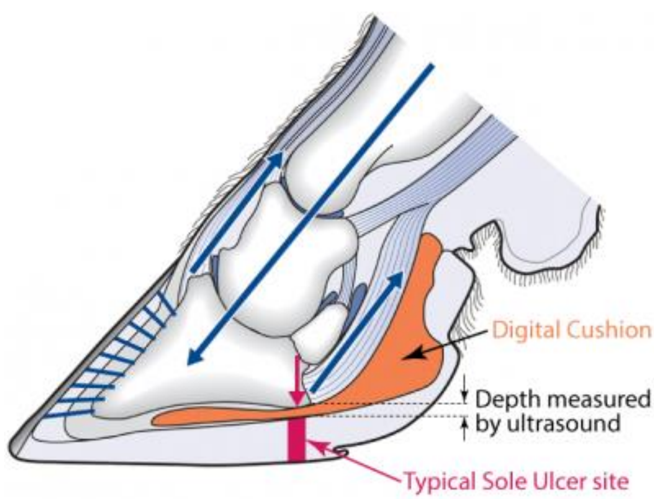

Figure 4. Representation showing the structures of the bovine hoof demonstrating the digital cushion that is responsible for absorbing impact. Figure from Greenough (2007).

After calving, the digital cushion thickness is reduced until the 120th day, when it is thinner (Bicalho et al., 2009). This moment corresponds with the period of greatest mobilization of energy reserves of milk cows and is the period with greatest occurrence of hoof lesions (Green et al., 2002; Casagrande, 2013).

4. Chapter 1 - Digital dermatitis: the main challenge for hoof health in grazing cattle under tropical condition

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Abstract

Lameness is a growing concern to the dairy industry worldwide. However, little is known about lameness and its causes in grazing cattle, especially in tropical climates. This study aimed to assess the prevalence of hoof lesions and lameness in dairy herds of all-year-round-grazing cattle under tropical condition, and to identify the main lesions associated with lameness. We visited forty-eight farms located in the Minas Gerais state, Brazil, equally divided into four groups based on daily milk production. All lactating cows in the visited farms were locomotion scored, and a representative sample was randomly chosen for hoof inspection. Among the 2267 lactating cows evaluated, 16.0% were scored as lame and 6.8% as severely lame. Nearly all cows presented at least one type of hoof lesion, of which heel horn erosion (90.1%), white line fissure (50.4%), and digital dermatitis (32.7%) were the most frequent. Heel horn erosion was present in all farms and digital dermatitis was present in all but two farms. Sole ulcer was observed in a single animal. Heel horn erosion and digital dermatitis presented the highest proportion of severe cases, while the majority of white line fissure were mild. Additionally, digital dermatitis was correlated to an increased chance of 2.5 times in mobility score. Collectively, our results demonstrate that digital dermatitis is the main concern and the biggest cause of lameness in grazing cattle under tropical condition independently of farm size, while claw horn disruption lesions are of secondary importance.

Keywords: Lameness; Heel horn erosion; Claw horn lesions; Mobility score; Pasture

Introduction

Lameness is a great concern to the dairy industry because of its high incidence worldwide and severe effects on productivity and animal welfare. Lameness leads to great economic loss through reduced milk yield, increased culling risk, reduced feeding time, low body condition score, impaired reproduction, and increased treatment expenses (Bruijnis et al., 2010; Cha et al., 2010). Additionally, lameness is an important welfare issue because of the increased pain and its effects on dairy cow well-being, preventing normal behaviour (O'Callaghan et al., 2003).

Lameness is a multifactorial condition, tough hoof lesions are responsible for 90% of lameness cases in dairy herds (Murray et al., 1996). Nevertheless, hoof lesions are normally presented sub clinically and regular hoof examination is needed to identify lesions and elucidate the causes of lameness and hoof health of the herd (Tadich et al., 2010; Shearer et al., 2012). This evaluation helps set goals and plan future actions and can be considered an initial step in a lameness control program (Tadich et al., 2010; Shearer et al., 2012).

Access to pasture is beneficial for the welfare of cattle, allowing them to express natural behaviour (Cook e Nordlund, 2009). Moreover, pasture-based systems usually have a lower lameness prevalence than housing systems do (Haskell et al., 2006; Barker et al., 2010; Adams et al., 2016). However, the reported prevalence of lameness among grazing cattle is highly varied, ranging from less than 10% to over 60% (Cook e Nordlund, 2009; Fabian et al., 2014; Gibbs, 2010). Furthermore, epidemiological studies on grazing cattle are scarce, and the prevalence of hoof disease in pasture-based systems is poorly characterised (Cook e Nordlund, 2009).

Digital dermatitis is a painful disease of infectious nature and one of the main cause of lameness in the dairy industry throughout the world (Cramer et al., 2008; Holzhauer et al., 2008). It has been described in all continents and its prevalence is reported to be ~30% , but can reach up to 83% in housing cattle (Holzhauer et al., 2006; Cramer et al., 2008). Because of its high prevalence DD represents the highest costs among hoof lesions and the biggest impact in cows welfare (Bruijnis et al., 2010, 2012). Although much attention has been addressed towards it, the epidemiology and aetiology of this disease hasn´t been well clarified yet (Berry et al., 2012).

The intensification of milk production, with a decrease in the number of farms and increase in herd size is a worldwide phenomenon made possible through the use of higher yielding cows, increased stocking density, more stock per person, and increased use of supplementary feeds (Bergsten et al., 2015; Von Keyserlingk et al., 2012). These factors influence the health status of the herd, normally increasing mastitis and lameness among other conditions and decreasing the incidence of milk fever (Flor e Tadich, 2008; Von Keyserlingk et al., 2012; Bergsten et al., 2015). Thus, farms of different sizes may present different prevalence of hoof lesions and lameness. In this scenario, it is critical to look at the effects of a more intensive production in hoof health.

Hence, the objectives of this cross sectional study were to 1) determine the prevalence of hoof lesions and lameness in dairy herds of all-year-round-grazing cattle, 2) establish the main lesions associated with lameness, and 3) elucidate the variance of hoof lesions and lameness between farms of different sizes.

Materials and Methods

Animals and Farms

The research was carried out in Prata municipality, situated in Minas Gerais state. The municipality of Prata has the largest cattle herd in the state of Minas Gerais with 356,491 heads and is the second largest milk producer with 78,900 cows milked (IBGE, 2012). The climate of the region is classified as tropical savanna (Aw) with dry winter and hot and rainy summer and the biome is Cerrado (predominantly) and the Semidecidual Seasonal Forest. Annual mean, minimum and maximum temperature of 24°C, 7°C and 40°C, respectively, and annual rainfall of 1.450 mm (Alvares et al., 2013).

Forty-eight dairy farms randomly selected from the Cooperative of Rural Producers of Prata (COOPRATA) database were divided into four equal groups based on their milk production. Group 1 comprised very small farms producing up to 300 L of milk/day; Group 2, small farms producing 301 to 600 L of milk/day; Group 3, medium-sized farms producing 601 to 1000 L of milk/day; and Group 4, large farms producing>1000 L of milk/day. This way of classify the farms was used because most farms did not have records regarding 305-day yield and there is no database were we can access the number of lactating cows in each farm. The farms were visited between July 2014 to March 2015. This time period encompasses the dry and rainy period in the region and an equal number of farms (24) were visited during each period. The selected farmers were contacted via telephone, informed of the study purpose, and enquired about interest in study participation.

The farms relied on pasture all year around, and 10 farms (20.8%) used rotational grazing. During the dry season (April to October), the grass quality drops significantly and in 58.3% of the visited farms sugar cane and corn silage are commonly supplemented. Concentrate (generally soy, corn and citrus pulp) and mineral salt supplements were provided all year round.

The cows were milked twice daily except on two farms where only one milking was conducted a day. Milking was done mechanically, except on six farms (12.5%). None of the visited farms took any preventive measures against lameness (i.e. footbath, mobility score or foot lesion record) or had a trimming routine, except from three farms that used a footbath with copper sulfate or formalin solution. Only two farms had a trimming chute used to treat lame animals.

Lameness score

All lactating animals in the visited farms were evaluated for mobility by using the four-point (0–3) DairyCo mobility scoring system (Barker et al., 2010), where 0 represents a sound animal with unchanged gait; 1, an animal with abnormal locomotion, but not favouring any particular limb; 2, a lame animal with uneven or arched back; and 3a severely lame animal that cannot walk as fast as a brisk human pace. Animals scoring 2 were considered lame and animals scoring 3 were considered severely lame. All mobility evaluations were performed by the same trained observer (MTF) taking care to observe all cows on flat and firm ground.

Animal and Clinical Examinations

This project was approved by the Committee for Animal Ethics on Animal Experimentation (CEUA) of the Universidade Federal de Minas Gerais, Brazil, under protocol number 121/2015.

The sample size to evaluate the prevalence of hoof lesions was calculated using the formula $n = p \times (p-1) \times (1.96/\Delta)^2$, admitting an expected prevalence (p) of 20% based on our previous work (Moreira et al., 2012), a confidence interval of 95%, and an error (Δ) of ±4%.The resulting sample size was 380 animals**.**

Six to 12 (mean, 8.16) randomly selected animals had their hoof inspected from among the lactating cows in each visited farm. The cows were crossbreeds, mainly a zebu breed (usually Gyr or Nelore) crossed with Holstein. All cows were between the first and tenth lactation, between three and 15 years old (average age ± standard deviation [SD], 7.19 ±3.6 years), and with body condition scores between 2 and 4.5 (mean ± SD, 3.37 ±0.42). In total, we examined 392 lactating cows for hoof lesions: 112 animals in very small herds, 88 in small herds , 92 in medium herd, and 100 in large herds.

The examined animals were confined in a transportable trimming chute and all four feet were evaluated. The claws were cleaned and the superficial layer of corneal tissue was removed to better identify lesions. The data from each cow were recorded, and the lesions were classified into 14 different types according to ICAR (2015), and scored from 1 to 3 depending on severity. Digital dermatitis (DD) lesions were scored according to Döpfer (1997) and adapted by Berry et al. (2012). Details about lesions descriptions and scores are given in Table 3.

Table 3. Description of the hoof lesions and used scoring system. The proposed classification was based on ICAR Claw Health Atlas (2015).

Abbreviation	Lesion	Description
HHE	Heel horn erosion	1: Superficial lesion like pits or pockmarks in the heel area 2: Mild to moderate, deeper or wider than 5mm 3: Severe and extensive V-shaped erosion
WLF	White line fissure	1: Separation of the white line which disappears after balancing both soles 2: Separation of the white line which remains after balancing both soles 3: Separation of the white line which remains after balancing both soles and present drainage at the coronary band
SH	Sole haemorrhage	1: Small and superficial red or yellow discolouration of the sole or white line 2: Large, but still superficial, haemorrhages 3: Extensive haemorrhages covering more than 1/3 of the sole with severe (dark) discolouration
SC	Scissor claws	Tip of toes crossing each other
DS	Double sole	Two or more layers of under-run sole horn
TS	Thin sole	Sole horn yields (feels spongy) when finger pressure is applied
SU	Sole ulcer	1: Severe sole haemorrhage at the SU spot 2: Ulcer exposing the corium 3: Infected ulcer with or without granulation tissue
ID	Interdigital dermatitis	1: Ulcerative lesion in the interdigital space 2: Extensive ulcerative lesion 3: Extensive area with ulcerative lesion and/or proliferative tissue
DD	Digital dermatitis	M1: Early stage with circumscribed erosive/ulcerative lesions< 2cm in diameter M2: Classical ulceration lesion >2 cm in diameter M3: Healing stage when the lesion is covered by a scab M4: Chronic stage normally presenting proliferative tissue and warts
TP	*Tunga* spp.	Presence of the female flea parasitizing the coronary band or accessory claw
IP	Interdigital Phlegmon	Symmetric painful swelling of the foot commonly accompanied with odorous smell with sudden onset of lameness
IH	Interdigital hyperplasia	Abnormal fibrous tissue growth in the interdigital space
OG/TRA	Overgrown claws/trauma	Overgrowth of the abaxial wall, cracks, or trauma in the wall or at the tip of the claw
OTH	Others	Foreign bodies, stones, ankyloses, amputated claw, corkscrew claws.

Statistical Analysis

Data were entered into Excel 2010 spread sheets (Microsoft Corp.) and were collapsed to evaluate total prevalence and the lesion-specific frequency for each herd. All data analysis was performing using Stata 12.1 (Stata Corp.). Differences in lesion prevalence between herd size were assessed using the two-group proportion test function. Model building was performed first using simple logistic regression to assess the association between hoof lesion and lameness. Hoof lesions associated to lameness at $P \leq 0.2$ were considered for further modelling. The final ordered logistic regression for lameness was performed using the OLOGIT procedure retaining lesions with $P \leq 0.05$.

Results

Herd Characteristics and Groups

Characteristics such as herd size, milk production, and average milk production per cow for each farm group are summarized in Table 4. The mean number of lactating cows in the visited farms was 57.9 (standard error [SE], ±4.3),ranging from 18 to 130 cows; total milk production ranged from 120 L/day to 2300 L/day, with an average of 775.2 L/day (SE, ±75.5); and daily milk yield per cow ranged from 4.8 to 21.3 L/day with an average of 12.7 L/day (SE, ±0.6). The number of lactating cows and daily milk production showed intergroup differences, while average milk yield per cow, amount of concentrate given to the higher yielding cows, and number of cows per herdsman were lower in very small herd than in medium and large herds. The values in small herd were lower than those in large herd. Supplementation of forage was more common in large than in very small herd. All farms in medium and large herd and 67% of farms in very small herds had concrete floors in the milking and waiting rooms. The average age of the lactating cows was similar among herds of different sizes.

Table 4. Farm characteristics at the time of visit. Average (Ave), maximum (Max), and minimum (Min) number of lactating cows, total milk production, average milk production per cow per day, highest amount of concentrate given to lactating cows, lactating cows/ herdsmen ratio, forage supplementation and the presence of concrete floor in the shed in very small, small, medium and large all year round grazing herds in Minas Gerais, Brazil. (±SE of the mean).

		Lactating cows	Total milk production	Average milk production[1]	Amount of concentrate	Cow/herdsman	Forage supplementation[2]	Concrete floor
Very small	Ave	26.6[a] ±2.1	240[a] ±18.8	9.4[a] ±0.9	7.9[a] ±1.0	19.0[a] ±2.2	17[a] ±0.11	67[b] ±0.14
	Max	40	300	16.7	16	30		
	Min	18	120	4.8	3	7		
Small	Ave	42.2[b] ±3.2	477[b] ±25.9	11.8[ab] ±0.7	9.8[ab] ±0.7	22.3[ab] ±2.4	50[ab] ±0.14	75[ab] ±0.12
	Max	60	600	16.1	13	44		
	Min	25	300	6.8	4.5	12.5		
Medium	Ave	66.8[c] ±4.6	867[c] ±35.5	13.6[bc] ±1	10.7[bc] ±0.5	29.7[bc] ±2.6	42[ab] ±0.14	100[a]
	Max	97	1100	20	13	48.5		
	Min	40	610	9.8	7	17.33333		
Large	Ave	96.2[d] ±5.3	1516[d] ±105	15.9[c] ±0.9	12.2[c] ±0.6	36.8[c] ±2.6	58[bc] ±0.14	100[a]
	Max	130	2300	21.3	16	49.5		
	Min	67	1150	12	9	21.6		
	Ave	57.9 (±4.3)	775 ±75.5	12.7 ±0.6	10.2 ±0.4	27.0 ±1.57	54.2 ±1.57	85 ±0.5

[1]Average milk production per cow per day (L).[2]Proportion of farms supplementing forage during the dry season.*Values with different letters (a, b, c) within each row differ significantly ($P < 0.05$).

Lameness

Of the 2267 lactating cows observed, 16.0% (SE, ±1.2) were scored as lame (scores 2 and 3) and 6.8% (SE, ±1.0) as severely lame (score 3). In total, 65.4% of animals scored 0, 18.5% scored 1. The frequency of lame animals within a farm ranged from 2.9% to 42.5% and of severely lame animals from 0 to 29.8%. No significant differences were observed in the prevalence of either lameness or severe lameness among the production herds of different sizes.

Hoof Lesions

In total, 97.2% of all cows had at least one lesion in one of their feet. The median was three different types of lesions per cow, and the maximum number of different lesions in one animal was nine. The prevalence of hoof lesions differed between herd size and is presented in Table 5. The hoof lesions that variated within farms of different size were heel horn erosion (HHE), white line fissure (WLF), sole haemorrhage (SH), interdigital dermatitis (ID) and *Tunga* spp.. HHE was more frequent in small and large hards than in medium herds; WLF was more frequent in small herds and less frequent in medium herds; SH was lower in very small herds than in small and large herds; ID was more frequent in very small and small herd than in the other herd sizes; the presence of *Tunga* spp. was more prevalent in very small and medium herds than in large herds; and traumas and overgrown claws were more frequent in very small herds than in large herds.

Table 5. Prevalence of hoof lesions and standard error (±SE) by production group in pasture based farms.

	Very small		Small		Medium		Large	
	Prevalence	SE	Prevalence	SE	Prevalence	SE	Prevalence	SE
Lameness	16.6a	2,1	14.4a	2,4	16.8a	3,1	16.1a	1,7
Severe lameness	7.0a	2,54	5.4a	1,4	7.7a	2,7	6.8a	1,2
HHE	86.6[ab]	3.23	98.9[c]	1.14	80.4[b]	4.16	94.0[a]	2.39
WLF	55.4[ab]	4.72	64.8[a]	5.12	35.9[c]	5.03	49.0[bc]	5.02
SH	17.9[b]	3.64	31.8[a]	4.99	28.3[ab]	4.72	31.0[a]	4.65
SC	19.6[a]	3.77	14.8[a]	3.8	15.2[a]	3.77	16.0[a]	3.68
DS	2.7[a]	1.53	1.1[a]	1.14	1.1[a]	1.09	5.0[a]	2.19
TS	0.9[a]	0.89	1.1[a]	1.14	1.1[a]	1.09	2.0[a]	1.41
SU	0	0	0	0	0	0	1.0	2.39
ID	16.1[a]	3.49	20.5[a]	4.32	4.3[b]	2.14	4.0[b]	4.76
DD	25.0[a]	4.11	30.7[a]	4.94	35.9[a]	5.03	34.0[a]	2.19
TP	17.0[a]	3.56	10.2[ab]	3.25	16.3[a]	3.87	5.0[b]	0
IP	0.9[a]	0.89	1.1[a]	1.14	2.2[a]	1.53	0.0[a]	1.41
IH	9.8[a]	2.82	4.5[a]	2.23	6.5[a]	2.59	6.0[a]	2.39
OG/TRA	26.8[a]	4.2	18.2[a]	4.14	19.6[a]	4.16	6.0[b]	1.97

*Values with different letters (a, b, c) within each row differ significantly ($P < 0.05$). Group 1: very small farms producing up to 300 L of milk/day; Group 2: small farms producing 301 to 600 L of milk/day; Group 3: medium-sized farms producing 601 to 1000 L of milk/day; Group 4: large farms producing >1000 L of milk/day. HHE: heel horn erosion; WLF: white line fissure; SH: sole haemorrhage; SC: scissor claws; DS: double sole; TS: thin sole; SU: sole ulcer; ID: interdigital dermatitis; DD: digital dermatitis; TP: *Tunga* ssp.; IP: interdigital phlegmon; IH: interdigital hyperplasia; OG/TRA: overgrown claws/trauma.

The prevalence and severity of hoof lesions is shown in Figure 5. The three most common lesions were HHE (90.1% ±2.12), WLF (50.4% ±4.0), and DD (32.7% ±2.93). Sole ulcer (SU) was found in only one animal, and thin sole (TS) and double sole (DS) were rarely found (1.2% and 2.5% of animals, respectively). Because of their low frequency, SU, SB, and DS were grouped as sole lesions (SL). Interdigital Phlegmon (IP) was found in only four animals (1.0%). At the farm level, HHE was found in all farms and DD was also found in all but two farms (95.8% positive farms; Figure 5), while SU, SB, DS, and PH were present in less than 10% of the farms (Figure 5).

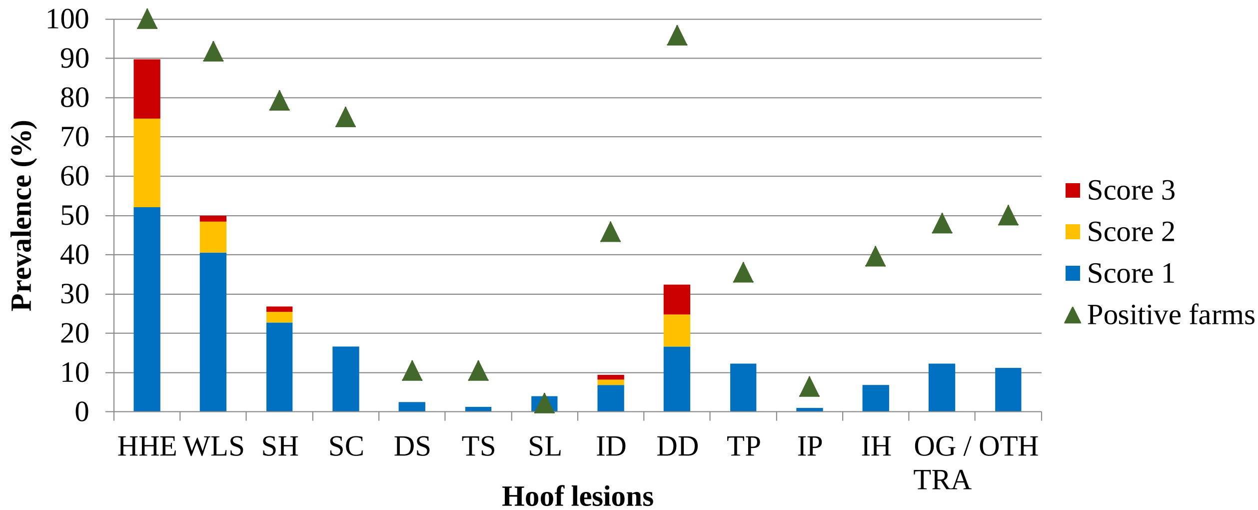

Figure 5. Prevalence of hoof lesions by score in crossbreed all year-round grazing cattle in Minas Gerais, Brazil. For DD, score 1 is analogous to M1 in the scoring system proposed by Döpfer (1997) and adapted by Berry et al. (2012), score 2 to M2, score 3 to M4; no M3 lesions were found. HHE: heel horn erosion; WLF: white line fissure; SH: sole haemorrhage; SC: scissor claws; SL: sole lesion including double sole, thin sole, and sole ulcer; ID: interdigital dermatitis; DD: digital dermatitis; TP: *Tunga* ssp.; IP: interdigital phlegmon; IH: interdigital hyperplasia; OG/TRA: overgrown claws/trauma; OTH: others.

Among the lesions, DD and HHE presented as the most severe types. Regarding DD, 76.4% of the lesions were classified as acute (51.2% of M1 plus 25.2% of M2) and 23.6% as chronic (M4), but no healing lesions (M3) were found. For HHE, 25.3% were scored as 2 and 16.8% scored as 3. Although WLF was highly prevalent, most were mild lesions and only 3.1% were scored as 3. All WLF lesions scored as 3 had drainage at the coronary band, but no white line abscess was found.

Lameness Ordered Logistic Regression

The ordered logistic regression represents the likelihood of an increase of one point in the mobility score (Table 6). Four lesions were present in the final model: PH, DD, ID, and WLF. Among the lesions, PH had the greatest increase in probability for a higher mobility score. ID increased the chance of intensification of gait impairment 4 times and DD and WLF increased it approximately 2.5 times.

Table 6. Ordered logistic regression of the hoof lesions as risk factor associated with the increase in one point the mobility score in cattle kept in pasture all year round in Minas Gerais, Brazil.

Hoof lesion	Odds Ratio	SE	*P*	95% CI
WLF	2.48	0.628	>0.001	1.51-4.07
ID	4.01	1.594	>0.001	1.91-8.77
DD	2.64	0.680	>0.001	1.59-4.37
PH	16.02	17.086	0.009	1.98-129.48

SE: standard error; CI: confidence interval; WLF: white line fissure; ID: interdigital dermatitis; DD: digital dermatitis; IP: interdigital phlegmon.

Discussion

To our knowledge, this is the first Brazilian study that describes lameness prevalence and relates it to hoof lesions. Although the study covered a limited area, it could represent a diverse subsection of Minas Gerais and even Brazilian dairy farms, because the selected farms represents the main management practices and daily farm operations at different scales present in pasture-based systems found in southeast Brazil.

The lameness prevalence of 16% found in the study region is very similar to reports from grazing cattle from other parts of the world (Amory et al., 2006; Haskell et al., 2006), but lower than most reports from housing cattle, which are normally higher than 25% (Barker et al., 2010; Capion et al., 2009; Von Keyserlingk et al., 2012). Access to pasture are beneficial to foot health, it ensures better pressure distribution as well as providing comfortable lying areas (Fabian et al., 2014; Olmos et al., 2009b; Somers et al., 2015; Adams et al., 2016). However, some reports on grazing cattle have documented prevalence as high as 26.2% in New Zealand's south island (Gibbs, 2010) while others have documented a lower prevalence of 8.3% (Fabian et al., 2014). The huge variation of lameness prevalence found in this and others studies (Barker et al., 2010; Capion et al., 2009; Von Keyserlingk et al., 2012) indicates that the reduction of lameness is a feasible goal.

Notably, a high percentage of cows presented a severe locomotion impairment, reaching the proportion of 30% in one herd. Nonetheless, the farms did not performed any proactive action nor control strategies for lameness even when more than one third of the herd were lame. These findings together with the high proportion of chronic lesions observed suggest that treatment is overdue or inadequate and cows spend long periods in poor welfare conditions.

DD was by far the most important cause for lameness in the studied region. Its high prevalence, combined with a more than two fold increase of the odds for a higher mobility score, makes it the lesion with the biggest impact on animal welfare and performance in this study. Furthermore, the herd-level prevalence of 32.7% of DD is very disturbing and even higher than reports from confined cattle (Holzhauer et al., 2006; Cramer et al., 2008). The high number of farms positive for DD indicates that this disease is endemic and widespread in the entire region. Similarly, other countries reported to have more than 90% positive farms (Holzhauer et al., 2006; Cramer et al., 2008). In Brazil, the first report of DD was made in 1992 by Borges et al. (1992), but the disease was probably present before that time. Since then, several Brazilian reports from single grazing herds have described DD with frequencies within herds varying from 0.92% (Silveira et al., 2009) to 44.2% (Moreira et al., 2012).

Other infectious lesions as HHE and ID were very prevalent. Nearly all cows presented HHE similarly to the findings by Capion et al. (2009). Despite the high prevalence of HHE, this lesion is not very painful and seldom causes clinical lameness (Manske et al., 2002). HHE and ID are strongly correlated with DD (Capion et al., 2009; Manske et al., 2002) because of its infectious nature. Possible reasons for the high prevalence of infectious lesions are the unhygienic environment conditions, such as the accumulation of manure, faeces and urine in corrals and waiting rooms observed in many farms (Manske et al., 2002). Other possible explanations for the high prevalence of DD were the lack of biosecurity and preventive measures (Barker et al., 2010; Berry et al., 2012). As typically seen, most farms enrolled in this study did not have a protocol for footbaths, routine trimming, or maintain any lameness or hoof lesion records. Furthermore, the common practice in this region of purchasing older animals produces a threefold increase in the odds for DD than in farms based exclusively on home-reared heifers (Rodriguez-Lainz et al., 1999).

The female of the sand flea *Tunga* spp. was found parasitizing 12.5% of the cows. *Tunga* spp. is found in tropical and sub-tropical regions and can parasitize the skin of several hosts including cattle and human (Linardi et al., 2013). In cattle, it is normally found parasitizing the skin next to the coronary band and in the teats (Ribeiro et al., 2007; Linardi et al., 2013). There are little reports of tungiasis in cattle and its real impact on claw health remains unknown. The authors observed that the parasite can cause inflammation at the site, leading to local laminitis and imperfect keratinisation. However, in this study as reported by others (Ribeiro et al., 2007) its presence was not associated with clinical lameness.

The lesions normally related to laminitis as WLF and SU were of secondary importance for grazing cattle in the study region. This finding differs greatly from those of previous reports from confined systems where SU and WLF are more frequently associated with clinical lameness (Cook and Nordlund, 2009). Although WLF and SH presented a high prevalence and WLF was present in the final ordered logistic model, their importance as a cause of lameness is questionable. The ordered logistic model represents the odds of a one-point increase in the mobility score due to a specific condition. This means that WLF can be associated with the increase in the mobility score from 0 to 1, which does not represent clinical lameness. Furthermore, both WLF and SH were mostly scored as 1 for severity of the lesion, suggesting these were very superficial and without great clinical significance. If lesion score 1 were excluded from the analysis, WLF prevalence would drop from 50% to only 9.44% and SH from 26.8% to 4.1%. Moreover, SU was found in only one animal out of 392 (0.26%). Furthermore, the horn quality of the examined animal did not show signs of chronic laminitis, such as horizontal lines, corkscrew claw, or yellow colouration (Greenough and Weaver, 1997), which may indicate that the cause for WLF is traumatic.

Farms of different sizes presented distinct characteristics; larger farms have cows with higher milk yield, and use more supplementation of forage during the dry season and higher amounts of concentrate. Nevertheless, the frequencies of lameness and severe lameness were similar between groups, but the prevalence of hoof lesions was distinct. In Chile, Flor and Tadich (2008) reported a higher prevalence of lameness in large herds (33.2%) than in small herds (28.7%), but provided no information about hoof lesions. Here, very small farms had higher frequency of overgrown claws/trauma, ID, and *Tunga* spp. than did larger farms, while SH was more frequent in bigger farms. All larger farms had concrete floors in their corrals and waiting rooms, which made flea growth more difficult. It also increased claw wear, preventing claw overgrowth (Greenough and Weaver, 1997). However, the hard surface combined with higher concentrate and forage suplementation in the diet probably contributed to the increased SH prevalence (Cook e Nordlund, 2009). Low-yielding farms normally use more extensive systems, where cows have access to larger areas and walk more, what can increase the chances of trauma and occurrence of ID (Cramer et al., 2009b). The cause of the lower prevalence of HHE and WLF in medium herds comparing to the others are unclear. Studies that evaluate risk factors for grazing cattle are scarce and the majority were not performed in tropical regions. In this context, further studies to elucidate the risk factors for hoof lesions and lameness in all year round grazing cattle are warranted.

Conclusions

This cohort study shows that average prevalence of lameness (visual locomotion score>2) was 16% in grazing farms and it did not differ between herds of different sizes. The main contributor for this situation was digital dermatitis, which was endemic in the region, present in 95.8% of the herds with a mean prevalence at herd-level of 32.7%. Its high prevalence combined with the correlation of more than two fold to higher mobility score make DD the biggest challenge for hoof health in grazing cattle under tropical condition independently of herd size. Other hoof lesions associated with an increase odds for poor mobility score were interdigital phlegmon, interdigital dermatitis and white line fissure. Further researches are necessary to identify the risk factors and the cause of the high prevalence of DD and others hoof lesions.

Conflict of interest statement

The authors declare that they have no conflict of interest, and this document is their original research work.

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5. Chapter 2 - Risk factor associated with lameness and hoof lesion in all year round grazing cattle in Brazil.

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Abstract

Husbandry practices and risk factors concerning lameness and hoof lesion were studied in dairy cows grazing all year round in Minas Gerais, Brazil. Multivariable models were build based on the observation of 2262 dairy cows for lameness and 392 for hoof lesions from 48 different farms. The vast majority of farmers did not perform any preventive measures for hoof health and only 6.3% use footbath. The factors associated with impaired mobility score were low BCS, longer time spend in corral, kept cows in paddocks during the drought period and a bad hygiene score. Track features was the most significant factor increasing more than threefold the odds for heel horn erosion, white line fissure and sole hemorrhage. Several factors related to unhygienic conditions such as frequency of corral cleaning, condition of corral exit, access to pile of manure and keep animals in paddocks during the dry period were identify as risk factors for hoof lesions. Poor human-animal relation was related with an increase odds for sole hemorrhage while patience of the farmer handling the cows on the track decrease in more than half the odds for interdigital hyperplasia. Our results furthermore rises a concern regarding animal wellbeing that should receive immediate attention. Results of this study improve our understanding of the risk factors presented in year round grazing farms and represent a first step to plan future action aiming to decrease lameness and hoof lesion in tropical farms.

Introduction

Lameness is considered one of the three most common occurrences in dairy cattle causing great economic loss by impairing milk production, reproduction, premature culling and increase treatment costs (Sogstad et al., 2007; Bruijnis et al., 2010; Bruijnis et al., 2012). It also represents the major welfare problem in dairy herds due to it high prevalence and effects in cows behavior (Bruijnis et al., 2012). Lameness is a sing of pain in which the affected animal attempts to reduce the weight borne by a particular limb (Leach et al., 2010; Whay et al., 1997, 1998). The main cause for lameness in cows are injuries in their hooves, which however can be present in subclinical form (Buch et al., 2011). Thereafter, for a more precise evaluation of hoof health and a better understanding of lameness causes, the analyses of hoof lesions and lameness together are necessary (Buch et al., 2011; Capion et al., 2009).

Several studies have emphasized the multifactorial nature of lameness and hoof lesions and their complex etiology. In the past few years, a large number of risk factors at herd and cow level were identified; i.g.: nutrition, hygiene, access to pasture, purchase of animals, cow comfort, trimming and footbath routine, genetics, age, social rank and physiological state (Barker et al., 2010; Galindo e Broom, 2000; Solano et al., 2015). However, the majority of these epidemiological studies were carried out in the north hemisphere with housing cattle where stall characteristics, bedding material and floor surface plays an important hole in lameness epidemiology (Barker et al., 2010; Adams et al., 2016; Westin et al., 2016). In this context, studies that examine risk factor for lameness in grazing cattle is scarce and even fewer investigated specific lesions.

With a complete different environment and management, grazing cattle has normally lower level of lameness compared to systems with limited outdoor access (Olmos et al., 2009a). Pasture provides soft and hygienic walking surfaces and better weight distribution (Olmos et al., 2009a; Schmid et al., 2009). Nonetheless, lameness can still be a big problem in grazing cattle as demonstrated by reports of lameness annual incidence of 26.2% in New Zealand south island (Gibbs, 2010) and prevalence of 22.9% in Ireland (Somers et al., 2015). This scenario demonstrates the necessity of research into the identification of risk factors for hoof health and lameness in cattle under tropical condition. Thereafter, the objective of this study was to investigate the potential risk factors for lameness and hoof lesions in all year round grazing cattle to provide a scientific basis for future control programs.

Materials and Methods

Local and farms

Between June 2014 and March 2015 we visited a total of 48 farms in Prata, Minas Gerais State, Brazil. These properties were drawn randomly from the database of the Cooperative of Rural Producers from Prata (COOPRATA). The only criteria was that farms had the dairy production as the main activity and practiced year-round grazing. Farmers were contacted by telephone and invited to participate in the study after explanation of research propose.

Animals and clinical examinations

All procedures was previously approved by the Committee for Animal Ethics on Animal Experimentation (CEUA) of the Universidade Federal de Minas Gerais, Brazil, under protocol number 121/2015.

All lactating cows in the visited farms were assessed for mobility score by the same trained observer using the four-point mobility score (0–3) by DairyCo (Barker et al., 2010). Besides that, in each farm, a sample from 7 to 12 animals was randomly selected for hoof examination, totalizing 392 cows. All animals were crossbreed manly composed by a Zebu breed, especially Gyr and Nelore crossed with Holstein. Selected animals were contained in a transportable trimming chute and had all four feet evaluated. The hoofs were washed with brush, water and soap if necessary and a thin layer of corneal tissue was removed for better lesion identification. The data from each cow were recorded on an individual recording sheet together with the identification of the animal, farm, date, mobility score, affected foot and claw, type of lesion and location. Hoof lesions in the 4 limbs were collapsed into a single record for each cow meaning that a cow was affected if at least 1 foot had the specific lesion. The lesions were classified in 14 different types adapted from ICAR (2015).Cows selected for hoof examination was also evaluated for presence of hock and knee lesions, hygiene condition and body condition score (BCS). The hygiene condition was evaluated based on leg cleanliness using a dichotomy answer of good or poor hygiene condition. The classification was adapted from Relun et al. (2012) and a good hygiene score refer to a clean cow with little or no manure contamination of the lower limb and a poor hygiene score refer to a dirty cow with the lower limb lightly splashed with manure or with distinct plaques of manure on the foot, progressing up the limb. Body condition score was measured using a 5-point scale with increments of 0.25 (Edmonson et al., 1989).

Husbandry practices

During the visit, a questionnaire and checklist were used to capture herd management data. The questionnaire and checklist covered various aspects of general management (i.g.: nutrition, farm size, number of cows, number of employees, cleaning frequency, how animals were guided), facilities (i.g.: flooring type, corral condition; water availability), environment condition (e.g.: track condition, shade availability) and hoof health management (i.g.: foot trimming routine and foot bath management). A list with the characteristics as well as their descriptive analysis can be seen in Table 1 and 2.

In order to group this diverse management practices found in the farms, the following classification were used: Intensive systems represent farms were the ones that used stored food all year round as silage as a supplementation or use high density grazing system as rotational grazing systems. Semi-intensive farms were the ones that use stored food in just part of the year as a supplementation because of the drought period. Extensive farms were those who rely only in pasture all year round and make it with a low grazing pressure.

Data Handling and Statistical Analyses

Data from questionnaire and animal examination were entered into Microsoft Excel (Microsoft Corp., Redmond, Washington, USA) sheet and exported to Stata 12.1 (Stata Corp., College Station, Texas, USA) for statistical analyses.

Multivariable models were created for each specific lesion as well as an ordered logistic model for mobility score. Hoff lesions with very low prevalence could not be used for modeling as well as the lesions classified as “others”. At the end multivariable models was built for heel horn erosion (HHE), digital dermatitis (DD), white line fissure (WLF), sole hemorrhage (SH), interdigital hyperplasia (IH), scissors claws (SC), interdigital dermatitis (ID), interdigital hyperplasia (IH) and *Tunga* spp. (TP).

Multivariable models were built in multiple steps. First, a simple logistic regression analysis was performed to assess the association between the outcome of interest and each predictor variable, applying the LOGIT procedure in Stata (StataCorp LP). After that, all variables with $P < 0.20$ were selected for inclusion in the next step of model building. Farm was included as a fixed factor into the models using the XTMELOGIT procedure in Stata (StataCorp LP).

Selected variables were included in the model and manual backward stepwise elimination was used retaining variables with $P \leq 0.05$. If any variables presented collinearity they were removed from the model and maintained the one with lower p value. All final models were assessed for proper fit by the assessment of residuals, model assumptions, and appropriate goodness of fit tests according to the methods described by Dohoo et al. (2003). Finally, previously excluded variables and biologically logical interaction terms were forced into the model again and retained if $P \leq 0.05$ or if they were judged as confounders (changed the estimate of any other variable by >30%).

Results

Descriptive information

Farms characteristics as herd size, milk production, average milk production per cow and cow characteristics as age and body condition score are summarized in Table 7. Visited farms presented a huge variation in their characteristics, especially regarding the cows milked/ employee ratio, number of lactating cows, average milk production/cow and the amount of concentrate given to higher yielding cows. All the visited farms can be considered medium to small farms with the biggest producing 2300L of milk/day and the smallest 120 L milk/day with low yielding cows (12.7 L/cow/day). Family members (54.2%) rule the majority of farms (Table 8).

Examined cows were between first and tenth lactation order, between tree and fifteen years old with an average of 7.19 years old (SD±3.6) and with body condition score between 2 and 4.5, average of 3.37 (SD±0.42) (Table 7). No animal presented hock or knees injuries and 20.8% of the cows had the lower leg considered dirty (Table 8).

Table 7. Description of farm and animals characteristics in visited farms with grazing cattle all year round in Minas Gerais State, Brazil.

Characteristics	Max	Min	Med	Aver	SD	SE
Cows milked/ employee ratio	49.5	7	26.4	27.0	10.9	1.6
Number of lactating cows	130	18	54.0	57.9	29.8	4.3
Daily milk production (L)	2300	120	605.0	775.2	523.2	75.5
Average milk production/cow (L)	21.3	4.8	12.5	12.7	3.9	0.6
Maximum amount of concentrate (Kg)	16	3	10.0	10.2	2.9	0.4
Age of the cows (years)	15	2	6.0	7.2	3.6	0.2
Body condition score	4.5	2	3.5	3.4	0.4	0.0

Max - Maximum; Min - Minimum; Med - Median; Aver - Average

Forage supplementation during the drought period (from Abril to October) was practiced in 58.3% of the visited farms, mainly using sugar cane and corn silage (Table 2). 45.8% of the farm kept their animals in small paddocks during this time of the year providing the majority of the food on feed pads. Concentrate were provided in all farms all continuously during the year, usually during the milking or together with the forage in the feeding pads. Cows were milked twice daily except for 2 farms where there was only one milking per day. Most of farms use mechanical milking, but in 6 farms (12.5%) it was still done manually. Mineral salt supplements were normally provided besides the concentrate. All information obtained through farm inspection and interviews were included in the statistical analysis for modeling.

As can be seen in Table 2, there were almost no preventive actions regarding lameness in the visited farms. No farm had a trimming routine and only in two there was a trimming chute that were used to treat lame animals. Only 6.25% used foot bath and in these farms cooper sulfate and formalin were the chemicals used for foot bath solution. Other relevant data were that most of the farmers declare to buy adult animals regularly without almost any disease tests and the majority of the farms weren't able to provide sufficient shade area and water for their animals.

Table 8. Description and frequency (%) of dichotomy characteristics in visited farms hypothesized as associated to lameness and hoof lesions in farms with all year round grazing cattle in Minas Gerais State, Brazil.

Clusters and factors	Yes	No	SD	SE
Production system [a]				
Intensive system	20.8	79.2	0.40	0.128
Semi-Intensive	58.3	41.7	0.49	0.093
Extensive	20.8	79.2	0.40	0.128
Manpower used				
Family	39.6	60.4	0.49	0.112
Family + employees	14.6	85.4	0.37	0.132
Employees	45.8	54.2	0.50	0.106
Animals are supplemented [b] (>6 months)	4.2	95.8	0.20	0.141
Animals are confined in drought period	45.8	54.2	0.50	0.106
Technical assistance	72.9	27.1	0.44	0.064
Use foot bath	6.3	93.7	0.24	0.14
Has trimming routine	0	100	-	-
Haslameness records	0	100	-	-
Has a trimming chute	4.2	95.8	0.20	0.288
Predominant floor type				
Concrete floor	39.6	60.4	0.49	0.070
Stamped ground	39.6	60.4	0.48	0.070

Paved ground	22.8	77.1	0.42	0.060
Obstacles and damaged facilities (step, holes ...)	52.1	47.9	0.50	0.10
Corral exit in bad condition (muddy)	56.3	43.7	0.50	0.095
Inadequate water availability	66.7	33.3	0.47	0.118
Distance from pasture to milking room > 500m	25	75	0.43	0.125
Inadequate shade availability	89.6	10.4	0.30	0.047
Tracks in bad condition (stones, mud, ridges and gullies)	27.1	72.9	0.44	0.123
Good hygiene condition of animals	79.2	20.8	0.41	0.066
Animals spend >3h at corral	56.3	43.7	0.49	0.095
Accumulation of moisture in the corral	47.9	52.1	0.50	0.104
Corral cleaning frequency <1x day [d]	45.8	54.2	0.498	0.106
Animal access to pile of manure	41.7	58.3	0.49	0.11
Hock and knees lesions	0.0	100	-	-
Animals are beaten	6.3	93.7	0.24	0.14
Animals are guided quietly	85.1	14.9	0.36	0.056
Use to supplement roughage	58.3	41.7	0.49	0.072
Roughage supplemented				
Sugar cane	27.1	72.9	0.44	0.123
Corn silage	33.3	66.7	0.47	0.118
Sorghum silage	4.2	95.8	0.20	0.141
Concentrate and roughage are mixed	39.6	60.4	0.49	0.112
There is different diet for each production group	89.4	10.6	0.31	0.048
There is specific diet for transition cows	53.2	46.8	0.50	0.10
Mineral supplementation at will	54.2	45.8	0.498	0.098
Purchase of animals	85.4	14.6	0.35	0.055

[a] Production system was classified depending of the technology and stock density used. Intensive systems were the ones that kept the animals in small paddocks and supplemented stored food (e.g. corn silage) all year round or use high density grazing system as rotational grazing systems. Semi-intensive is the ones that use stored food in just part of the year as a supplementation because of the drought period. Extensive systems are those who rely only in pasture and make it with a lower grazing pressure and can supplement a small amount of concentrate. [b] Animals are confined outside in small paddocks with stamped ground and received their diet in feed pads, but they still have access to pasture areas all day. [c] When the average cleanliness score of the herd was ≤2. Cleanliness score was evaluated using a 4-point scale (Cook, 2006) observing only the lower legs of the animal. [d] The cleaning of the facilities was normally performed by manual scrape or with water.

Lameness and hoof lesion prevalence

In total 2267 lactating cows were evaluated for mobility score and 392 were also examined for hoof lesions distributed in the 48 farms. The mean herd lameness (visual locomotion score >2) prevalence for all the 48 farms was 16.0% (SE ±1.2) and varied from 2.9% to 42.5%. Severe lameness (score 3) was observed in 6.8% (SE ±1.0) of the cows, ranging from 0.0% to 29.8%. In total, 65.4% of animals were scored as 0; 18.5% as 1; 9.2% as 2 and 6.8% as score 3. Hoof lesions were very prevalence being found in 97.2% of the cows. The lesions in descending order were HHE (mean % ± SE; 90.1 ±2.12), WLF (50.4 ±4.0), DD (32.7 ±2.93) , SH (26.8 ±3.0), SC (16.6 ±2.5), overgrow and trauma (12.2 ±1.6), *Tunga* spp. (12.2 ±2.0), others (11.2 ±1.4), ID (9.4 ±2.4), IH (6.9 ±2.0), sole bruising (1.3 ±0.6), interdigital phlegmon (1.0 ±0.5), sole ulcer (0.3 ±0.3). These results demonstrate that infectious diseases were the most important in the region and that, differently from housing cattle claw horn lesions (excluding WLF) were very uncommon, especially SU which was found in only 2 animals.

Lameness ordered logistic model

The final ordered logistic model for lameness was based on 2267 observed cows from 48 farms and it has included four variables as can be seen in Table 9. In the ordered logistic model, the odds ratio represents the chance for increasing in one point the mobility score, which means that it does not necessarily cause clinical lameness. The four factors associated with impaired mobility score were low BCS, longer time spend in corral, cattle confined in paddocks during the drought period and a bad hygiene score.

Table 9. Ordered logistic model representing the odds ratio (OR) for the increase in one point the mobility score.

Factors	OR	SE	95% CI	P
BCS[a]	0.26	0.0926	0.1275-0.5214	>0.001
Cows are confined[b]	2.07	0.1564	1.098-3.918	0.025
Animals stay in corral >3h/day	2.36	0.7126	1.3034-4.2629	0.005
Poor hygiene condition	2.61	0.1304	1.340-5.102	0.005

[a] Body condition score; [b] Cows are kept in small paddocks during the drought period. OR: odds ratio; SE: standard error; CI: confidence interval; BCS: body condition score.

Hoof lesions models

The final model of risk factors for all infectious hoof lesions are summarized in Table 10 and it is also represented in Fig. 6. Logistic models were built for HHE, DD, ID, IH and *Tunga* spp.. Factors associated with an increase in risk for heel horn erosion were tracks in bad condition, a low frequency of cleaning the corral

(<1x/day) and the bad condition in corral exit; while the use of foot bath and farms with an extensive breeding system characterized by low stock density were protective factors. Longer waiting period in the corral and a bad hygiene score were correlated with an increase in more than two folds the chance of finding DD. The risk of interdigital dermatitis increased with the decrease of BCS and the presence of damage concrete, presenting loose ends or leaving holes on the floor. It were identified two protector factors for interdigital hyperplasia, being the animals guided quietly and cleaning the corral more than one time per day. The risk of finding animals parasitized by *Tunga* spp. decreased in farms with paved floor and with the increase in each liter of daily production. The rainy season, with in the regions is in summer, was another strong protective factor.

Although the condition of the tracks presented a p value of 0.066 in the model for HHE, we keep this factor because it is excluded from the model other factors also became non-significant, such as the use of footbath. Keeping this way, the model is capable to explain more the variety in HHE occurrence.

Table 10. Risk factors present in final models for noninfectious hoof lesions (Heel horn erosion, digital dermatitis, interdigital dermatitis, interdigital hyperplasia and *Tunga* spp. in grazing cattle in Brazil.

Factors	**OR**	**SE**	**95% CI**	**P**
Hell horn erosion				
Cleaning corral <1x/day	5.24	0.089	2.096 - 12.987	<0.001
Use of footbath	0.15	0.084	0.049 - 0.447	0.001
Bad condition of corral exit	2.81	0.157	1.180 – 6.67	0.020
Extensive system	0.29	0.113	0.139 - 0.629	0.002
Tacks in bad condition	7.10	0.150	0.876 - 58.8	0.066
Digital dermatitis				
Animals stay in corral >3h /day	2.06	0.55	1.227 - 3.465	0.01
Poor hygiene condition	2.38	0.13	1.299 – 4.348	0.01
Interdigital dermatitis				
BCS	0.27	0.16	0.081 - 0.890	0.03
Damage concrete	3.18	1.15	1.565 – 6.460	0.00
Interdigital hyperplasia				
Animals are guided quietly	0.36	0.17	0.146 - 0.887	0.03
Cleaning the corral < 1 x day	0.31	0.14	0.126 - 0.766	0.01
Tunga spp.				
Rain season	0.04	0.04	0.008 – 0.246	>0.001
Milk production of the farm [a]	0.98	0.007	0.997 – 1.000	0.03
Paved floor	0.11	0.11	0.015 – 0.805	0.03

[a] The increase of 1L of milk produced/day. OR: odds ratio; SE: standard error; CI: confidence interval; BCS: body condition score.

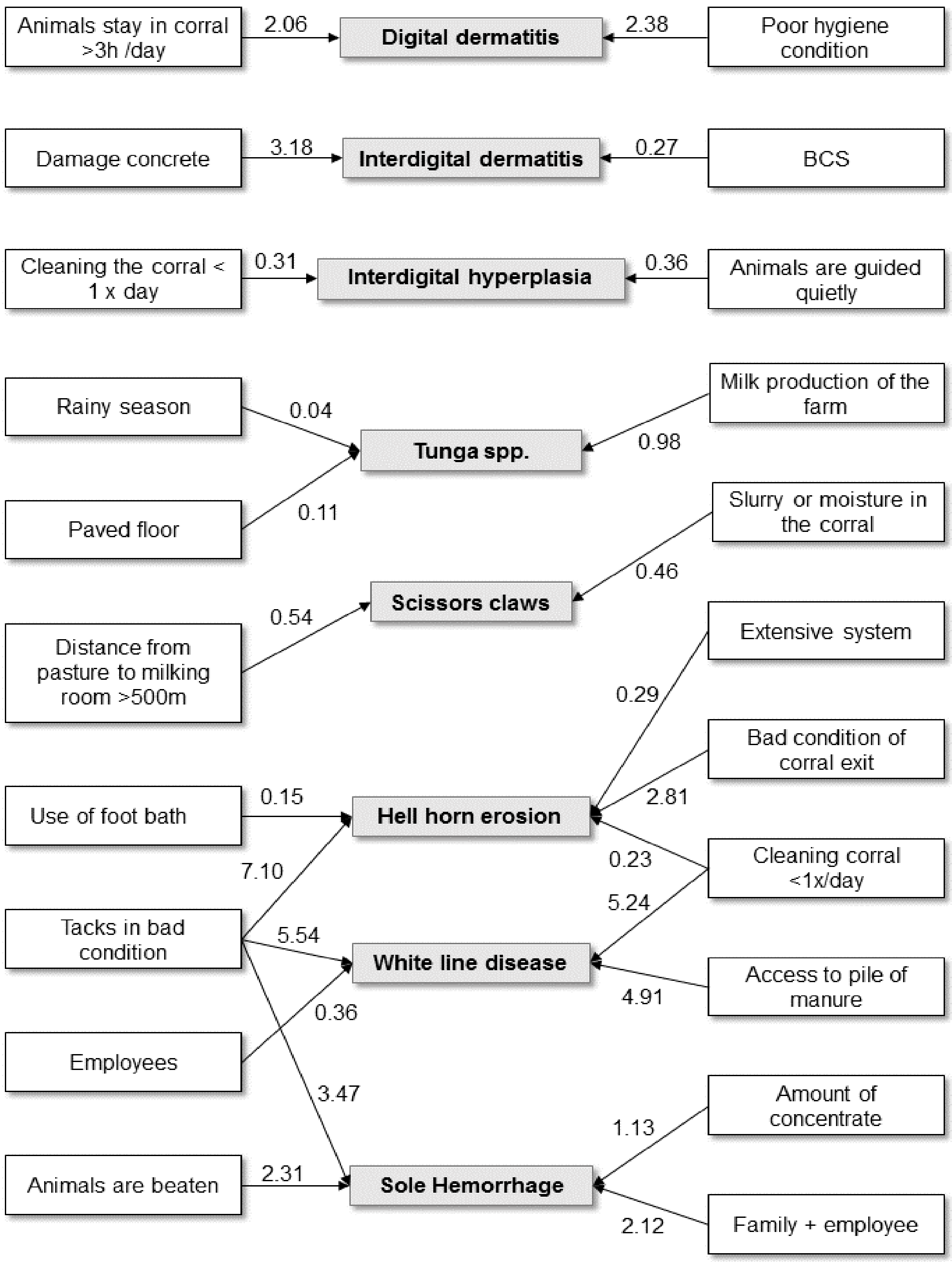
The final models for WLF, SH and SC are described in Table 11 and is represented in Fig. 6. White line disease was positive associated with age, the presence of piled up manure with animals access and condition of the tracks whereas there were stones, mud, ridges or gullies and. A higher cleaning frequency and the use of employees were correlated with a decrease of chance for WLF. The risk of SH increased with the amount of concentrate and presence of stones, mud, ridges or gullies in the tracks. SH was also positively associated with farms where family and employee work together and in farms where animals were beat. Protective factors associated with scissors claws were long distance between pasture and milking room and the presence of slurry or moisture in the corral.

Table 11. Risk factors present in final models for noninfectious hoof lesions (White line fissure, sole hemorrhage and scissors claws) in grazing cattle in Brazil.

Factors	OR	SE	95% CI	P
White line fissure				
Age	1.12	0.05	1.036 - 1.219	0.01
Employees	0.36	0.13	0.182 - 0.722	0.001
Cleaning corral <1x/day	0.23	1.68	0.108 - 0.488	<0.001
Track in bad condition	5.54	3.38	1.674 - 18.330	0.01
Animal access to pile of manure	4.91	1.95	2.256 - 10.699	0.001
Sole Hemorrhage				
Amount of concentrate in the diet	1.13	0.05	1.038 - 1.232	0.01
Family + employee	2.12	0.64	1.174 - 3.836	0.01
Track in bad condition	3.47	1.43	1.542 - 7.794	0.00
Animals are beaten	2.31	0.71	1.259 - 4.224	0.01
Scissors claws				
Distance from pasture to milking room >500m	0.54	0.16	0.299 - 0.962	0.04
Slurry or moisture in the corral	0.46	0.13	0.264 - 0.819	0.01

[a]Animals need to walk more than 500 meters to go to milking room. OR: odds ratio; SE: standard error; CI: confidence interval.

Figure 6. Diagram illustrating the correlations between risk factors, hoof lesions and lameness in grazing cattle. The number next to the arrow represents the increase or decrease in the odds ratio for the occurrence of the hoof lesions.



Discussion

To successfully reduce lameness and hoof lesions incidence on farms, it is essential that interventions be targeted at the risks factors. Aiming for that this study provide the initial step for a better comprehension for risk factors for hoof lesions and lameness in grazing cattle under tropical condition. The studies population was randomly selected from a limited area in Minas Gerais state. However, the visited farms presented a great variation in herd sizes, different management practices and farm daily operations, although none of them kept the animals indoors at any time of year. Therefore, we believe that the visited farms represents a diverse subsection of Brazilian dairy farms and provide a basis for a study of the epidemiology of lameness and hoof lesions for a wider region.

Our results revealed management practices and environment elements that may influence animal welfare in the visited farms. The continuous access to pasture have a benefic effect for cow comfort and it permit animals to express its normal behaviors (Haskell et al., 2006; Holzhauer et al., 2012; Krohn e Munksgaard, 1993; Stafford e Gregory, 2008). This is perceived by the public opinion who associated acces to pasture with a good animal welfare (Cardoso et al., 2016). The inexistence of hock injuries in the visited farms corroborate with this statement. The high proportion of animals considered clean compare to most of housed cattle (Sant'anna and Paranhos da Costa, 2011) and the fact that in the majority of farms the animals were guided quietly were also indication of good animal welfare. However, even though in a minority of farms, we could experience animals be beaten. This is intolerable and advocates against the dairy industry and its image by the public opinion and consumers (Cardoso et al., 2016). Inadequate water and shade availability was also frequently observed. These factors go against the "five freedoms" (freedom from hunger and thirst; discomfort; pain, injury and disease; the ability to express normal behavior; and freedom from fear and distress) established in the Farm Animal Welfare Council (1992), and represent a serious jeopardy for animal wellbeing and should receive swiftly attention.

The lack of preventive measures for hoof health emphasize the importance of a work focusing in increasing awareness of the farmers to lameness and its consequences. It may indicate that those farmers are underestimating the prevalence of lameness on their farms and that lame cows on some farms have to wait for prolonged periods before they are identified as being lame and receive treatment for lameness.

The mean lameness prevalence of 16% are similar from reports from housing (Foditsch et al., 2016; Westin et al., 2016) and grazing cattle (Haskell et al., 2006). However, the percentage of cows with a severe locomotion score (6.8%) is higher than found by Foditsch et al. (2016) in the US, who reported a prevalence of 2%, and similar to Von Keyserlingk et al. (2012), who reported a prevalence of severe lameness ranged from 4% to 8% farms of Canada and US.

In the final ordered logistic model for lameness the increase in body condition is associated with a better mobility score (OR=0.26). Several researches have associated low BCS with lameness because of the direct relation between BCS and the thickness of the digital cushion (Green et al., 2014; Machado et al., 2010). Cows with thinner digital cushion are more likely to have claw horn lesions, but the same is not true for infectious lesions (Green et al., 2014). The relationship observed in the present study has the limitation inherent from a cross-sectional study; it is not possible to determine the temporal relationship between exposure factors and disease (Dohoo et al., 2003). Taking into account this limitation and the results that infectious lesions were the most important, the lower BCS is probably a consequence from the lameness rather than a causative factor. The same interpretation is valid for the correlation between BCS and ID.

The other three factors associated with an increase in the mobility score are related with an unhygienic environment and increase in animal density. Unhygienic condition was also high correlated with infectious lesions as DD and HHE. Animals exposed to longer times in the corral and presenting a poor hygiene score had a two-fold increase in the odds for lameness and DD. A direct relationship between the cleanliness of dairy cows and lameness prevalence and the presence of DD have been described in housing cattle (Relun et al., 2013; Westin et al., 2016). Confine the animals in paddocks during the dry period was also related with a worse mobility score (OR=2.07). The poor condition of the corral exit and cleaning the corral less frequently were associated with an increase in 2.8 and 5.2 times the odds for HHE. These areas are a bottleneck and are often with mud, moisture and sometimes boulders and small stones are placed to prevent water accumulation. The association of more HHE in poor hygiene condition is in agreement with Manske et al. (2002) and Sogstad et al. (2005). All these exposed factors increase the contact of the hoof to slurry, urine and manure. In this situation, the digital horn became more soft and susceptible to abrasion and bacterial invasion (Gregory et al., 2006; Winkler e Margerison, 2012).

HHE was less frequent in extensive farms (OR=0.29). These farms are characterized by low stock density and low yielding cows. In this kind of system, the animals normally spend longer times at pasture, what is associated with a decrease in HHE because of less exposed to the unfavorable environment of sheds and even HHE might heal spontaneously when cows are on pasture (Toussaint Raven et al., 1985; Somers et al., 2005).

The use of footbath was significant as a preventive measure for HHE (OR=0.15). The use of formalin solution was the most common for footbath in the regions and it provide a hardening and disinfection effect on the hoof (Cook et al., 2012; Gregory et al., 2006). Footbath is a preventive tool used with success for laminitis related and infectious lesions, decreasing the frequency and helping in the healing process (Cook et al., 2012; Fjeldaas et al., 2014; Speijers et al., 2012). Thus, the use of footbath was expected to be a protective factor for more hoof lesions. Probably the reason why footbath was only significant for HHE is that only 6.3% farm used it, limiting the sample size.

Cleaning the corral less frequently was identified to be risk factors for HHE (OR=5.24). However, it acted as a protective factor for IH (OR=0.31) and WLF (OR=0.23). A possible explanation is that depending on the floor type as paved floors, gravel and boulders became looser and exposed when cleaned frequently and may increase the chance for traumas, while the dry manure may protect the hoofs against it.

Factors related with human-animal relationship were present in the model for SH and IH. Good human-animal relationship as patience of the farmer handling the cows on the track decrease in more than half the odds for HI. Conversely, poor human-animal relationship by hitting animals were related with an increase chance for SH (OR=2.31). Chesterton et al. (1989) demonstrated that the patience of the farmer handling the cows on the track is one of the most important factors affecting lameness prevalence in grazing cattle in New Zealand. The negative human-animal relationship will increase fear of humans and consequently animals are likely to be stressed and are more likely to sustain injuries trying to avoid humans during routine inspections and handling (Hemsworth, 2011). The interaction between stockperson and animals was described to be related with an increase lameness rate in Australia (Harris et al., 1988; Hemsworth et al., 1995).

In our study, tracks features was the factor affecting adversely the largest number of hoof lesions. It was associated with an increase odds of 7.1, 5.5 and 3.5 times the chances of finding HHE, WLF and SH respectively, highlighting the importance of track maintenance in farms with grazing cattle. This is much in accordance with the studies performed by Chesterton et al (1989) and Harris et al. (1988), which the tracks maintenance was described as one of the two most importance factors for lameness prevalence in grazing cattle together with the patience of the stockperson whilst bringing cows in for milking. Burow et al. (2014) described that prepared tracks with asphalt, gravel, slag, concrete, and/or rubber was correlated with lower lameness rates than tracks in plain soil with or without grass cover. The majority of tracks in the visited farms were without preparations.

Obstacle in the cow’s path way was also present in the model for ID. In this case, the presence of damaged concrete increase in three times the odds for ID. Even though ID is an infectious lesion, its pathology is yet not well describe and a damage skin can be a initiate feature for the lesion to develop (Somers et al., 2005a). Barker et al. (2010) found that damaged concrete was related with an increase in general lameness prevalence, without association with an specific condition..

Tunga spp. is a hematophagous ectoparasite sand flea that can parasite livestock animals and man (Linardi et al., 2013). The epidemiology of this ectoparasitosis is far from being understood as well as its consequences for hoof health (Marin et al., 2015; Muehlen et al., 2003; Ribeiro et al., 2007). Although *Tunga* spp. lesions can be painful, it is normally associated with an itching sensation in humans (Feldmeier et al., 2004) and is not associated with clinical lameness in cattle (Ribeiro et al., 2007). Our results demonstrate that this parasite present a seasonal occurrence and the rainy season act as a strong preventive factor (OR=0.04), what is also described by (Ribeiro et al., 2007). The other factors associated with a decrease chance for *Tunga* spp. are the presence of stamped floor (OR=0.11), what difficulties the development of the flea and the increase in milk productions (OR=0.98 per liter of increase in milk production). The eggs and larvae of this sand flea develop a few centimeters deep in dry manure and sand (Linardi et al., 2010), what explain the association with the stamped floor and wet environment. The negative association with high yielding farms probably has to do with a combination of characteristics. Larger farms normally present more paved area, some use of footbath, and in smaller farms there is a greater chance of other stock animals as pigs and chickens are present and they also can serve as a reservoir of the parasite (Feldmeier et al., 2004).

An increase of WLF was associated with increased age (OR=1.12) and access to pile of manure (OR=4.91). The increased risk of white line disease with increasing parity was similarly observed by Barker et al. (2009) and is possibly because older cows tend to have a worst claw conformation (Vermunt and Greenough, 1995). After cleaning the corral, some farmers just pile the manure up waiting for a larger amount is gather until remove it. If animals have access to this area, they are often observed stepping and laying down in this pile of dry manure. This condition may predispose WLF due to a prolonged contact to contaminated environment. Nonetheless, no other hygiene related factor was associated with WLF supporting this hypothesis.

The etiology of WLF is complex and its causes can involve chronic laminitis, penetration of foreign bodies or greater stress in hoof structure by twisting the foot (Barker et al., 2009; Bicalho e Oikonomou, 2013; Hemsworth et al., 1995). However, in our study, the risk factors associated with WLF suggest that the separation of the white line is caused mainly by trauma and unappropriated hoof conformation. This observation is corroborated by the very low prevalence of SU and other signs often seen in chronic laminitis conditions such as horizontal lines, corkscrew claw, or yellow colouration (Greenough and Weaver, 1997). The decrease odd for WLF in farms with employees is possible associated with a confounding effect that is not clear.

The risk factors identified for sole hemorrhage in our study are related with trauma or known predisposing factors to laminitis (Nielsen et al., 2013; Thomas et al., 2015). The increase in one kilo of concentrate produce the increase in 1.13 the chance of finding SH. High energetic diet have often been related with laminitis consequences (Cook et al., 2004; Nocek, 1997a). The features of the tracks including the presence of stones, mud or other obstacle and the fact that

The statistical model for SC included only protective factors. The long walk distance between the pasture and milking parlor and the presence of slurry and moisture in the corral are both correlated with an increase in horn wear (Rodríguez-Lainz et al., 1996; Vermunt e Greenough, 1995). This probably prevent the SC balancing the claw grow and wear (Vermunt and Greenough, 1995).

Conclusion

The identified risk factors for lameness and hoof lesions encompass a wide aspect of husbandry practices, facilities maintenance and human-animal relationship, what highlight the multifactorial nature of these conditions. Track features and maintenance emerge as the single most important factor increasing in more than three folds the odds for HHE, WLF and SH. The hygiene score of the animals and longer hours spend in the corrals were identified as risk factors for lameness as well for DD. Several other factors related to environmental hygiene conditions were identify as risk factors for hoof lesions including the frequency of corral cleaning, condition of corral exit, access to pile of manure and keep animals in paddocks during the dry period. Identified risk factors related with the human-animal interaction were the patience of herdsmen conducting the cows from pasture to milking and if the animals were hit. These factors were related to an increase odds for IH and SH respectively. Nevertheless, some risks factors for WLF and SH were less clear, and further researches are warranted.

Acknowledgement

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6. Chapter 3 -Pathology and bacteria related to digital dermatitis in dairy cattle in all year round grazing system in Brazil

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Abstract

Digital dermatitis (DD) is one of the main causes of lameness in dairy cattle worldwide, and it is frequently reported in high-yielding, free stall dairy herds from regions with a temperate climate. However, DD is also observed with high prevalence in grazing cattle with a low milk yield in tropical regions. To clarify whether these differences have an impact on the etiology of the disease, we studied DD lesions from all year round grazing cattle of mixed breed in Brazil using high-throughput 16S rRNA gene sequencing and fluorescent *in situ* hybridization. The study included samples from 66 skin lesions and 5 healthy skins collected from five farms. Both techniques showed *Treponema* spp. to be the most abundant bacteria, represented in all but one of the samples with minimal epidermal alterations. We identified eleven different *Treponema* strains belonging to the six major phylotypes of *Treponema* which have all previously been identified in DD lesions. Furthermore, this is the first study to identify *Dichelobacter nodosus* in DD lesions by gene sequencing. *D. nodosus* was also identified by fluorescent *in situ* hybridization in almost half of biopsy specimens in areas with mild epithelial damage and together with *Treponema*. The present data support the hypothesis that *Treponema* constitutes the main pathogen responsible for DD, independent of the environment and region where cows are kept, and it further suggests *D. nodosus* as another potentially important pathogen.

Key words: Next generation sequencing; Fluorescence *in situ* hybridization; Bovine digital dermatitis; *Treponema*; *Dichelobacter nodosus*

Introduction

Since first being reported in 1974, bovine digital dermatitis (DD) has spread all over the world to reach an endemic situation in several countries (Evans et al., 2016; Palmer and O'Connell, 2015). In the last decades, it has become a growing issue in dairy cattle and presently represents the main cause of lameness in cattle (Evans et al., 2016; Klitgaard et al., 2008; Palmer and O'Connell, 2015). DD is an inflammatory skin lesion characterized by ulceration and/or papillomatous growths, usually located on the plantar surface of the hind foot and normally circumscribing the heel bulbs (Evans et al., 2016). Lesions are painful and may result in lameness, reduced milk yields, impaired reproductive performance and premature culling (Bruijnis et al., 2012). The severe consequences of this disease makes it a great concern for the dairy industry worldwide and a subject of much attention (Bruijnis et al., 2012).

DD is a polymicrobial disease for which the etiology still remains to be elucidated (Krull et al., 2014). Spirochetes of the genus *Treponema* are the most prevalent bacteria in the lesions, and they are normally found in the deep part of the lesions in the layer between damaged and healthy tissue (Klitgaard et al., 2008; Nielsen et al., 2016; Zinicola et al., 2015). To date, more than 20 different *Treponema* phylotypes have been identified from DD lesions and among these, *Treponema phagedenis-like*, *Treponema refringens-like Treponema medium*/ *vincentii-like*, *Treponema denticola- like*, and *Treponema pedis* were the most prevalent. Other bacteria which have been identified from DD lesions are *Mycoplasma*, *Fusobacterium, Porphyromonas* (Krull et al., 2014; Nielsen et al., 2016) *Bacteroides* spp., *Campylobacter* spp., *Guggenheimella* spp (Wyss et al., 2005; Zinicola et al., 2015) and *D. nodosus* (Rasmussen et al., 2012), but the role played by each of these bacterial groups is not well known. As many *Treponema* spp. are not yet cultivable, culture independent methods, such as next generation sequencing (NGS) and fluorescent *in situ* hybridization (FISH), have been used with success to study DD etiology (Klitgaard et al., 2013; Nielsen et al., 2016).

The first report of DD in Brazil was in 1992, by Borges et al. (1992). Since then, several authors have reported DD with prevalence ranging from 0.92% (Silveira et al., 2009) to 44.2% (Moreira et al., 2012). A recent study using nested PCR identified three *Treponema* phylogroups in DD lesions in Brazil, *T. medium*/*T. vincentii*-like, *T. phagedenis*-like, and *T. denticola*/*T. putidum*-like, but no details about the housing were provide (Nascimento et al., 2015). Previous studies of digital dermatitis have focused on samples from dairy herds with Holstein cows using free stalls in temperate climates (Capion et al., 2013; Evans et al., 2012; Klitgaard et al., 2014; Palmer and O'Connell, 2015). However, despite the great differences in environmental and management conditions, DD is also observed with high prevalence in grazing cattle (Moreira et al., 2012). These differences may have an impact on the epidemiology and the etiology of the disease. In this context, the aim of this study was, therefore, to identify DD-associated pathogens from the lesions of all year round grazing cattle in Brazil in well-described housing systems, using high-throughput 16S rRNA gene sequencing and fluorescence *in situ* hybridization (FISH).

Materials and methods

The set-up of this study was in accordance with the recommendations of The Animal Welfare Act of 1966 (AWA) (P.L. 89–544) and its amendments 1970 (P.L. 91–579); 1976 (P.L. 94– 279), 1985 (P.L. 99–198). The project was previously approved by the Ethics Committee in Animal Experimentation from Universidade Federal de Minas Gerais (CEUA/UFMG) under protocol number 121 / 2015. All farm owners were in agreement with the observational study to be performed in their properties.

Farms

Samples were collected from five different farms, all presenting a history of DD problems, in different regions of Minas Gerais state, Brazil. The details of the farms are listed in Table 12. All farms kept the animals in pastures all year round and provided concentrate feed during milking or in feed pads after milking. Roughage supplementation with sugar cane and/or corn silage was provided during the dry period in all the farms. The daily milk production of the farms varied from 250L to 2000L and the average milk yield per cow from 9L to 20L per day. All animals on the farms were crossbred Holstein x Gyr cows. No preventive measures were taken regarding lameness and hoof lesions other than the use of a foot bath with copper sulfate or formalin solution in farms 4 and 5.

A representative sample from all animals from the farms was examined for hoof lesions using a stand-up trimming chute. DD lesions were sampled for later analysis. All lactating animals in the visited farms were evaluated for mobility using the four-point (0–3) DairyCo mobility scoring method (Barker et al., 2010).

Table 12 Overview of characteristics of the 5 visited farms

Characteristic	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5
Lactating cows	100	28	56	83	86
Milk prod (L/day)	1200	250	900	1400	1600
Milk prod./cow (L)	12	9	16	16.9	19
DD prevalence (%)	15.8	41.2	36.6	42.3	46.5
Lameness prevalence (%)	18	31.4	42.5	12	35.1
Foot bath [a]	-	-	-	+	+

[a] Use (+) or no use (-) of foot bath in the farm

Sample collection

DD lesions were cleaned thoroughly with water and dried off with paper towels, and biopsies were surgically excised after local anesthesia with lidocaine 2% subcutaneously or by bier block anesthesia. After this procedure, the surgical lesions were treated with topical tetracycline and bandages until the lesions were healed. The DD lesions sampled were classified according to Barry et al. (2012). Briefly, score M1 is a small active ulcer (<2 cm across) with moist surface and mottled red–gray coloring; M2 is a larger ulcerative active stage (>2 cm across) and normally painful upon manipulation; M3 is the healing stage where the lesion is covered by a dry brown scab, normally with no pain on manipulation; M4 is the chronic stage with proliferative hyperkeratotic growths that vary from papilliform to mass-like projections; M4.1 is the chronic stage with small active painful M1 focus.

The biopsies were subdivided, one half was placed in 10% neutral buffered formalin and then prepared for histological and FISH examination. The other half of the sample was stored in a nucleic acid stabilization solution (RNA*later*®, Ambion, Austin TX) for sequencing, and kept for 24 h at 5°C and then stored at -20°C until use.

Fluorescent in situ hybridization (FISH)

For FISH analysis, serial sections of 4 µm were cut from the paraffin blocks and mounted on SuperFrost+ slides (Menzel-Gläser, Braunschweig, Germany). The oligonucleotide probes used in this study are listed in Table S1 and included probes specific for Domain Bacterium, *Treponema spp.*, *F. necrophorum*, *D. nodosus*, *P. levii*, and nine *Treponema* phylotypes, *T. pedis*, *T. refringens*, *T. denticula*, *T. phagedenis*, *T. medium*, PT1, PT3, PT12, PT13. The probe for the domain Bacterium was used on all slides in combination with one other probe for individual bacterial species. The slides were mounted in a Sequenza slide rack (Thermo Shandon, Cheshire, United Kingdom) and kept for 14h in 45°C with 100µl of hybridization buffer (10 µl of 1 M Tris [pH 7.2], 18 µl of 5 M NaCl, 1 µl of 10% sodium dodecyl sulfate, 71 µl of H2O) with a concentration of 5 ng/µl of each applied oligonucleotide probe. The probe for the domain Bacterium was 5' labeled with fluorescein isothiocyanate (FITC) and all other bacteria probes were 5' labeled with the isothiocyanate derivative Cy3 (Eurofins MWG Operon, Ebersberg, Germany). The sections were then washed three times in pre-warmed (45 °C) hybridization buffer for 5 minutes and subsequently washed three times in pre-warmed (45 °C) washing buffer solution (10 ml of 1 M Tris [pH 7.2], 18 ml of 5 M NaCl, 72 ml of H2O) with the identical time interval. The sections were rinsed in water, air dried, and mounted in Vectashield (Vector Laboratories Inc., Burlingame, CA) for epifluorescence microscopy. An Axioimager M1 epifluorescence microscope equipped for epifluorescence with a 100-W HBO lamp and filter sets 43 and 38 was used to visualize Cy3 and FITC, respectively. Images were obtained using an AxioCam MRm version 3 FireWiremonocrome camera and AxioVision software, version 4.5 (Carl Zeiss, Oberkochen, Germany).

All biopsy specimens were scored from 0 to 3 according to the total bacteria invasion and the prevalence of each individual bacterial and *Treponema* phylotype, according to Nielsen et al. (2016) and Rasmussen et al. (2012). Briefly, total bacteria invasion score 0 represents no invasive bacteria; score 1, low number of invasive bacteria; score 2, moderate number of invasive bacteria; score 3, high number of invasive bacteria. For individual bacteria score 0 signifies no bacteria; score 1, the specific bacteria represents up to 5% of the total number of bacteria; score 2, represents between 5% and 10% of the total number of bacteria; and score 3 represents more than 10% of the total number of bacteria.

Histopathology

The slides used to perform FISH analysis were also used for histopathological evaluation afterwards. All biopsies were stained with hematoxylin and eosin (H&E). Epidermises were evaluated according to the presence and severity of acanthosis, parakeratosis, papillomatous proliferation, bacterial colonization showed by pale-staining and ballooning keratinocytes (swollen noneosinophilic cytoplasm and enlarged or condensed nuclei) and exocytosis, erosion, and/or ulceration of dermal papillae. Dermises were evaluated based on the inflammatory response present. Thereafter, the epidermal damage and inflammatory response in the dermis were scored from 0 to 3, adapted from Nielsen et al. (2016). Normal epidermis was defined as score 0, mild or focal epithelial proliferation and hyperkeratosis was defined as score 1, moderate epithelial proliferation and acanthosis was considered score 2 and extensive or diffuse damage with severe epithelial proliferation, acanthosis and exudation, erosion or necrosis of the dermal papilla was scored as 3. Inflammatory response was scored as 0 when there was no alteration, score 1 when there was a mild increase in the number of lymphocytes and mononuclear cells score 2 when the increase in inflammatory cells was moderate and score 3 when it was severe and diffuse. Scorings were based according to most severe alterations found in the biopsies. A Leica DMRB microscope was used for reading and images were obtained using a Leica MC120 HD camera and Leica Application Suite software, version 4.7.0 (Leica Microsystems, Wetzlar, Germany).

DNA extraction

Bacterial DNA was extracted from lesions using a DNeasy tissue kit (Quiagen, Hilden, Germany). A 22 mg piece of fixed tissue was cut in small pieces with a scalpel. A sterile 5-mm steel bead (Quiagen, Hilden, Germany) was added, and samples were run two times on a TissueLyser II (Qiagen) at 20 Hz for 3 min per run in 180µl of ATL buffer. All the subsequent procedures were performed according to the protocols of the supplier. The concentrations and purity of the samples were evaluated using a Nanodrop 1000 spectrophotometer (Fisher Scientific, Wilmington, MA) and only samples with A260/A280 ratios of >1.8 were used for further analyses.

Preparation of libraries and sequencing.

Amplification of DNA and library preparation were accomplished as described by Nielsen et al. (2016). Briefly, the DNA was amplified by PCR using a universal bacterial primer set which targets the V1–V2 region (Wilmotte et al., 1993) and a *Treponema*-specific primer set targeting the V3–V4 hyper variable regions of the 16S rRNA gene (Klitgaard et al., 2008). The prepared mixture for PCR contained 5 ml of 10xPCR Gold Buffer (Applied Biosystems, Foster City, CA, USA), 1.5 mM MgCl2 solution (Applied Biosystems), 200 mM of each deoxynucleoside triphosphate (Amersham Biosciences, Piscataway, NJ), 0.4 mM of each specific primer, 2.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems), and 2 ml of template DNA. The PCRs were performed in a T3 thermocycler (Biometram, Göttingen, Germany) following the steps of denaturation at 94°C for 6 min; 30 cycles of denaturation at 94°C for 45 s, annealing at 57°C for 45 s, extension at 72°C for 90s; elongation step of 10 min and cooling to 4°C. The primers had unique hexameric barcodes added at their 5' ends to enable multiplexing.

DNA quality and concentration was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc.). The resulted amplicons were pooled in equal amounts and purified with the Qiagen Mini Elute kit (Qiagen) according to the protocol. The DNA was submitted to the National High-Throughput DNA Sequencing Centre at the University of Copenhagen, Denmark for sequencing on the Illumina MiSeqTM platform. Sequences generated by Illumina MiSeq are available under the accession number SUB2355933 in the NCBI Sequence Read Archive (SRA).

Sequence analysis

Sequence analysis of 16S rRNA gene was performed by BION-meta software (http://box.com/bion). The de-multiplexing step was performed according to the primer and barcode sequences. Forward and reverse sequences were joined, allowing no gaps, a minimum match percentage of 80% and an overlap length of minimum 35 bp. Amplicons with qualities lower than 98%, which is equivalent to a Phred score of 17, were excluded. Sequences of at least 250 nucleotides in length were classified against the Ribosomal Database Project database II (RDP II;

http://rdp.cme.msu.edu/index.jsp) using a word length of 8 and a match minimum of 90%. Then, the number of reads for each barcode was normalized for further analysis.

Statistical analysis

Analysis was conducted using R software. To elucidate the microbial patterns involved in DD etiology, multivariate analysis of the resulting microbial profiles was carried out by Analysis of Similarities (ANOSIM) and comparison between control and DD samples regarding diversity of species were estimated using the Shannon index. A heatmap visualizing the Spearman correlations between majorly abundant families was generated in R.

Results

In total, we evaluated 66 DD lesions and five control samples of healthy skin. The majority of the lesions were characterized macroscopically as being in classical active ulcerative stages. In 25.4% of the lesions the affected area was no more than 2cm across (M1) and in 43.3% of the lesions the affected area was >2cm across (M2). Healing stages (M3) represented 10.4% of the sampled lesions and were only found in farms with footbaths. The proliferative and wart-like conditions (M4) accounted for 20.9%. Three of the control samples were taken from healthy animals, whereas the last two control samples were taken from DD-affected animals, but from a healthy area of the hind feet 2 cm from the lesion area.

Histopathology

Thirty-eight of the 66 DD biopsies, (57.6%) were characterized by severe epithelial proliferation, acanthosis, with some presenting papiliform projections. Furthermore, the epidermis had areas of erosion, ulcer, and the dermis presented exudation, necrosis of dermal papilla and extensive inflammatory infiltration (score 3). Twenty-six samples, (39.4%) presented a moderate increase in epidermis thickness without ulceration and moderate infiltration of inflammatory cells in the dermis (score 2), while the last two DD samples (3%) had only minor alterations manly represented by mild acanthosis. The healthy skin samples presented normal epidermises, besides the two control samples taken from affected feet, which presented mild hyperkeratosis (score 1). A biopsy revealing the different scoring stages of epidermal/dermal alterations from unaffected to ulcerative digital dermatitis, macroscopically graded M2, is shown in Fig. 7.

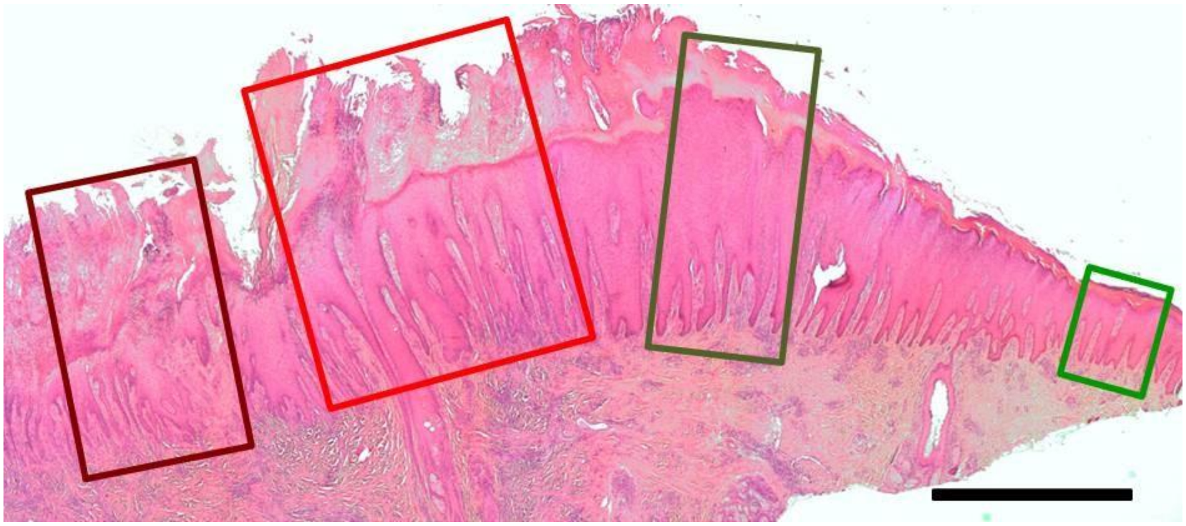

Figure 7. Histology of skin biopsy from a dairy cow with ulcerative digital dermatitis macroscopically graded M2 according to Barry et al (2012). Within the sample different segments of the skin show typical epidermal and dermal alterations varying from left to right unaffected/healthy (green) score 0, moderate epithelial proliferation and acanthosis (score 2, dark green), extensive epidermal degeneration with severe epithelial proliferation, exudation and erosion (score 3, red). To the left, pointing to the center of the lesions, a score 3 segment with severe exudation and ulceration (dark red). H&E, bar 2mm.

Fluorescent in situ hybridization FISH

We evaluated the 66 samples using double *in situ* hybridization targeting the domain Bacterium plus one species or genus specific probe, totalling 14 different probes. Fig 8 illustrates the prevalence and score for all bacteria and *Treponema* phylotypes investigated by FISH. Of the 66 samples, only one was negative for colonizing/invading bacteria, presenting only minimal epidermal alterations. *Treponema* spp. were the most frequent and abundant bacteria, present in 64 of the 66 (97%) samples. Furthermore, in 64% of the cases, treponemes represented the majority of the observed bacteria. *P. levii* was the second most prevalent bacteria with 64.6% positive samples, followed by *D. nodosus* (48.5%) and *F. necrophorum* (23.5%). Although very frequent, these three bacterial species represented less than 5% of the total bacteria present in most cases (Fig.8). Regarding the *Treponema* phylotypes, each lesion presented a median of 4 phylotypes and a maximum of 7 from the 9 tested phylotypes. The prevalences were, in decreasing order *T. phagedenis* (71%), *T. refringens* (68%), *T. pedis* (67.2%), *T. medium* (54.1%), PT1 (40.9%), PT13 (38.2%), *T. denticula* (37.5%), PT3 (30.4%), PT12 (3.3%). Two samples were negative for *Treponema* in FISH analysis. One of these was the sample negative for all tested probes. The other was classified as score 2 for dermal and epidermal damage and was positive *P. levii* in FISH. Additionally, *Treponema* were identified in this sample by sequencing analysis. One sample, which was taken from apparently healthy skin two cm from a lesion, was positive for *T. pedis*, *T. medium* and *T. phagadenis*. This sample had a score of 2 in the histopathological examination with altered dermis and epidermis and was likewise positive for *Treponema* in the NGS analyses.

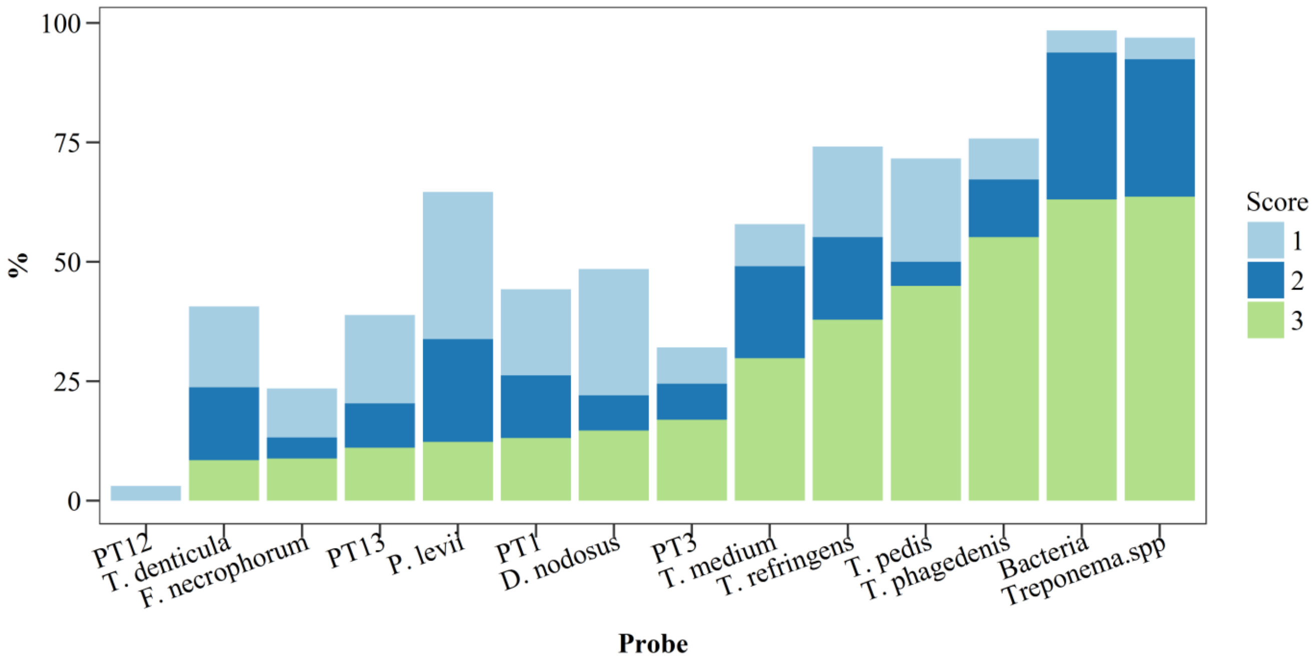

Figure 8. Prevalence and abundance score of different bacteria and *Treponema* phylotypes investigated by Fluorescent *in situ* hybridization in biopsy specimens of bovine digital dermatitis (n=66) from crossbreed dairy cows kept in pasture all year round. For total bacteria invasion score 1 represent a low number of invasive bacteria; score 2, moderate number of invasive bacteria; score 3, high number of invasive bacteria. For individual bacteria: score 1, bacteria represents up to 5% of the total number of bacteria; score 2, between 5% and 10% of the total number of bacteria; and score 3 represent more than 10% of the total number of bacteria.

Usually, spirochetes colonized the deeper parts of lesions with severe epidermal damage (score 3), forming a bright red fluorescing band between the healthy and affected tissue, as shown in Fig. 9. Among the *Treponema* phylotypes, *T. pedis*, *T. medium*, *T. phagedenis*, and *T. refringens* were normally the ones present in the deeper parts of the lesions while *T. denticola*, and the uncultured *Treponema* PT1 were located superficially and PT12 were rarely found. *D. nodosus* were present as sparse single cells or as diplobacilli in areas of the skin with imperfect keratinization. Usually, *Treponema* and *D. nodosus* were the only bacteria present in areas where the epidermis and keratin layer where more preserved. *F. necrophorum* were present as single cells and as clusters, and normally together with other bacteria. *F. necrophorum* was only present in one sample without ulceration. *P. levii* formed small clusters in the very superficial layers of the lesions in distinguishing areas from *Treponema*. *F. necrophorum* and *P. levii* were found together occasionally once both bacteria were mainly present within ulcerated areas, with exudation or in more degradable epidermis.

Fig 7 illustrates the diverse histopathological aspects of the DD and the bacterial colonization pattern described previously. Compared to the bacterial abundance as revealed by FISH, invading bacteria were not observed in the green segment, whereas the superficial layers of the dark green segment commonly were invaded by varying numbers of *Dichelobacter nodosus* and treponemes. The red segments were characterized by a high number of invasive bacteria systematically with treponemes on the borderline between diseased and healthy tissue. *Fusobacterium necrophorum* and *Porphyromonas levii* were predominantly found in the ulceration of the dark red segment.

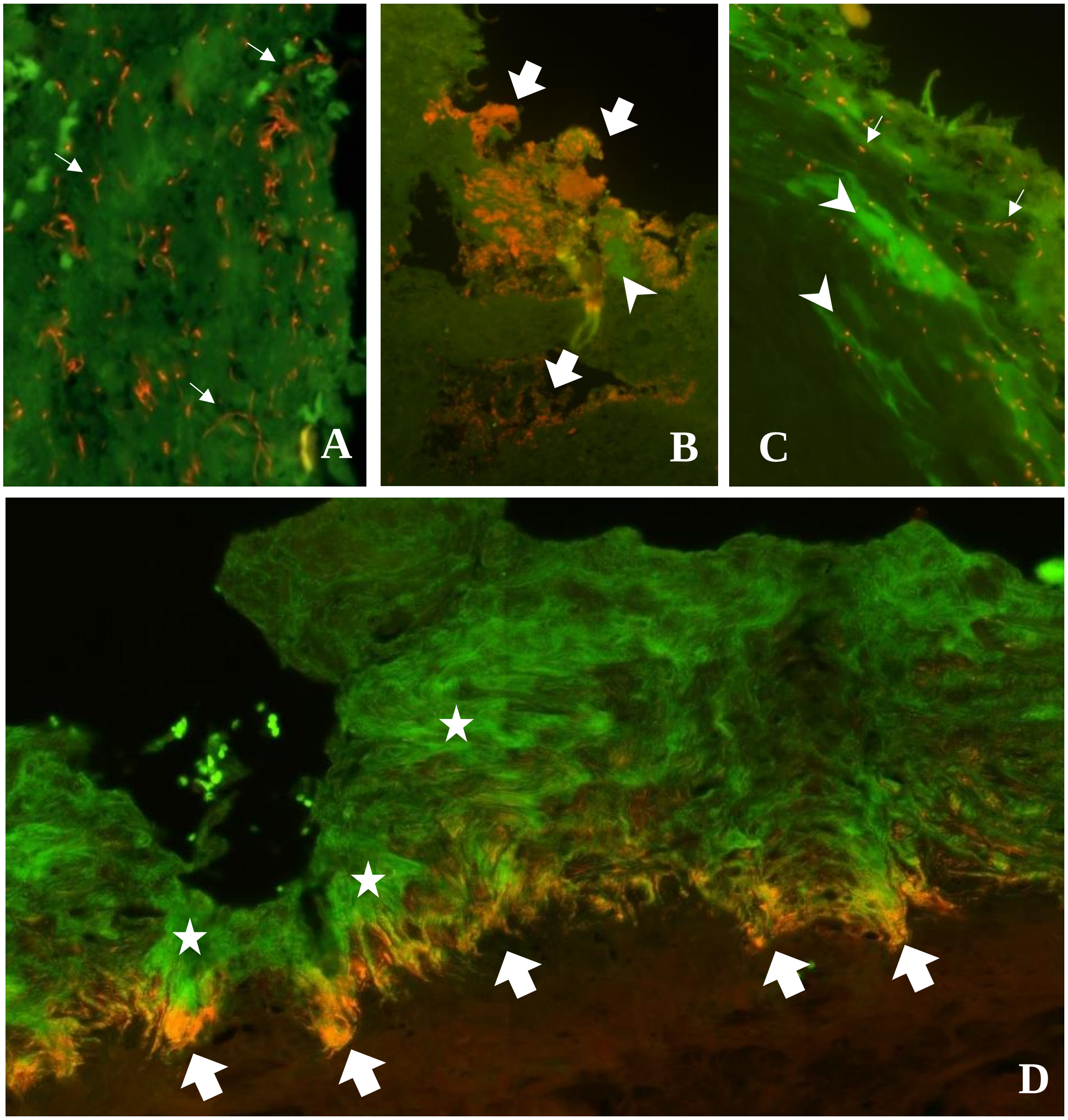

Figure 9. Fluorescent in situ hybridization on digital dermatitis biopsies demonstrating several bacteria. A) Fluorescent in situ hybridization demonstrating *Fusobacterium necrophorum* (red) (arrows) in small clusters and individual bacteria B) Double hybridization showing *Porphiromonas levii* (red) (arrows) organized in cluster in superficial layer and other bacteria in green (arrow head). C) Fluorescent in situ hybridization demonstrating *Dichelobacter nodosus* (red) (arrows) in sparse individual bacteria and other bacteria in green (arrow head). D) Digital dermatitis biopsy demonstrating massive and deep colonization of Spirochete-like bacteria (green) (stars) and *Treponema refringens* (red/orange) (arrows) in between damage and health tissue.

16S rRNA sequencing (NGS)

We successfully sequenced all 44 DD-lesions samples submitted and 4 of the 5 controls samples with the primers V1–V2 and 42 DD-lesions and 1 control sample with the primers V3–V4. The lower number of samples for sequencing compared to FISH is due to logistical matters and the availability of the nucleic acid stabilization solution. The reason some samples were not sequenced was that no PCR product could be amplified. The sequences were de-multiplexed according to the sequences of the barcodes and primers, and subsequently chimeras and sequences with quality below 98% were discarded. In total, 2,486,038 (V1–V2 pool) and 2,472,912 (V3–V4 pool) joined sequences were used for taxonomic classification. Of these sequences, 1,610,008 of the V1–V2 pool and 1,578,668 of the V3–V4 pool were taxonomically classifiable according to the RDPII database (http://rdp.cme.msu.edu/).

The diversity of bacterial genera present in DD samples was lower than in the control samples (Fig 10). The number of different genera representing more than 1% of the total reads in DD samples was 23, while in controls this number was 35.

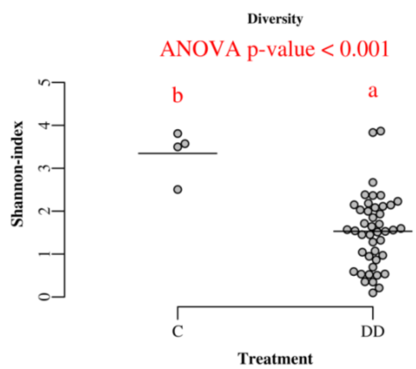

Figure 10. Diversity of bacteria genus in control and digital dermatitis specimens measured by Shannon-index.

We were not able to detect any correlations between the bacterial species identified in NGS analysis and all the environmental parameters which might influence the composition of the DD microbiota, e.g.: macroscopic score of the lesion, use of footbath, sample region and farm.

The most representative phyla represented in the sequencing results were in decreasing order *Spirochaetes*, *Bacteroidetes*, *Firmicutes* and *Fusobacteria*. The predominant genera in the DD samples were *Treponema* and *Porphyromonas* (Fig 11) with a prevalence of 86.36% for both genera. *Treponema* was the most abundant genus present in the DD lesions, representing more than 50% of total sequence reads in 52.3% of the samples with a mean abundance of 44.9%. *Porphyromonas* was the second most abundant bacteria and accounted for more than 20% of total reads in 31.8% of the samples with a mean abundance of 17.5%. Other genera that accounted for a high proportion of total reads were *Fusobacterium* (mean density of 5.15%), unclassified bacteria from class Clostridia (2.8%), *Helcococcu*s (2.5%), *Mycoplasma* (2.4%), *Dichelobacter* (2.1%), *Catonella* (2.1%) and

Campylobacter (2.0%). Six DD samples were negative for *Treponema* in the results from V1-V2 primer. However, *Treponema* 16S RNA was successfully amplified by V3-V4 primer in four of these six samples, while in the other two samples *Treponema* was identified by FISH examination. In the six samples negative for *Treponema* in V1-V2 primer, *Porphyromonas* was the most abundant bacteria. In the control samples, *Corynebacteriaceae, Ruminococcaceae* were the predominant family.

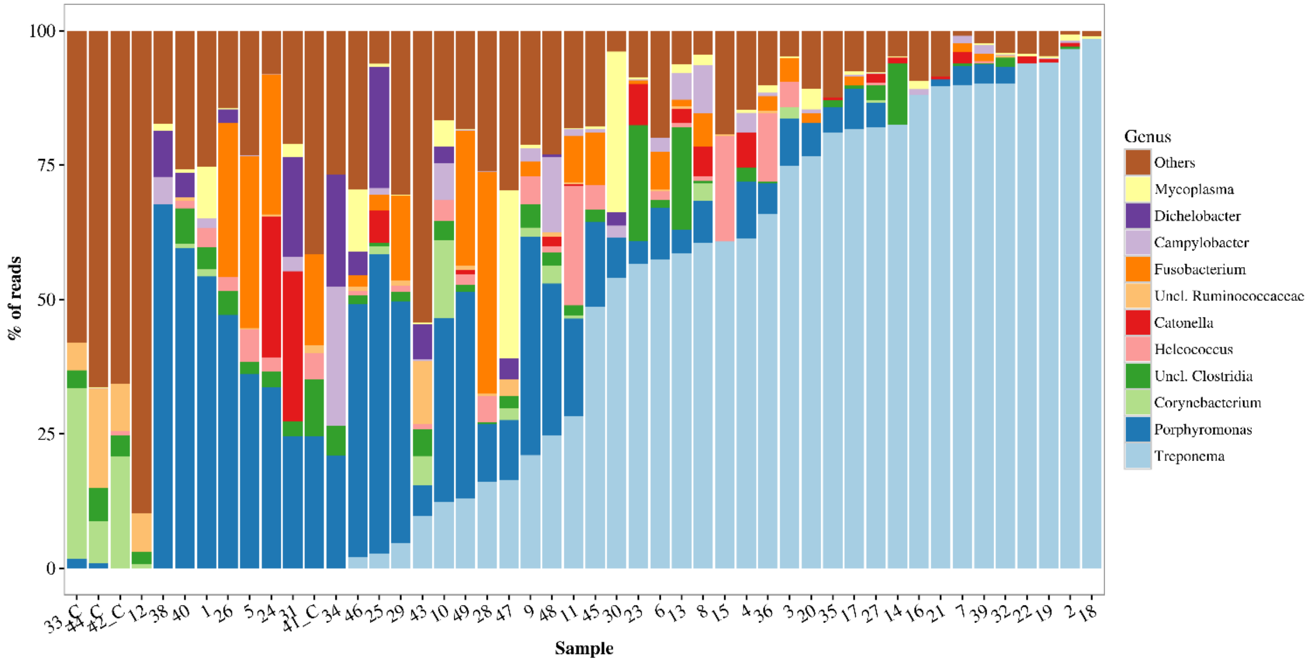

Figure 11. Relative abundance of bacteria genus amplified by high-throughput sequencing of V1 and V2 hypervariable regions of 16S rRNA in 44 biopsy specimens of bovine digital dermatitis and 4 controls samples from crossbreed dairy cows kept in pasture all year round. Control samples are identified by _C.

Treponema species identified by V3-V4 primers were *T. pedis*, *T. refringens*, *T. denticula*, *T. phagedenis*, *T.medium*, *T. porcinum* and *T. zuelzerae* (Fig 12). Other bacteria amplified by the V3-V4 primers were *Sphaerochaeta globosa*.

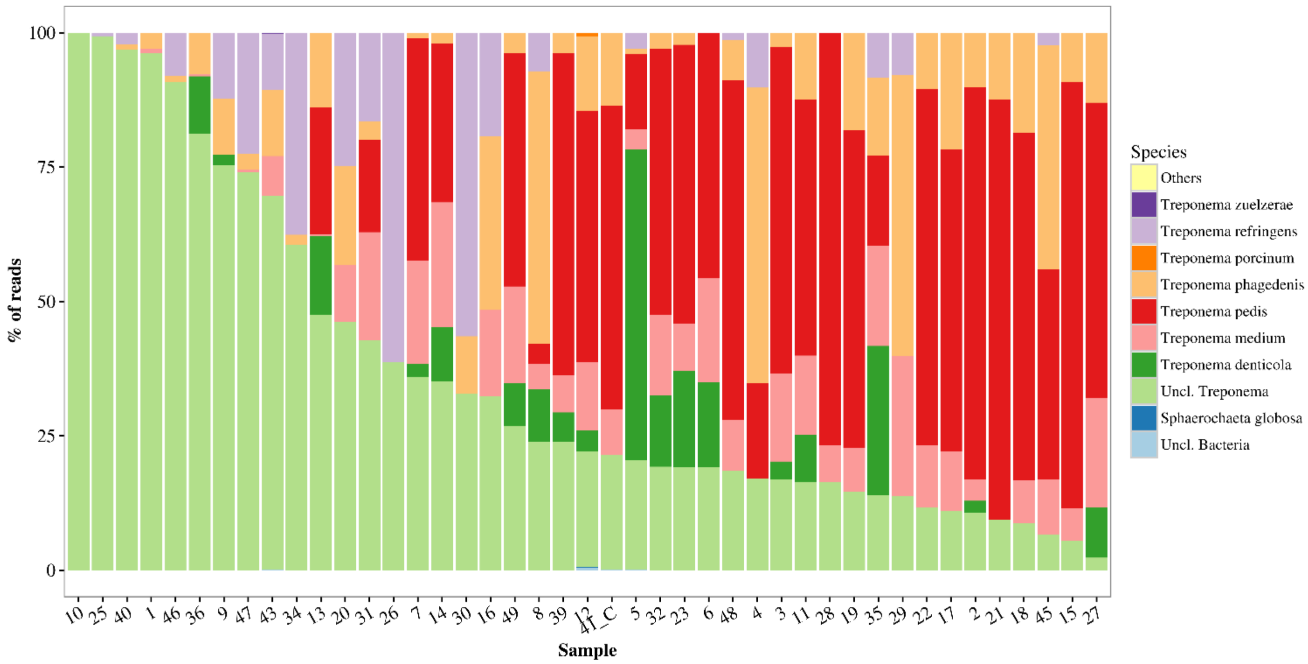

Figure 12. Relative abundance of Treponema phylotypes and other bacterias amplified by high-roughput sequencing of V3 and V4 hypervariable regions of 16S rRNA in 43 biopsy specimens of bovine digital dermatitis and 1 control sample from crossbreed dairy cows kept in pasture all year round. Control sample is identified by _C.

A correlation analysis of the families identified from the deep sequencing analysis is shown in Fig 13. In the heathy skin samples, the *Corynebacteriaceae* and *Ruminococcaceae* were moderately correlated (r^2=0.48). The family *Cardiobacteriacea* which *D. nodosus* belongs to was moderately and positively correlated with *Campylobacteraceae* (r^2=0.48) and *Porphyromonadaceae* (r^2=0.38). The *Porphyromonadaceae* was also positive correlated with *Fusobacteriaceae* (r^2=0.38). *Spirochaetaceae* was negatively correlated with almost all other bacterial families, showing a medium to low coefficient and a strong (r^2=-0.68) negative coefficient towards *Porphyromonadaceae*.

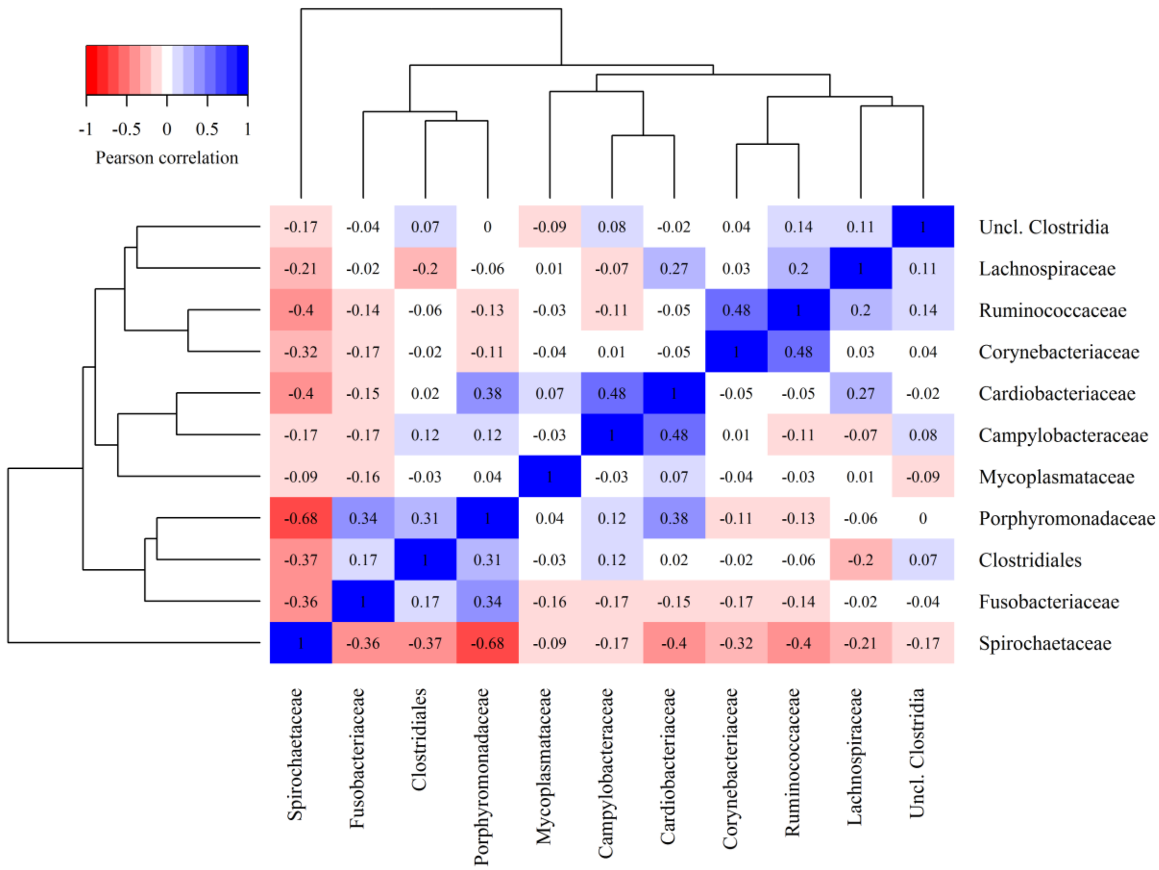

Figure 13. Heat map demonstrating the correlation of bacterial families across bovine digital dermatitis and control samples.

Discussion

Although DD is found worldwide, attempts to study its etiology have been restricted to temperate climate regions where Holstein is the principal breed and most herds are housed in free stalls (Evans et al., 2008; Klitgaard et al., 2013, 2008; Krull et al., 2014; Yano et al., 2010; Zinicola et al., 2015). In this study, we investigated samples from Brazilian grazing cattle by molecular methods to determine whether differences in environment, climate and geographical distance could reveal significant factors that determine the composition of the bacterial populations present in DD lesions.

In the DD lesions, *Treponema* was the dominant bacteria representing more than 50% of total reads in 52.3% of biopsy specimens. We identified eleven different *Treponema* strains belonging to the six major *Treponema* phylotypic clusters which have previously been described from DD lesions in other parts of the world; *T.*

phagedenis-like, *T. refringens*-like, *T. denticola*/*pedis*-like, *T. medium*/*vicentii*-like, *T. porcinum*-like and *T. zuelzerae*-like (Evans et al., 2008; Rasmussen et al., 2012; Zinicola et al., 2015; Nielsen et al., 2016). The most frequent and abundant types were *T. pedis*, *T. medium*, *T. phagedenis*, and *T. refringens*, which were also found in deeper parts of the lesions by FISH, much in accordance with other reports (Cruz et al., 2005; Nielsen et al., 2016). Only one DD sample was negative for Treponema. This sample was only analyzed by FISH and in histopathological evaluation presented only mild alteration (score 1), characterized by epidermal hyperplasia, what may indicate that this lesion was in a final healing stage.

Besides the known DD pathogens, the phylogenetic analysis of the amplicons sequenced revealed a large number of *Treponema* reads which could only be classified to genus level. The possible reason is the existence of a larger number of not-yet classified *Treponema* species in this part of the world. However, is not possible to determine whether this unknown population constitutes an important contributor to DD development in this scenario, or that it is mainly composed of environmental treponemes without pathological importance.

Biopsy specimens from DD lesions presented lower bacterial diversity than control samples in the Shannon index. As can be seen in Fig 11, *Treponema* and *Porphyromonas* normally dominated the bacterial population in lesions specimens, while in control samples it was not possible to distinguish a predominant genus. This was described previously by Krull et al. (2014) and Nielsen et al. (2016), who also found *Treponema* and *Porphyromonas* to be the genera that differentiate control and DD samples most, together with *Mycoplasma* and *Fusobacterium*. One control sample which was taken from a macroscopically healthy skin 2 cm away from DD lesion, was positive for *Treponema*, *Porphyromonas* and *Fusobacterium*, similar to DD lesions. Additionally, FISH and histopathology analyses showed an increase in thickness of the epidermis with a high abundance of *T. refringens*, *T. medium* and *T. phagadenis*. The biopsy area was partially covered by hair. This may indicate that the DD lesion in that foot was expanding and microscopic changes were taking place, although there was no visible alteration on the surrounding area. A similar technique for collecting healthy skin samples was used by Zinicola et al. (2015) with success.

In Brazil, a previous study was successful in demonstrating the groups *T. medium/T. vincentii*-like, *T. phagedenis*-like, and *T. denticola/T. putidum*-like in DD lesions using a nested-PCR method (Nascimento et al., 2015). However, no information about the environment where the animals were kept is available, nor taxonomic origin of the other bacteria present. Pasture is advocated to be a less contaminated environment compared to confined facilities, and therefore generally presents lower hoof lesions and lameness prevalence (Haskell et al., 2006). Nonetheless, the visited farms presented a high prevalence of DD and all the main *Treponema* phylotypes and the majority of other bacteria frequently reported in DD could be found. This highlights the importance of DD worldwide, independent of housing conditions.

FISH analyses revealed a shift in bacterial population between preserved and degraded epidermis areas. In areas with mild epithelial damage, characterized by hyperkeratosis and acanthosis, the bacteria population was composed of sparse presence of spirochetes and *D. nodosus.* The *Treponema* population increased toward areas where the epidermis was more degraded, with partial loss of the epithelium and parakeratosis. The observed microbiota then shifted to a predominance of *P. levii* and *F. necrophorum*, and rare *Treponema* in areas with ulcers, hemorrhages, and exudate. The bacterial family correlation shown in Fig 13 corroborate with the spatial observation from FISH, indicating that *Treponema* and *P. levii* colonize different areas of the lesion. These finding are in accordance with previous work by our research group and others (Cruz et al., 2005; Klitgaard et al., 2008; Rasmussen et al., 2012; Nielsen et al., 2016).

To our knowledge, this is the first study that successfully found *D. nodosus* in DD lesions using 16S rRNA gene sequencing. *D. nodosus* has been described (Blowey et al., 1994) and identified by FISH from DD lesions before (Rasmussen et al., 2012). *D. nodosus* is associated with several diseases in ruminants foot, and was advocated to participate in early lesions in bovine DD (Rasmussen et al., 2012; Sullivan et al., 2015). In our results, *D. nodosus* was highly prevalent in FISH specimens, with almost 50% of positive samples. Although normally found superficially and in epidermal lesions, it could also be detected deeper, and besides that, it was observed in all DD developing stages, from the very beginning, to acute and chronic forms. These finding indicate the participation of *D. nodosus* in DD etiology, not only in beginning stages, but also in all the different macroscopic stages. In this context, one could speculate that *D. nodosus* has a role in the development and expansion of the lesion, together with *Treponema* species.

The spatial distribution of *P. levii*, despite its high prevalence, and *F. necrophorum*, suggest these species to be secondary invaders, and not the main etiological organisms. This is also suggested by other authors (Krull et al., 2014; Nielsen et al., 2016; Zinicola et al., 2015). However, in a recent study using gene expression to clarify DD etiology, *P. levii* was indicated to participate in disease development, while the involvement of *F. necrophorum* was not sustained (Marcatili et al., 2016). Therefore, although our results do not support the participation of either of these two bacteria in DD progression, further studies are warranted.

Among the other bacteria found in DD lesions, *Mycoplasma* has been advocated to participate in the transition between early phases to active lesions (Krull et al., 2014; Nielsen et al., 2016), but this study had no observation supporting this theory. Similarly, we did not identify the bacteria *Guggenheimella bovis* nor *Candidatus*

Amoebophilus asiaticus, which were described to be part of DD microbiota in studies by Schlafer et al. (2008) and Zinicola et al. (2015), respectively. *Helcococcu*s, *Campylobacter, Catonella* and an unclassified bacterium from class *Clostridia* were abundant bacteria present in biopsy specimens in this study. However, neither of these bacteria seem to be essential to DD development. In this context, it seems very likely that, except for *Treponema* spp., the bacterial species involved in DD vary enormously, but their importance for disease development is yet to be clarified.

Conclusions

We have demonstrated that the dominant microbiota of DD lesions from all year grazing cattle are similar to previous studies from all year free stall housing, and possess all the six main *Treponema* phylotypes described previously. Furthermore, our results corroborate with the theory that DD is a polymicrobial disease and *Treponema* is the main bacteria responsible for disease development. In this case, *T. pedis*, *T. medium*, *T. phagedenis*, and *T. refringens* were the most abundant treponemes found. Among the other bacteria present in lesions, *D. nodosus* seems to play an important role in the development and expansion of the lesion, possibly acting together with *Treponema*. Additionally, we could not find any evidence of the participation of *P. levii*, despite their great abundance, and *F. necrophorum*, or any other bacteria, in disease development.

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References

ADAMS, A. E.; LOMBARD, J. E.; FOSSLER, C. P.; et al. Associations between housing and management practices and the prevalence of lameness, hock lesions, and thin cows on US dairy operations. J. Dairy Sci., v. 100, n. 3, p. 3758–3765, 2016.

ALVARES, C. A.; STAPE, J. L.; SENTELHAS, P. C.; et al. Köppen's climate classification map for Brazil. Meteorologische Zeitschrift, v. 22, n. 6, p. 711–728, 2013.

AMORY, J. R.; KLOOSTERMAN, P.; BARKER, Z. E.; et al. Risk factors for reduced locomotion in dairy cattle on nineteen farms in The Netherlands. J. Dairy Sci., v. 89, n. 5, p. 1509–15, 2006.

BARKER, Z. E.; LEACH, K. A; WHAY, H. R.; et al. Assessment of lameness prevalence and associated risk factors in dairy herds in England and Wales. J. Dairy Sci., v. 93, n. 3, p. 932–41, 2010.

BARKER, Z. E.; AMORY, J. R.; WRIGHT, J. L.; et al. Risk factors for increased rates of sole ulcers, white line disease, and digital dermatitis in dairy cattle from twenty-seven farms in England and Wales. J. Dairy Sci., v. 92, n. 5, p. 1971–1978, 2009.

BARTLETT, P.C.; KANEENE, J. B.; KIRK, J.H. et al Development of a computerized dairy herd health data base for epidemiologic research. Prev. Vet. Med., v. 4, n.1, p. 3-14, 1986.

BERGSTEN, C.; CARLSSON, J.; JANSSON MÖRK, M. Influence of grazing management on claw disorders in Swedish freestall dairies with mandatory grazing. J. Dairy Sci., v. 98, n. 9, p. 6151–6162, 2015.

BERGSTEN, C.; FRANK, B. Sole hemorrhages in tied heifers in early gestation as an indicator of laminitis: Effects of diet and flooring. Acta Vet. Scand, v. 37, p. 375–382, 1996.

BERRY, S. L.; READ, D. H.; FAMULA, T. R.; et al. Long-term observations on the dynamics of bovine digital dermatitis lesions on a California dairy after topical treatment with lincomycin HCl. Vet. J., v. 193, n. 3, p. 654–8, 2012.

BICALHO, R. C.; OIKONOMOU, G. Control and prevention of lameness associated with claw lesions in dairy cows. Livest. Sci., v. 156, p. 96–105, 2013.

BICALHO, R. C.; MACHADO, V. S.; CAIXETA, L. S. Lameness in dairy cattle: A debilitating disease or a disease of debilitated cattle? A cross-sectional study of lameness prevalence and thickness of the digital cushion. J. Dairy Sci., v. 92, n. 7, p. 3175–84, 2009.

BLOWEY, R.W., DONE, S.H., COOLEY, W. Observations on the pathogenesis of digital dermatitis in cattle. Vet. Rec. v.135, p.115–117, 1994.

BOOGAARD, B. K.; BOCK, B. B.; OOSTING, S. J.; et al. Social Acceptance of Dairy Farming: The Ambivalence Between the Two Faces of Modernity. J. Agric. Envir. Ethics, v. 24, n. 3, p. 259–282, 2011.

BOOTH, C. J.; WARNICK, L. D.; GRÖHN, Y. T.; et al. Effect of lameness on culling in dairy cows. J. Dairy Sci., v. 87, p. 4115–4122, 2004.

BORGES, J.R.J., PITOMBO, C.A., SANTIAGO, S.S., et al. Incidência de afecções podais em bovinos leiteiros submetidos a diferentes sistemas de manejo (Port.). Arq. Esc. Med. Vet. Univ. Fed. Bahia, v.15, p. 34-42, 1992.

BRANDT, S.; APPRICH, V.; HACKL, V.; et al. Prevalence of bovine papillomavirus and Treponema DNA in bovine digital dermatitis lesions. Vet. Microbiol., v. 148, n. 2–4, p. 161–167, 2011.

BRUIJNIS, M. R. N.; BEERDA, B.; HOGEVEEN, H.; et al. Assessing the welfare impact of foot disorders in dairy cattle by a modeling approach. Animal, v. 6, n. 6, p. 962–970, 2012.

BRUIJNIS, M. R. N.; HOGEVEEN, H.; STASSEN, E. N. Assessing economic consequences of foot disorders in dairy cattle using a dynamic stochastic simulation model. J. Dairy Sci., v. 93, n. 6, p. 2419–32, 2010.

BUCH, L. H.; SØRENSEN, A C.; LASSEN, J.; et al. Hygiene-related and feed-related hoof diseases show different patterns of genetic correlations to clinical mastitis and female fertility. J. Dairy Sci., v. 94, n. 3, p. 1540–1551, 2011.

BUROW, E.; THOMSEN, P. T.; ROUSING, T.; et al. Track way distance and cover as risk factors for lameness in Danish dairy cows. Prev. Vet. Med., v. 113, n. 4, p. 625–628, 2014.

CAPION, N., BOYE, M., EKSTRØM, C., et al. A Study into Digital Dermatitis Transmission and Bacterial Associated Pathological Changes Involved in the Disease. Open J. Vet. Med. 3, 192–198, 2013.

CAPION, N.; THAMSBORG, S. M.; ENEVOLDSEN, C. Prevalence and severity of foot lesions in Danish Holstein heifers through first lactation. Vet. J., v. 182, n. 1, p. 50–8, 2009.

CARDOSO, C. S.; HÖTZEL, M. J.; WEARY, D. M.; et al. Imagining the ideal dairy farm. J. Dairy Sci., v. 99, n. 2, p. 1663–1671, 2016.

CASAGRANDE, F.P. *Perfíl metabólico e mineral de vacas no período peri-parto: qualidade e conformação dos cascos*. 2013. 125f. (Tese de Doutorado) – Escola de Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte.

CHA, E.; HERTL, J. A.; BAR, D.; et al. The cost of different types of lameness in dairy cows calculated by dynamic programming. Prev. Vet. Med., v. 97, n. 1, p. 1–8, 2010.

CHESTERTON RN, PFEIFFER DU, MORRIS RS, TANNER CM. Environmental and behavioural factors affecting the prevalence of foot lameness in New Zealand dairy herds – a case-control study. New Zealand Vet. J.37, 135–42, 1989.

CLARKSON, M. J.; DOWNHAM, D. Y.; FAULL, W. B.; HUGHES, J. W.; MANSON, F. J., MERRITT, J. B., MURRAY, R. D., RUSSELL, W. B., SUTHERST, J. E. AND WARD, W. R. Incidence and prevalence of lameness in dairy cattle. Vet. Rec., v. 138, p. 563-567, 1996.

COOK, N. B.; RIEMAN, J.; GOMEZ, A; et al. Observations on the design and use of footbaths for the control of infectious hoof disease in dairy cattle. Vet. J., v. 193, n. 3, p. 669–73, 2012.

COOK, N. B.; NORDLUND, K. V. The influence of the environment on dairy cow behavior, claw health and herd lameness dynamics. Vet. J., v. 179, n. 3, p. 360–369, 2009.

COOK, N. B.; NORDLUND, K. V; OETZEL, G. R. Environmental Influences on Claw Horn Lesions Associated with Laminitis and Subacute Ruminal Acidosis in Dairy Cows . J. Dairy Sci., v. 87, n. June 2003, p. 36–46, 2004.

CRAMER, G.; LISSEMORE, K. D.; GUARD, C. L.; et al. Herd- and cow-level prevalence of foot lesions in Ontario dairy cattle. J. Dairy Sci., v. 91, n. 10, p. 3888–3895, 2008.

CRAMER, G.; LISSEMORE, K. D.; GUARD, C. L.; et al. The association between foot lesions and culling risk in Ontario Holstein cows. J. Dairy Sci., v. 92, n. 6, p. 2572–2579, 2009a.

CRAMER, G.; LISSEMORE, K. D.; GUARD, C. L.; et al. Herd-level risk factors for seven different foot lesions in Ontario Holstein cattle housed in tie stalls or free stalls. J. Dairy Sci., v. 92, n. 4, p. 1404–11, 2009b.

CRUZ, C.E., PESCADOR, C.A., NAKAJIMA, Y., DRIEMEIER, D., 2005. Immunopathological investigations on bovine digital epidermitis. Vet. Rec. 157, 834–840. doi:10.1136/vr.157.26.834

DANSCHER, A. M.; ENEMARK, H. L.; ANDERSEN, P. H.; AALBÆK, B.; NIELSEN, O. L., Polysynovitis after oligofructose overload in dairy cattle. J. Comp. Pathol., v. 142, p. 129–138, 2010.

DEWES, H.F. Some aspects of lameness in dairy herds. New Zealand Vet. J., v. 26, n. 6, p. 147-157, 1978.

DÖPFER, D.; KOOPMANS, A; MEIJER, F. A; et al. Histological and bacteriological evaluation of digital dermatitis in cattle, with special reference to spirochaetes and Campylobacter faecalis. Vet. Record, v. 140, n. 24, p. 620–623, 1997.

EDDY, R. G.; SCOTT, C. P. Some observations on the incidence of lameness in dairy cattle in Somerset. Vet. Rec. v. 106, p. 140-144, 1980.

EDMONSON, A.J., LEAN, I.J., WEAVER, L.D. et al. A body condition scoring chart for Holstein dairy cows. J. Dairy Sci., v. 72, p. 68–78. 1989.

ESSLEMONT, R. J.; SPINCER, I. The incidence and costs of the diseases in dairy herds. DAISY, Dep. Agr. Univ. Reading, UK, Rep., v. 2, p.58, 1993.

ETTEMA, J. F.; ØSTERGAARD, S. Economic decision making on prevention and control of clinical lameness in Danish dairy herds. Livest. Sci., v. 102, p. 92–106, 2006.

EVANS, N. J.; BROWN, J. M.; DEMIRKAN, I.; et al. Three unique groups of spirochetes isolated from digital dermatitis lesions in UK cattle. Vet. Microbiol., v. 130, p. 141–150, 2008.

EVANS, N. J.; MURRAY, R. D.; CARTER, S. D. Bovine digital dermatitis: current concepts from laboratory to farm. The Vet. J., v. 211, p. 3–13, 2016.

EVANS, N. J.; TIMOFTE, D.; ISHERWOOD, D. R.; et al. Host and environmental reservoirs of infection for bovine digital dermatitis Treponemes. Vet. Microbiol., v. 156, n. 1–2, p. 102–109, 2012.

FABIAN, J.; LAVEN, R. A.; WHAY, H. R. The prevalence of lameness on New Zealand dairy farms: A comparison of farmer estimate and locomotion scoring. Vet. J., v. 201, n. 1, p. 31–38, 2014.

FARM ANIMAL WELFARE COUNCIL. FAWC updates the five freedoms. Vet. Record, v. 131, p. 357, 1992.

FELDMEIER, H.; EISELE, M.; VAN MARCK, E.; et al. Investigations on the biology, epidemiology, pathology and control of Tunga penetrans in Brazil: I. Natural history of tungiasis in man. Parasit. Resea., v. 94, n. 4, p. 275–282, 2004.

FERREIRA, P. M.; LEITE, R. C.; CARVALHO, A. U.; et al. Custo e resultados do tratamento de seqüelas de laminite bovina : relato de 112 casos em vacas em lactação no sistema free-stall. Arq. Bras. Med. Vet. Zoo., v. 56, n. 5, p. 589–594, 2004.

FJELDAAS, T.; BØE, K. E.; LARSSEN, R. B. Water footbath , automatic flushing , and disinfection to improve the health of bovine feet. J. Dairy Sci., v. 97, n. 5, p. 2835–2846, 2014.

FLOR, E.; TADICH, N. Claudicaciones en vacas de rebaños lecheros grandes y pequeños del sur de Chile. Arch. Med. Vet., v. 40, n. 2, p. 125–134, 2008.

FODITSCH, C.; OIKONOMOU, G.; MACHADO, V. S.; et al. Lameness Prevalence and Risk Factors in Large Dairy Farms in Upstate New York. Model Development for the Prediction of Claw Horn Disruption Lesions. Plos One, v. 11, n. 1, p. e0146718, 2016.

GALINDO, F.; BROOM, D. M. The Effects of Lameness on Social and Individual Behavior of Dairy Cows The Effects of Lameness on Social and Individual Behavior of Dairy Cows. J. Appl. Anim. Welf. Sci., v. 5, n. 3, p. 193–201, 2010.

GALINDO, F.; BROOM, D. M. The relationships between social behaviour of dairy cows and the occurrence of lameness in three herds. Res. Vet. Sci.**,** v. 69, n. 1, p. 75–9, 2000.

GIBBS, S.J., 2010. Dairy lameness in the South Island. In: Proceedings of the Australasian Dairy Science Symposium, Christchurch, New Zealand, 31st August-2nd September 2010 pp. 424-427.

GOMEZ, A; COOK, N. B.; BERNARDONI, N. D.; et al. An experimental infection model to induce digital dermatitis infection in cattle. J. Dairy Sci., v. 95, n. 4, p. 1821–30, 2012.

GREEN, L. E.; HUXLEY, J. N.; BANKS, C.; et al. Temporal associations between low body condition, lameness and milk yield in a UK dairy herd. Prev. Vet. Med., v. 113, n. 1, p. 63–71, 2014.

GREENOUGH, P. R.; VERMUNT, J. J. Evaluation of subclinical laminitis in a dairy herd and observations on associated nutritional and management factors. Vet. Record, v. 128, p. 11-17, 1991.

GREENOUGH, P.R., WEAVER, A.D., 1997. Lameness in Cattle, Third Edn. W.B. Saunders, London, UK.

GREENOUGH, P.R., 2007. Bovine Laminitis and Lameness, First Ed. Saunders, London, UK.

GREGORY, N.; CRAGGS, L.; HOBSON, N.; et al. Softening of cattle hoof soles and swelling of heel horn by environmental agents. Food Chem. Toxico., v. 44, n. 8, p. 1223–1227, 2006.

HARRIS, D. J.; HIBBURT, C. D.; ANDERSON, G. A.; et al. The incidence, cost and factors associated with foot lameness in dairy cattle in south-western Victoria. Australian Vet. J., v. 65, n. 6, p. 171–176, 1988.

HASKELL, M. J.; RENNIE, L. J.; BOWELL, V. A.; et al. Housing System, Milk Production, and Zero-Grazing Effects on Lameness and Leg Injury in Dairy Cows. J. Dairy Sci., v. 89, n. 11, p. 4259–4266, 2006.

HEDGES, J.; BLOWEY, R.W.; PACKINGTON, A.J.; et al. A longitudinal field trial of the effect of biotin on lameness in dairy cows. J. Dairy Sci., v. 84, p. 1969–1975, 2001.

HEMSWORTH, P. H.; BARNETT, J. L.; BEVERIDGE, L.; et al. The welfare of extensively managed dairy cattle: A review. Appl. Ani. Behav. Sci., v. 42, n. 3, p. 161–182, 1995.

HEMSWORTH, P. Stockmanship and animal welfare, with reference to lamenessProceedings of the 16th International Symposium & 8th Conference on Lameness in Ruminants. Anais...2011

HERNANDEZ, J.; SHEARER, J. K.; WEBB, D. W. Effect of papillomatous digital dermatitis and other lameness disorders on reproductive performance in a Florida herd. In: Proceedings of 11th International Symposium on Disorders of the Ruminant Digit, Parma: Itália, p. 353–357, 2002.

HOBLET, K.H.; WEISS, W. Metabolic hoof horn disease. Claw horn disruption. Vet. Clin. North Am. Food. Anim. Pract., v. 17, p. 111-127, 2001.

HOLZHAUER, M.; BARTELS, C. J. M.; DÖPFER, D.; et al. Clinical course of digital dermatitis lesions in an endemically infected herd without preventive herd strategies. Vet. J.(London, England : 1997), v. 177, n.2, p. 222–30, 2008.

HOLZHAUER, M.; BRUMMELMAN, B.; FRANKENA, K.; et al. A longitudinal study into the effect of grazing on claw disorders in female calves and young dairy cows. Vet. J., v. 193, n. 3, p. 633–8, 2012.

HOLZHAUER, M.; HARDENBERG, C.; BARTELS, C. J. M.; et al. Herd- and Cow-Level Prevalence of Digital Dermatitis in The Netherlands and Associated Risk Factors. J. Dairy Sci., v. 89, n. 2, p. 580–588, 2006.

IBGE, INSTITUTO BRASILEIRO DE GEOPROCESSAMENTO E ESTATÍSTICA 2012. Produção da Pecuária Municipal. http://www.cidades.ibge.gov.br/xtras/temas.php?lang=&codmun=315280&idtema=121&search=minas-gerais|prata|pecuaria-2012. Accessed 31st August of 2014.

IBGE, Instituto Brasileiro de Geoprocessamento e Estatística 2014. Perfil de cidades brasileiras http://www.cidades.ibge.gov.br/xtras/perfil.php?lang=&codmun=315280&search=||infogr%E1ficos:-informa%E7%F5es-completas. Accessed 31st August of 2014.

ICAR (International Committee for Animal Recording) Claw Health Atlas. Rome, Italy, p.45, 2015.

ITO, K.; KEYSERLINGK, M. A. G. VON; LEBLANC, S. J.; et al. Lying behavior as an indicator of lameness in dairy cows. J. Dairy Sci., v. 93, n. 8, p. 3553–60, 2010.

KANEENE, J.B.; HURD, H.S. The National Animal Health Monitoring System in Michigan. I. Design, data and frequencies of selected dairy cattle diseases. Prev. Vet. Med., v. 8, p. 103-114, 1990.

KARLSSON, F.; KLITGAARD, K.; JENSEN, T. K. Identification of Treponema pedis as the predominant Treponema species in porcine skin ulcers by fluorescence in situ hybridization and high-throughput sequencing. Vet. Microbiol., v. 171, p. 122–131, 2014.

KLITGAARD, K.; BOYE, M.; CAPION, N.; et al. Evidence of multiple Treponema phylotypes involved in bovine digital dermatitis as shown by 16S rRNA gene analysis and fluorescence in situ hybridization. J. Clin. Microbiol., v. 46, n. 9, p. 3012–3020, 2008.

KLITGAARD, K.; BRETÓ, A. F.; BOYE, M.; et al. Targeting the treponemal microbiome of digital dermatitis infections by high-resolution phylogenetic analyses and comparison with fluorescent in situ hybridization. J. Clin. Microbiol., v. 51, n. 7, p. 2212–2219, 2013.

KLITGAARD, K.; NIELSEN, M. W.; INGERSLEV, H. C.; et al. Discovery of bovine digital dermatitis-associated Treponema spp. in the dairy herd environment by a targeted deep-sequencing approach. Appl. Environ. Microbiol., v. 80, p. 4427–4432, 2014.

KNAPPE-POINDECKER, M.; JØRGENSEN, H. J.; JENSEN, T. K.; et al. Experimental infection of cattle with ovine Dichelobacter nodosus isolates Maren. Acta Vet. Scand., v. 57, n. 55, 2015.

KOSSAIBATI, M. A; ESSLEMONT, R. J. The costs of production diseases in dairy herds in England. Vet. J., v. 154, n. 2, p. 41–51, 1997.

KROHN, C. C.; MUNKSGAARD, L. Behavior of Dairy-Cows Kept in Extensive (Loose Housing Pasture) or Intensive (Tie Stall) Environments .2. Lying and Lying-Down Behavior. Appl. Anim. Behav. Sci., v. 37, n. 1, p. 1–16, 1993.

KRULL, A. C.; SHEARER, J. K.; GORDEN, P. J.; et al. Deep Sequencing Analysis Reveals the Temporal Microbiota Changes Associated with the Development of Bovine Digital Dermatitis. Infec. Immu., v. 82, n. 8, p. 3359–3373, 2014.

KRULL, A. C.; SHEARER, J. K.; GORDEN, P. J.; et al. Digital dermatitis: Natural lesion progression and regression in Holstein dairy cattle over 3 years. J. Dairy Sci., n. 1997, p. 1–14, 2016.

LAVEN, R. A; LOGUE, D. N. Treatment strategies for digital dermatitis for the UK. Vet. J., v. 171, n. 1, p. 79–88, 2006.

LEACH, K. A; WHAY, H. R.; MAGGS, C. M.; et al. Working towards a reduction in cattle lameness: 2. Understanding dairy farmers' motivations. Research in Veterinary Science, v. 89, n. 2, p. 318–23, 2010.

LEAN, I. J.; WESTWOOD, C. T.; GOLDER, H. M.; et al. Impact of nutrition on lameness and claw health in cattle. Livest. Sci., v. 156, n. 1–3, p. 71–87, 2013.

LEECH, F. B.; DAVIES, M. E.; MACRAE, W. D.; WITHERS, F. W. Disease, Wastage and Husbandry in the British Dairy Herd. London, Her Majesty's Stationery Office, 1960.

LINARDI, P. M.; CALHEIROS, C. M. L.; CAMPELO-JUNIOR, E. B.; et al. Occurrence of the off-host life stages of Tunga penetrans (Siphonaptera) in various environments in Brazil. Annals of tropical medicine and parasitology, v. 104, n. 4, p. 337–345, 2010.

LINARDI, P. M.; DE AVELAR, D. M.; FACURY FILHO, E. J. Establishment of Tunga trimamillata (Siphonaptera: Tungidae) in Brazil. Parasit. Res., v. 112, n. 9, p. 3239–3242, 2013.

LISCHER, C. J.; OSSENT, P. PATHOGENESIS OF SOLE LESIONS ATTRIBUTED TO LAMINITIS IN CATTLEProceedings of the 12th International Symposium on Lameness in Ruminats. Anais...2002

LOGUE, D. N.; OFFER, J. E.; LAVEN, R. A; et al. Digital dermatitis--the aetiological soup. Vet. J.(London, England : 1997), v. 170, n. 1, p. 12–3, 2005.

MACHADO, V. S.; CAIXETA, L. S.; MCART, J. A A; et al. The effect of claw horn disruption lesions and body condition score at dry-off on survivability, reproductive performance, and milk production in the subsequent lactation. J. Dairy Sci., v. 93, n. 9, p. 4071–4078, 2010.

MAIN, D. C. J.; LEACH, K. A; BARKER, Z. E.; et al. Evaluating an intervention to reduce lameness in dairy cattle. J. Dairy Sci., v. 95, n. 6, p. 2946–54, 2012.

MANSKE, T.; HULTGREN, J.; BERGSTEN, C. Prevalence and interrelationships of hoof lesions and lameness in Swedish dairy cows. Prev. Vet. Med., v. 54, n. 3, p. 247–263, 2002.

MARCATILI, P.; NIELSEN, M. W.; SICHERITZ-PONTÉN, T.; et al. A novel approach to probe host-pathogen interactions of bovine digital dermatitis, a model of a complex polymicrobial infection. BMC Genomics, v. 17, n. 1, p. 987, 2016.

MARCH, S., BRINKMANN, J., WINCKLER, C. Reducing lameness in organic dairy herds by implementation of farm-individual intervention measures. 15th International Symposium on Lameness in Ruminants, 09.-13.06.2008, Kuopio, Finland, 47-48, 2008.

MARIN, R. E.; HOUSTON, R.; OMANSKA-KLUSEK, A.; et al. Pathology and diagnosis of proliferative and ulcerative dermatitis associated with Tunga penetrans infestation in cattle. J. Vet. Diag. Invest., v. 27, n. 1, p. 80–85, 2015.

MAUCHLE, U. Afecções podais e conformação dos cascos em bovinos. 2001. 80 f. Dissertação (Mestrado) – Escola de Veterinária da Universidade Federal de Minas Gerais, Belo Horizonte.

MCLENNAN, M.W. The incidence of lameness requiring veterinary treatment in Queensland. Aust. Vet. J., v. 65, n. 5, p. 144-147, 1988.

MOLINA, L. R.; CARVALHO, A. U.; FACURY FILHO, E. J.; FERREIRA, P.M.; FERREIRA, V.C.P. Prevalência e classificação das afecções podais em vacas lactantes na bacia leiteira de Belo Horizonte. Arq. Bras. Med. Vet. Zootec., v. 51. n. 2. p. 149-152, 1999.

MOREIRA, T.F., MOLINA, L.R., CARVALHO, et al. 2012. Prevalence of hoof lesions in dairy cows in semi-intensive system in Brazil. In: Proceedings of the XXVII World Buiatrics Congress, Lisbon, Portugal, 3rd – 8th June 2012 pp. 265.

MUEHLEN, M.; HEUKELBACH, J.; WILCKE, T.; et al. Investigations on the biology, epidemiology, pathology and control of Tunga penetrans in Brazil: II. Prevalence, parasite load and topographic distribution of lesions in the population of a traditional fishing village. Parasit. Res., v. 90, n. 6, p. 449–455, 2003.

MÜLLING, C. K. W.; GREENOUGH, P. R. Applied physiopathology of the foot. In: Proceedings of the XXIV World Buiatrics Congress, Nice, France. Anais...2006

MURRAY, R. D.; DOWNHAM, D. Y.; CLARKSON, M. J.; et al. Epidemiology of lameness in dairy cattle: description and analysis of foot lesions. Vet Rec., v. 138, n. 1978, p. 586–591, 1996.

NASCIMENTO, L. V.; MAUERWERK, M. T.; DOS SANTOS, C. L.; et al. Treponemes detected in digital dermatitis lesions in Brazilian dairy cattle and possible host reservoirs of infection. J. Clin. Mic., v. 53, n. 6, p. 1935–1937, 2015.

NIELSEN, B. H.; THOMSEN, P. T.; GREEN, L. E.; et al. A study of the dynamics of digital dermatitis in 742 lactating dairy cows. Prev. Vet. Med., v. 104, n. 1–2, p. 44–52, 2012.

NIELSEN, C.; STENGÄRDE, L.; BERGSTEN, C.; et al. Relationship between herd-level incidence rate of energy-related postpartum diseases, general risk factors and claw lesions in individual dairy cows recorded at maintenance claw trimming. Acta Vet. Scandinavica, v. 55, p. 55, 2013.

NIELSEN, M. W.; STRUBE, M. L.; ISBRAND, A.; et al. Potential bacterial core species associated with digital dermatitis in cattle herds identified by molecular profiling of interdigital skin samples. Vet. Microbiol., v. 186, p. 139–149, 2016.

NOCEK, J. E. Bovine acidosis: implications on laminitis. J. Dairy Sci., v. 80, n. 5, p. 1005–1028, 1997.

O'CALLAGHAN, K. A.; CRIPPS, P. J.; DOWNHAM, D. Y.; et al. Subjective and objective assessment of pain and discomfort due to lameness in dairy cattle. Ani. Wel., v. 12, p. 605–610, 2003.

OIKONOMOU, G.; TROJACANEC, P.; GANDA, E. K.; et al. Association of digital cushion thickness with sole temperature measured with the use of infrared thermography. J. Dairy Sci., v. 97, n. 7, p. 4208–4215, 2014.

OLMOS, G.; BOYLE, L.; HANLON, A.; et al. Hoof disorders, locomotion ability and lying times of cubicle-housed compared to pasture-based dairy cows. Livest. Sci., v. 125, n. 2–3, p. 199–207, 2009a.

OLMOS, G.; BOYLE, L.; HORAN, B.; et al. Effect of genetic group and feed system on locomotion score, clinical lameness and hoof disorders of pasture-based Holstein–Friesian cows. Animal, v. 3, n. 1, p. 96, 2009b.

OSSENT, P.; GREENOUGH, P. R.; VERMUNT, J. J. LAMINITIS. In: Greenough, P.R., Weaver, A.D. (Eds.), Lameness in Cattle, WB Saunders Company, Philadelphia, p. 277–292, 1997.

PALMER, M.; O'CONNELL, N. Digital Dermatitis in Dairy Cows: A Review of Risk Factors and Potential Sources of Between-Animal Variation in Susceptibility. Animals, v. 5, n. 3, p. 512–535, 2015.

POTTERTON, S.L.; BELL, N.J.; WHAY, H.R.; BERRY, E.A.; ATKINSON, O.C.D.; DEAN, R.S.; MAIN, D.C.J.; HUXLEY, J.N. A descriptive review of the peer and non-peer reviewed literature on the treatment and prevention of foot lameness in cattle published between 2000 and 2011. Vet. Journal, v.193, p. 612–616, 2012.

PRENTICE, D. E.; NEAL, P. A. Some observations on the incidence of lameness in dairy cattle in west Cheshire. Vet. Rec., v. 91, p. 1-7, 1972.

RÄBER, M.; LISCHER, C. J.; GEYER, H.; et al. The bovine digital cushion - A descriptive anatomical study. Veterinary Journal, v. 167, p. 258–264, 2004.

RABOISSON, D.; MOUNIÉ, M.; MAIGNÉ, E. Diseases, reproductive performance, and changes in milk production associated with subclinical ketosis in dairy cows: a meta-analysis and review. J. Dairy Sci., v. 97, n. 12, p. 7547–63, 2014.

RASMUSSEN, M.; CAPION, N.; KLITGAARD, K.; et al. Bovine digital dermatitis: Possible pathogenic consortium consisting of Dichelobacter nodosus and multiple Treponema species. Vet. Microbiol., v. 160, n. 1–2, p. 151–161, 2012.

REFAAI, W.; VAN AERT, M.; ABD EL-AAL, A. M.; et al. Infectious diseases causing lameness in cattle with a main emphasis on digital dermatitis (Mortellaro disease). Livest. Sci., v. 156, n. 1–3, p. 53–63, 2013.

RELUN, A.; LEHEBEL, A.; BAREILLE, N.; et al. Effectiveness of different regimens of a collective topical treatment using a solution of copper and zinc chelates in the cure of digital dermatitis in dairy farms under field conditions. J. Dairy Sci., v. 95, n. 7, p. 3722–35, 2012.

RELUN, A.; LEHEBEL, A.; BRUGGINK, M.; et al. Estimation of the relative impact of treatment and herd management practices on prevention of digital dermatitis in French dairy herds. Prev. Vet. Med., v. 110, n. 3–4, p. 558–562, 2013.

RIBEIRO, J. C. V. C.; COELHO, S. C.; RUAS, J. R. M.; et al. Infestação de Tunga penetrans siphonaptera: Tungidae em cascos de vacas leiteiras F1 Holandês-Zebu. Arq. Bras. Med. Vet. Zoot., v. 59, n. 2, p. 520–522, 2007.

RODRIGUEZ-LAINZ, A., MELENDEZ-RETAMAL, P., HIRD, D.W., et al. Farm- and host-level risk factors for papillomatous digital dermatitis in Chilean dairy cattle. Prev. Vet. Med., v. 42, p. 87-97, 1999.

RODRÍGUEZ-LAINZ, A.; HIRD, D. W.; CARPENTER, T. E.; et al. Case-control study of papillomatous digital dermatitis in Southern California dairy farms. Prev. Vet. Med., v. 28, n. 96, p. 117–131, 1996.

ROMANI, A. F.; SILVA, L. A. F.; FIORAVANTI, M. C. S.; et al. Ocorrência de lesões podais em fêmeas bovinas leiteiras no estado de goiás. ARS VETERINARIA, v. 20, p. 322–329, 2004.

RUSSELL, A. M.; ROWLANDS, G. J.; SHAW, S. R. et al. Survey of lameness in British dairy cattle. Vet. Rec., v. 111, p. 155-160, 1982.

SANT'ANNA, A C.; PARANHOS DA COSTA, M. J. R. The relationship between dairy cow hygiene and somatic cell count in milk. J. Dairy Sci., v. 94, n. 8, p. 3835–44, 2011.

SANTOS, T. M. A.; PEREIRA, R. V.; CAIXETA, L. S.; et al. Microbial diversity in bovine Papillomatous digital dermatitis in Holstein dairy cows from upstate New York. FEMS Microb. Eco., v. 79, n. 2, p. 518–529, 2012.

SCHLAFER, S.; NORDHOFF, M.; WYSS, C.; et al. Involvement of Guggenheimella bovis in digital dermatitis lesions of dairy cows. Vet. Microbiol., v. 128, n. 1–2, p. 118–125, 2008.

SCHMID, T.; WEISHAUPT, M. A.; MEYER, S. W.; et al. High-speed cinematographic evaluation of claw-ground contact pattern of lactating cows. Vet. J., v. 181, n. 2, p. 151–157, 2009.

SHEARER, J. K.; VAN AMSTEL, S. R.; BRODERSEN, B. W. Clinical diagnosis of foot and leg lameness in cattle. The Veterinary clinics of North America. Food animal practice, v. 28, n. 3, p. 535–56, 2012.

SHEARER, J. K.; VAN AMSTEL, S. R.; MELENDEZ, P. *Manual para el Programa de Recortador de Pezuñas*, Cojeras em el Ganado Lechero, marzo 4-6, 1999.

SILVEIRA, J. A. S.; ALBERNAZ, T. T.; OLIVEIRA, C. M. C.; et al. Afecções podais em vacas da bacia leiteira de Rondon do Pará. Pesq. Vet. Bras., v. 29, n. 11, p. 905–909, 2009.

SOGSTAD, A M.; FJELDAAS, T.; OSTERÅS, O. Lameness and claw lesions of the Norwegian red dairy cattle housed in free stalls in relation to environment, parity and stage of lactation. Acta Veterinaria Scandinavica, v. 46, n. 4, p. 203–17, 2005.

SOLANO, L.; BARKEMA, H. W.; MASON, S.; et al. Prevalence and distribution of foot lesions in dairy cattle in Alberta, Canada. J. Dairy Sci., v. 99, n. 8, p. 6828–6841, 2016.

SOLANO, L.; BARKEMA, H.; PAJOR, E.; et al. Prevalence of lameness and associated risk factors in Canadian Holstein-Friesian cows housed in freestall barns. J. Dairy Sci., v. 98, n. 10, p. 6978–6991, 2015.

SOMERS, J. G. C. J.; FRANKENA, K.; NOORDHUIZEN-STASSEN, E. N.; et al. Prevalence of claw disorders in Dutch dairy cows exposed to several floor systems. J. Dairy Sci., v. 86, n. 6, p. 2082–93, 2003.

SOMERS, J. G. C. J.; FRANKENA, K.; NOORDHUIZEN-STASSEN, E. N.; et al. Risk factors for interdigital dermatitis and heel erosion in dairy cows kept in cubicle houses in the Netherlands. Prev. Vet. Med., v. 71, n. 1–2, p. 23–34, 2005a.

SOMERS, J. G. C. J.; FRANKENA, K.; NOORDHUIZEN-STASSEN, E. N.; et al. Risk factors for digital dermatitis in dairy cows kept in cubicle houses in The Netherlands. Prev. Vet. Med., v. 71, n. 1–2, p. 11–21, 30 set. 2005b.

SOMERS, J. R.; HUXLEY, J.; LORENZ, I.; et al. The effect of Lameness before and during the breeding season on fertility in 10 pasture-based Irish dairy herds. Irish Vet. J., v. 68, n. 1, p. 14, 2015.

SOUZA, R. C. *Perfil epidemiológico e clínico das afecções podais em vacas nas bacias leiteiras de Belo Horizonte e Pedro Leopoldo.* 57f. Dissertação (Mestrado) – Escola de Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte, 2002.

SOUZA, R. C.; FERREIRA, P. M.; MOLINA, L. R.; et al. Perdas econômicas ocasionadas pelas enfermidades podais em vacas leiteiras confinadas em sistema. Arq. Bras. Med. Vet. Zootec, v. 58, n. 6, p. 982–987, 2006.

SPEIJERS, M. H. M.; FINNEY, G. A; MCBRIDE, J.; et al. Effectiveness of different footbathing frequencies using copper sulfate in the control of digital dermatitis in dairy cows. J. Dairy Sci., v. 95, n. 6, p. 2955–64, jun. 2012.

SPEIJERS, M. H. M.; LOGUE, D. N.; NIAMH, E.; et al. Treatment Strategies for Digital Dermatitis f or t he UK The Digital Dermatitis Problem in the UK. WCDS Adv. Dairy Tech., v. 25, p. 283–294, 2013.

STAFFORD, K.; GREGORY, N. Implications of intensification of pastoral animal production on animal welfare. New Zealand Vet. J., v. 56, n. 6, p. 274–280, 2008.

SULLIVAN, L. E.; CLEGG, S. R.; ANGELL, J. W.; et al. High-level association of bovine digital dermatitis treponema spp. with contagious ovine digital dermatitis lesions and presence of fusobacterium necrophorum and dichelobacter nodosus. Journal of Clinical Microbiology, v. 53, n. 5, p. 1628–1638, 2015.

SWEENEY M, WATTS J, PORTIS E, et al. Identification of *Porphyromonas levii* isolated from clinical cases of bovine interdigital necrobacillosis by 16S rRNA sequencing. Vet. Ther., v. 10, n. 4, p. E1-10, 2009

TADICH, N., FLOR, E., GREEN, L. Associations between hoof lesions and locomotion score in 1098 unsound dairy cows. Vet. J., v. 184, p. 60-65, 2010.

TARLTON, J. F.; HOLAH, D. E.; EVANS, K. M.; et al. Biomechanical and histopathological changes in the support structures of bovine hooves around the time of first calving. Vet. J., v. 163, p. 196–204, 2002.

THOMAS, H. J.; MIGUEL-PACHECO, G. G.; BOLLARD, N. J.; et al. Evaluation of treatments for claw horn lesions in dairy cows in a randomized controlled trial. J. Dairy Sci., v. 98, n. 7, p. 4477–4486, 2015.

THOMSEN, P. T.; MUNKSGAARD, L.; SØRENSEN, J. T. Locomotion scores and lying behaviour are indicators of hoof lesions in dairy cows. Vet. J., v. 193, n. 3, p. 644–647, 2012.

TOMLINSON, D. J.; MÜLLING, C. H.; FAKLER, T. M. Invited review: formation of keratins in the bovine claw: roles of hormones, minerals, and vitamins in functional claw integrity. J. Dairy Sci., v. 87, n. 4, p. 797–809, 2004.

TRANTER, W.; MORRIS, R. A case study of lameness in three dairy herds. New Zealand Vet. J., v. 39, p. 88-96, 1991.

VERMUNT, J. J.; WEST, D. M. Predicting copper status in beef cattle using serum copper concentrations. N. Z. Vet. J., v. 42, p. 194–195, 1994.

VAN NUFFEL, A.; ZWERTVAEGHER, I.; PLUYM, L.; et al. Lameness detection in dairy cows: Part 1. How to distinguish between non-lame and lame cows based on differences in locomotion or behavior. Animals, v. 5, n. 3, p. 838–860, 2015.

VERMUNT, J. J. One step closer to unravelling the pathophysiology of claw horn disruption: For the sake of the cows' welfare. Vet. J., v. 174, p. 219–220, 2007.

VERMUNT, J. J.; GREENOUGH, P. R. Structural characteristics of the bovine claw: Horn growth and wear, horn hardness and claw conformation. British Vet. J., v. 151, p. 157–180, 1995.

VON KEYSERLINGK, M. A G.; BARRIENTOS, A.; ITO, K.; et al. Benchmarking cow comfort on North American freestall dairies: lameness, leg injuries, lying time, facility design, and management for high-producing Holstein dairy cows. J. Dairy Sci., v. 95, n. 12, p. 7399–408, 2012.

WARNICK, L. D.; JANSSEN, D.; GUARD, C. L.; GRÖHN, Y. T. The effect of lameness on milk production in dairy cows. J. Dairy Sci., v. 84, p. 1988–1997, 2001.

WELLS, S. J.; TRENT, A. M.; MARSH, W. E.; et al. Individual cow risk factors for clinical lameness in lactating dairy cows. Prev. Vet. Med., v. 17, p. 95–109, 1993.

WESTIN, R.; VAUGHAN, A.; DE PASSILLÉ, A. M.; et al. Cow and farm-level risk factors for lameness on dairy farms with automated milking systems. J. Dairy Sci., p. 1–12, 2016.

WHAY, H. R.; MAIN, D.C.; GREEN, L.E.; et al. Assessment of the welfare of dairy cattle using animal-based measurements: Direct observations and investigation of farm records. Vet. Rec., v. 153, p. 197–202, 2003.

WHAY, H. R.; WATERMAN, A. E.; HOUSE, L.; et al. The Influence of Lesion Type on the Duration of Hyperalgesia Associated with Hindlimb Lameness in Dairy Cattle. The Vet. J., v. 156, p. 23–29, 1998.

WHAY, H. R.; WATERMAN, A. E.; WEBSTER, A. J. F. Associations Between Locomotion, Claw Lesions and Nociceptive Threshold in Dairy Heifers During the. The Vet. J., v. 154, p. 155–161, 1997.

WHITAKER D., KELLY J., SMITH E.. Incidence of lameness in dairy cows. Vet. Rec., v. 113, p. 60-62, 1983.

WINKLER, B.; MARGERISON, J. K. Mechanical properties of the bovine claw horn during lactation. J. Dairy Sci., v. 95, n. 4, p. 1714–28, 2012.

WYSS, C.; DEWHIRST, F. E.; PASTER, B. J.; et al. Guggenheimella bovis gen. nov., sp. nov., isolated from lesions of bovine dermatitis digitalis. International Journal of Systematic and Evolutionary Microbiology, v. 55, n. 2, p. 667–671, 2005.

YANO, T.; MOE, K. K.; YAMAZAKI, K.; et al. Identification of candidate pathogens of papillomatous digital dermatitis in dairy cattle from quantitative 16S rRNA clonal analysis. Vet. Microbiol., v. 143, n. 2–4, p. 352–362, 2010.

YANO, T.; YAMAGAMI, R.; MISUMI, K.; et al. Genetic heterogeneity among strains of Treponema phagedenis-like spirochetes isolated from dairy cattle with papillomatous digital dermatitis in Japan. Journal of Clinical Microbiology, v. 47, n. 3, p. 727–733, 2009.

ZINICOLA, M.; LIMA, F.; LIMA, S.; et al. Altered microbiomes in bovine digital dermatitis lesions, and the gut as a pathogen reservoir. PLoS ONE, v. 10, n. 3, p. 1–23, 2015.

Supplementary Material

Table S1. Names and sequences of 16S rRNA-targeting oligonucleotide probes used in this study.

Probe name (no.)	Sequence	Target	Reference or source
S-S-Trep-Dig3-432	5'-CAT CCC AGT ATC ATT CCC-3'	Uncultured Treponema PT1	Klitgaard et al. (2008)
S-S-Trep-T16-432	5'-CAT CTC ACA GGC ATT CCC-3'	T. denticula	Klitgaard et al. (2008)
S-S-Trep-I:B:C7-432	5'-CAT CAG ATG AGC ATT CCC-3'	T.medium	Klitgaard et al. (2008)
S-S-TrepGenus-725	5'-CAG AAA CYC GCC TTC GCC-3'	Treponema	Klitgaard et al. (2008)
S-S-F.necro-183-Cy3 (	5'-GAT TCC TCC ATG CGA AAA-3'	Fusobacterium necrophorum	Boye et al. (2006)
Eub-338	5'-GCT GCC TCC CGT AGG AGT'-3	Bacteria	(Amann et al., 1990)
S-D.nodosus-443	5'-CAT GCA CCG TTC TTC ACT'-3	*D. nodosus*	(Rasmussen et al., 2012)
P. levii-443	5'-TACCTACGTTTACTCGCC-3'	*P. levii*	Nielsen (2016)
PT12-216	5'-CG AGC CCA TCT TTA GGC GAA G-3'	Uncultured Treponema PN20	(Rasmussen et al., 2012)
PT13	5'-GT AGC TCC TTT CCC TTC ACC TTA A- 3'	Uncultured Treponema PT13	(Rasmussen et al., 2012)
T. pedis	5'-AG AGT CCT CAA CCT TTA CGT GTT-3'	T. pedis	(Rasmussen et al., 2012)
T. refringens	5'-GC TCC CTT TCC TTA CAT GAT-3'	T. refringens	(Rasmussen et al., 2012)
S-S-Trep-Dig4-432	5'-CAT CTC AGT GTC ATT CCC-3'	Uncultured Treponema PT3	Klitgaard et al. (2008)
Probe/primer name (no.)	**Sequence**	**Target**	**Reference or source**
V1-V2 forward	AGAGTTTGATCCTGGCTCAG	Bacteria	(Wilmotte et al., 1993)
V1-V2 reverse	CTGCTGCCTYCCGTA	Bacteria	(Wilmotte et al., 1993)
V3-V4 forward	GGGAGGCAGCAGCTAAGAA	Treponema spp.	(Klitgaard et al., 2008)
V3-V4 reverse	ATCTACAGATTCCACCCCTA	Treponema spp.	(Klitgaard et al., 2008)

Reference List

AMANN, R.I.; KRUMHOLZ, L.; STAHL, D.A. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J. Bacteriol*., v. 172, p. 762-770, 1990.

BOYE, M.; AALBAEK, B.; AGERHOLM, J.S. *Fusobacterium necrophorum* determined as abortifacient in sheep by laser capture microdissection and fluorescence in situ hybridization. *Mol. Cell Probes*, v. 20, p. 330-336, 2006.

KLITGAARD, K.; BOYE, M.; CAPION, N. et al. Evidence of multiple *Treponema* phylotypes involved in bovine digital dermatitis as shown by 16S rDNA analysis and fluorescent in situ hybridisation. *J. Clin. Microbiol.*, v. 46, p. 3012-3020, 2008.

RASMUSSEN, M.; CAPION, N.; KLITGAARD, K. et al. Bovine digital dermatitis: possible pathogenic consortium consisting of *Dichelobacter nodosus* and multiple *Treponema* species. *Vet. Microbiol*., v. 160, p. 151-161, 2012.

WILMOTTE, A.; VAN DER, A.G.; DE, W.R. Structure of the 16 S ribosomal RNA of the thermophilic cyanobacterium Chlorogloeopsis HTF ('Mastigocladus laminosus HTF') strain PCC7518, and phylogenetic analysis. *FEBS Lett*., v. 317, p. 96-100, 1993.

NIELSEN, M.W.; STRUBE, M.L.; ISBRAND, A. et al. Potential bacterial core species associated with digital dermatitis in cattle herds identified by molecular profiling of interdigital skin samples. *Vet. Microbiol.*, v. 186, p. 139–149, 2016.