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Ovine footrot: new insights into bacterial colonisation

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Abstract:	Ovine footrot is characterised by interdigital dermatitis (ID) and by the separation of the skin and hoof horn (underrunning footrot). Dichelobacter nodosus is the essential pathogen causing footrot; the role of other microorganisms in this disease remains unclear. The aims of this study were: (i) to investigate the colonisation of D. nodosus, Fusobacterium necrophorum and Treponema spp. in biopsies from the ovine interdigital skin of healthy, ID and footrot affected feet and (ii) to characterize the virulence of D. nodosus strains in those biopsies. Post-slaughter biopsy samples (n=241) were collected and analysed by real-time PCR to determine prevalence and load of the different bacterial species. The highest prevalence and load of D. nodosus were found on feet with ID. The vast majority of samples contained virulent D. nodosus and some samples contained both virulent and benign D. nodosus. Notably, the more pathogenic subspecies of F. necrophorum was found in samples from UK sheep. Our findings provide further insights into the role bacterial colonisation may play in the early stage of ID and in the progression towards footrot.

Ovine footrot: new insights into bacterial colonisation

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ABSTRACT

Ovine footrot is characterised by interdigital dermatitis (ID) and by the separation of the skin and hoof horn (underrunning footrot). *Dichelobacter nodosus* is the essential pathogen causing footrot; the role of other microorganisms in this disease remains unclear. The aims of this study were: (i) to investigate the colonisation of *D. nodosus, Fusobacterium necrophorum* and *Treponema* spp. in biopsies from the ovine interdigital skin of healthy, ID and footrot affected feet and (ii) to characterize the virulence of *D. nodosus* strains in those biopsies. Post-slaughter biopsy samples (n=241) were collected and analysed by real-time PCR to determine prevalence and load of the different bacterial species. The highest prevalence and load of *D. nodosus* were found on feet with ID. The vast majority of samples contained virulent *D. nodosus* and some samples contained both virulent and benign *D. nodosus*. Notably, the more pathogenic subspecies of *F. necrophorum* was found in samples from UK sheep. Our findings provide further insights into the role bacterial colonisation may play in the early stage of ID and in the progression towards footrot.

INTRODUCTION

Ovine footrot is a major cause of lameness affecting sheep welfare worldwide (Goddard and others 2006), it is characterized by two different clinical presentations, interdigital dermatitis (ID) and underrunning footrot. ID is an initial inflammation of the interdigital skin where the superficial epidermal layers are inflamed, damaged and slough off irregularly and it may develop into underrunning footrot, which is characterized by the separation of the hoof horn from the sensitive underlying tissue (Beveridge 1941, Egerton and others 1969). In Australia, mild/benign footrot is also used synonymously for ID and underrunning footrot is called virulent footrot (Raadsma and Dhungyel 2013).

Footrot is a complex disease with *Dichelobacter nodosus,* a Gram negative anaerobic bacterium, as the essential pathogen causing underrunning footrot (Egerton and others 1969, Kennan and others 2001, Han and others 2008, Kennan and others 2010). *D. nodosus* load was found to be already increased in ID prior to the development of underrunning footrot, therefore suggesting that *D. nodosus* load drives the early stages of infection (Witcomb and others 2014, Witcomb and others 2015). Additionally, the occurrence of this disease is associated with different factors such as the virulence of *D. nodosus* strains (Kennan and others 2010), farm management (Green and others 2007), environmental conditions (Wassink and others 2005, Muzafar and others 2016) and initial damage in the epithelium of the interdigital skin (Beveridge 1941, Egerton and others 1969).

Whole genome sequencing demonstrated that *D. nodosus* has a global conserved bimodal population, correlating with virulent and benign phenotypes (Kennan and others 2014). A large number of virulent *D. nodosus* strains were identified in Australia (Kennan and others 2014), while in Scandinavian countries, such as Sweden, mainly benign strains have been found (Frosth and others 2015). In UK flocks, virulent *D. nodosus* was more prevalent than benign in swabs from sheep with ID and footrot (Moore and others 2005). Virulent and benign *D. nodosus* strains differ in their ability to degrade the extracellular matrix of the host due to enzymatic activity of extracellular proteases (Riffkin and others 1995). The acidic protease AprV2 is responsible for the overall elastase activity of virulent strains and was shown to be essential for the development of footrot, while the acidic protease AprB2 is associated with a benign phenotype (Kennan and others 2010). Importantly, presence of virulent *D. nodosus* strains does not always correlate with severity of clinical presentations since virulent *D. nodosus* has also been identified in sheep without any clinical sign and in ID cases (Stäuble and others 2014, Moore and others 2005).

In addition, *Fusobacterium necrophorum*, *Treponema* spp. and a range of other bacterial genera have been identified in the ovine interdigital skin (Roberts and Egerton 1969, Bennett and others 2009, Calvo-Bado and others 2011, Frosth and others 2015). The role of *F. necrophorum* in this disease still needs to be fully understood, with two hypothesis currently discussed: (1) *F. necrophorum* is important to

establish ID prior to *D. nodosus* infection and hence initiates the disease (Egerton and others 1969), or (2) *F. necrophorum* is involved in the persistence and severity of footrot, once the underrunning lesion has developed, playing a role as an opportunistic, secondary pathogen (Witcomb and others 2014, Witcomb and others 2015). *F. necrophorum* is divided into subspecies *necrophorum* and *funduliforme*, the first is described to be more pathogenic (Tan and others 1996).

Treponema spp. are usually free living spirochetes, but they have been associated with contagious ovine digital dermatitis (CODD) (Sullivan and others 2015) and bovine digital dermatitis (BDD) (Gomez and others 2012). BDD and CODD have polytreponemal aetiology with different *Treponema* species involved in their pathogenesis (Sayers and others 2009, Sullivan and others 2015). Initial identification of spirochetes in ovine footrot lesions was reported by Beveridge (1941. Recent studies identified *Treponema* spp. in a sheep with ID lesions from a flock with footrot history (Calvo-Bado and others 2011) and were found in both flocks and feet, with and without footrot (Frosth and others 2015). Hence it suggests that further investigation to elucidate their role and whether different species of *Treponema* can be identified in ovine footrot is warranted.

Taken together, current data suggest that footrot is a polymicrobial disease and *D. nodosus* and other microorganisms might have a synergistic relationship. However, the role of bacterial diversity and load and how that differ between healthy, ID and footrot feet remains unclear. In this

context, the aims of this study were (i) to investigate the colonisation of *D. nodosus, F. necrophorum, Treponema* spp. and eubacteria, and (ii) to characterize the virulence of *D. nodosus* strains in a cross section of healthy, ID and footrot abattoir biopsies from the ovine interdigital skin.

MATERIAL AND METHODS

Collection of tissue biopsies

This study included 241 ovine interdigital post-slaughter biopsies collected at an abattoir using a convenience sampling approach due to variable availability of the clinical conditions at slaughter. The entire sample set included 79 healthy, 39 mild interdigital dermatitis (slight lesion with ≤5% of the interdigital skin space affected), 26 moderate/severe interdigital dermatitis (interdigital skin lesions with ≥5% of the interdigital space affected), and 97 footrot samples (Table 1). Of these, 40 animals had all four feet sampled, total of 160 samples. During two visits to the abattoir (01/11/2013 and 04/11/2013) it was not possible to follow the same animal on the processing line, therefore 78 feet biopsies were collected without the information if they belonged to the same sheep (Table 1). Since the animals were sampled in the processing line of the abattoir, no information regarding sheep breed or other characteristics were available for this study.

At the abattoir, all feet disease status was scored by two different scorers with one of the scorers present during all visits in order to standardise the sampling and scoring method, for details see Table 1. The

scoring system was adapted from Parsonson and others (1967), allowing classification into healthy, ID or footrot feet according to established scoring criteria: absence of interdigital skin lesion = healthy; slight interdigital skin lesion (≤5% affected) = mild ID; moderate to severe ID lesion (>5% affected); presence of underrunning lesion = footrot.

Tissues were collected as described previously (Davenport and others 2014) and placed into RNALater® (Sigma-Aldrich, Saint Louis, USA) at 4°C prior to long term storage at -20°C.

DNA extraction and real-time PCR assays

For enzymatic digestion, each tissue was cut into small pieces and incubated with 180µl of ATL buffer and 20µl of proteinase K (20mg/ml) (QIAGEN, Hilden, Germany) at 56°C for 3 hours. DNA was isolated using the QIAamp cador® kit according to manufacturer's recommendations and eluted in 50µl AVE buffer (QIAGEN, Hilden, Germany). The final DNA concentration was determined using NanoDrop® (ND-1000, (Thermo Fisher Scientific Inc., Waltham, USA). Bacterial load was quantified using real-time PCR based on *16S rRNA* gene for eubacteria (Strub and others 2007) and *D. nodosus* (Frosth and others 2012) and the intergenic spacer region 2 (ISR2) containing a tRNAIle gene for *Treponema* spp. (Frosth and others 2015). Real-time PCR for *F. n.* subsp. *necrophorum* and *F. n.* subsp. *funduliforme* targeted the *gyrB* gene (Frosth and others 2015). Differentiation between virulent and benign *D. nodosus* was performed based on the presence of *aprV2* (virulent) and *aprB2* (benign) genes

(Frosth and others 2015). *D. nodosus* and eubacteria assays were performed using PCR Lightcycler® 480 (Roche Applied Science, Penzberg, Germany). Virulent and benign *D. nodosus*, *F. necrophorum* and *Treponema* spp. assays were carried out in an Applied Biosystems® 7500 Fast Real-Time PCR System (Thermo Fisher Scientific Inc., Waltham, USA).

Statistical analysis

Fisher's exact test was performed for bacterial prevalence and One-way ANOVA followed by Dunn's multiple comparisons test for bacterial load using GraphPad Prism® (Version 6.0, La Jolla, USA). Confidence intervals of the prevalence data were calculated using Graphpad Software (http://graphpad.com/quickcalcs/confInterval2/). A P-value ≤0.05 was considered significant.

RESULTS

Prevalence and load of *D. nodosus*, *F. necrophorum* and *Treponema* spp. in tissues from the ovine interdigital skin

The prevalence of *D. nodosus*, *F. necrophorum*, *Treponema* spp. and eubacteria was investigated in the ovine interdigital skin biopsies. All samples were positive for eubacteria (100% of prevalence). Both total *D. nodosus* and virulent *D. nodosus* were significantly more prevalent in mild ID ($P<0.05$ and $P<0.01$, respectively), moderate/severe ID ($P<0.001$ and $P<0.0001$, respectively) and footrot ($P<0.05$ and $P<0.01$,

respectively) in comparison with healthy feet, with highest prevalence in moderate/severe ID samples. Moreover, total *D. nodosus* and virulent *D. nodosus* were significantly more prevalent in moderate to severe ID than in footrot samples (Fig 1a, see online supplementary appendix 1). In contrast, benign *D. nodosus* was only detected in 7% (17/241) of the samples (Fig 1b). Mixed populations of benign and virulent *D. nodosus* strains were found in a small number of samples across all clinical conditions (Fig 1b).

F. necrophorum was detected in 15% (36/241) of the samples, with 14.5% (35/241) positive for *F. necrophorum* subsp. *necrophorum*, only two samples positive for *F. necrophorum* subsp. *funduliforme* and one sample positive for both subspecies. *F. necrophorum* was significantly more prevalent in footrot than in healthy feet ($P<0.05$) (Fig 1c). Presence of both *D. nodosus* and *F. necrophorum* in the same tissue sample or virulent *D. nodosus* and *F. necrophorum* in the same tissue was significantly higher in footrot compared to healthy feet ($P<0.01$ and $P<0.01$, respectively). *Treponema* spp. prevalence was very low (8%, 20/241) and similar across all clinical conditions (Fig 1d) (see online supplementary appendix 1).

Similar proportion of eubacterial DNA was detected at around 0.06%±0.020 (mean ± standard error of the mean) of total DNA for all samples, with 0.07%±0.041 for healthy samples, 0.028%±0.007 mild ID, 0.027±0.011 for moderate/severe ID and 0.073±0.039 for footrot samples. *D. nodosus* load was significantly higher in moderate/severe ID and footrot in comparison to healthy feet ($P<0.0001$ for both) (Fig 2a).

Virulent *D. nodosus* load was significantly increased in mild ID, moderate/severe ID and footrot compared with a healthy feet (P=0.001, P<0.0001 and P<0.0001, respectively), with highest load in moderate/severe ID (Fig 2b). *F. necrophorum* load was significantly increased in footrot but not in ID samples (P=0.022) (Fig 2c). The highest *Treponema* spp. load was found in footrot followed by healthy feet (Fig 2d).

In summary, while eubacterial load were similar in all feet, both prevalence and load of total and virulent *D. nodosus* were highest in moderate to severe ID, while *F. necrophorum* were highest in footrot samples.

DISCUSSION

In this study we provided further insights into the bacterial colonisation present in healthy, ID and footrot ovine feet. We found similar patterns regarding the prevalence and load of *D. nodosus* and *F. necrophorum* in post slaughter biopsies from the interdigital space as previous studies in UK sheep flocks using swabs and biopsies (Moore and others 2005, Calvo-Bado and others 2011, Witcomb and others 2014, Witcomb and others 2015).

As expected, the highest *D. nodosus* prevalence and load found in this study was in ID samples, hence supporting the hypothesis that *D. nodosus* drives the development of the early stages of footrot (Witcomb and others 2014, Witcomb and others 2015). Interestingly, *D. nodosus* was

found in a large proportion of biopsy samples from healthy feet (58%, 46/79), suggesting it might be present in the stratum corneum (horny layer) but not necessarily causing disease. It is also possible that these visibly healthy feet might have had subclinical footrot and may have developed underrunning lesions in the following days. Risk factors for the development of underrunning footrot in addition to the presence of virulent *D. nodosus* include poor foot conformation (Kaler and others 2010), superficial skin damage (Egerton and others 1969), sheep breed (Emery and others 1984) and presence of co-infecting bacteria such as *F. necrophorum* (Egerton and others 1969, Roberts and Egerton 1969).

In this study, the majority of *D. nodosus* present in the ovine interdigital skin biopsies were virulent strains. Similarly, high prevalence of virulent *D. nodosus* in the UK sheep was identified previously using gelatinase gel protease assay (Moore and others 2005). Therefore, these studies demonstrate that virulent strains are currently circulating in UK flocks. In contrast, in Sweden where underrunning footrot is not endemic, most of the *D. nodosus* were found to be benign (Frosth and others 2015). We found a mixed population of benign and virulent *D. nodosus* strains in the same feet, a potential synergistic role of benign and virulent strains needs still to be investigated.

F. necrophorum prevalence and load were higher in footrot than in ID and healthy samples. These results, together with other published data that also found an increased presence of *F. necrophorum* in footrot lesions (Beveridge 1941, Bennett and others 2009, Witcomb and others 2014,

Witcomb and others 2015), support the hypothesis that *F. necrophorum* contributes to the pathogenesis of underrunning footrot. *F. necrophorum* was presumed to facilitate *D. nodosus* invasion (Egerton and others 1969), in the present study we found that the presence of both *D. nodosus* and *F. necrophorum* in the same tissue was significantly higher in footrot than in healthy feet; nevertheless, the exact nature and the role of the synergy between *F. necrophorum* and *D. nodosus* remains unclear.

Only a small number (9%, 7/79) of healthy biopsy samples were positive for *F. necrophorum* in this study. Similarly, Witcomb and others (2015) found low prevalence of *F. necrophorum* in swabs (8%, 1/12) and biopsies (8%, 1/12) from healthy feet, but in an earlier study where swabs were repeatedly collected from 18 sheep during 5 weeks, *F. necrophorum* was found in 62% (140/225) of healthy feet (Witcomb and others 2014). This suggests that the prevalence of *F. necrophorum* in healthy feet varies according to sampling structure and collection methods. *F. necrophorum* is a commensal in the alimentary tract (Smith and Thornton 1997) and might be present in faeces contaminating ovine feet. Moreover, it was also detected on swabs taken from the oral cavity of sheep and suggested it might be transmitted from the mouth of sheep to the paddock (Bennet and others 2009); hence, the significance of *F. necrophorum* in healthy ovine interdigital skin remains unclear, it may colonise healthy skin as a commensal microorganism without causing any skin disease, while in damaged skin, *F. necrophorum* colonisation may initiate ID and, thus, predispose the invasion of *D. nodosus*. Whether *D. nodosus* essentially

requires *F. necrophorum* colonisation to facilitate its skin invasion remains unclear.

F. necrophorum is divided into subspecies *necrophorum* and *funduliforme*, the first is described to be more pathogenic due to a higher lipopolysaccharide content and higher production of leukotoxin (Tan and others 1996). In this study, the majority of positive samples for *F. necrophorum* was subsp. *necrophorum*. Previous studies investigating this bacterium in UK flocks did not differentiate the subspecies of *F. necrophorum* (Witcomb and others 2014, Witcomb and others 2015). Therefore, despite the fact that this sample set is small, this is the first study suggesting that *F. necrophorum* subsp. *necrophorum* may be the more prevalent subspecies circulating in UK flocks. Since *F. n.* subsp. *necrophorum* is described to be more virulent than *F. n.* subsp. *funduliforme* (Tan and others 1996) and considering the fact that this bacterium may exacerbate footrot lesions, there might be an association between the high prevalence of severe footrot lesions in the UK and the presence of *F. n*. subsp. *necrophorum*. In contrast, in Swedish flocks where most of the footrot lesions were associated with mild footrot, *F. n.* subsp. *funduliforme* was more prevalent than *F. n.* subsp. *necrophorum* (Frosth and others 2015).

Spirochaetes have also been identified in ID and/or footrot lesions (Beveridge 1941, Calvo-Bado and others 2011, Frosth and others 2015). In the present study, a small number of biopsies were positive for *Treponema* spp. with similar prevalence in healthy, ID and footrot feet.

Similarly, low detection of *Treponema* spp. in ovine biopsies from UK sheep was also reported by Calvo-Bado and others (2011); moreover, no significant association between *Treponema* spp. and footrot was reported by Frosth and others (2015). Hence, whether the low detection of *Treponema* spp. reflects its importance in the footrot pathogenesis remains an open question to be further elucidated. We amplified treponemal DNA using a genus-specific qPCR and not a species-specific assay, hence detecting free living as well as pathogenic *Treponema* spp.*;* therefore more studies are warranted to characterize the *Treponema* species commonly present in ovine footrot. In contrast to early investigations reporting that an initial infection with *D. nodosus* is often followed by an infection with *Treponema* spp. (Beveridge 1941, Thomas 1962), we only found 3% of the biopsies (8/241) positive for both virulent *D. nodosus* and *Treponema* spp.

A limitation of using abattoir samples is that it is impossible to investigate the progression of the disease and thus verify whether healthy or ID feet positive for *D. nodosus* would develop footrot lesions. On the other hand, an advantage of using abattoir samples is the ability to collect biopsies from animals that are slaughtered for other purposes than this study and detect bacteria localized within tissues.

Conclusions

The results presented in this study, together with other published data confirm that *D. nodosus* is mainly associated with ID stage, and

F. necrophorum with footrot stage; therefore supporting that *D. nodosus* drives the early stages of footrot and *F. necrophorum* plays a role in the pathogenesis of ovine footrot. Moreover, virulent *D. nodosus* population is more prevalent than benign in UK flocks. *Treponema* spp. was detected in few samples; hence further studies are warranted to provide more detailed information about the role *Treponema* spp. may have in ovine footrot. Additionally, this study reports novel results regarding the higher prevalence of *F. necrophorum* subsp. *necrophorum* than subsp. *funduliforme* in sheep from the UK, and a mixed population of virulent and benign *D. nodosus* present in the same skin biopsy.

Conflicts of interest

Authors declare that they have no conflicts of interest.

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Figure legends

FIG 1: Bacterial prevalence in the ovine interdigital skin from healthy, interdigital dermatitis and footrot feet biopsies. Prevalence and confidence intervals (CI 95%) are shown for total *Dichelobacter nodosus* (a); virulent and benign *D. nodosus* (b); *Fusobacterium necrophorum* (c); *Treponema* spp. (d). Mild ID (interdigital dermatitis score 1); m/s ID (moderate to severe ID scores 2, 3 and 4). Data were analysed by Fisher's Exact Test. *P ≤0.05, **P ≤0.01, ***P ≤0.001, ****P ≤0.0001

FIG 2: Bacterial load in the ovine interdigital skin from healthy, interdigital dermatitis and footrot feet biopsies. Load of total *Dichelobacter nodosus* (a), virulent *D. nodosus* (b), *F. necrophorum* (c) and *Treponema* spp. (d) as percentage of total eubacterial DNA. Due to very low numbers of positive samples, mild ID and ms ID have been pooled together as ID (interdigital dermatitis) for *F. necrophorum* (c), and for *Treponema* spp. (d). Healthy= 79 samples; mild ID= 39 samples; m/s ID= 26 samples; footrot= 97 samples. Mean is indicated by a black horizontal line. Data were analysed by Dunn's multiple comparisons test. *P ≤0.05, **P ≤0.01, ***P ≤0.001, ****P ≤0.0001. Number 0.001 (y axis): results below of the detection limit. mild ID score 1; ms ID: moderate to severe ID scores 2, 3 and 4.

TABLE 1: Number of visits to the abattoir and number of biopsies collected from healthy, interdigital dermatitis and footrot ovine feet.

Date of visit to abattoir	N° of healthy feet	N° of ID feet	N° of footrot feet	N° of sheep with all four feet sampled	Total n° of samples collected
21/10/2013 [1,2]	32	6	2	10	40
01/11/2013*[1,2]	0	20	20	0	40
04/11/2013*[1,2]	4	4	30	0	38
13/12/2013[1,2]	14	7	19	10	40
16/12/2013[1,2]	10	19	10	10	39
19/01/2015[1,3]	19	9	16	10	44
Total	**79**	**65**	**97**	**40**	**241**

ID= interdigital dermatitis

*It was not possible to follow the same animal in the processing line

[1-3] Scorer 1 (GM), scorer 2 (MB), scorer 3 (MA)

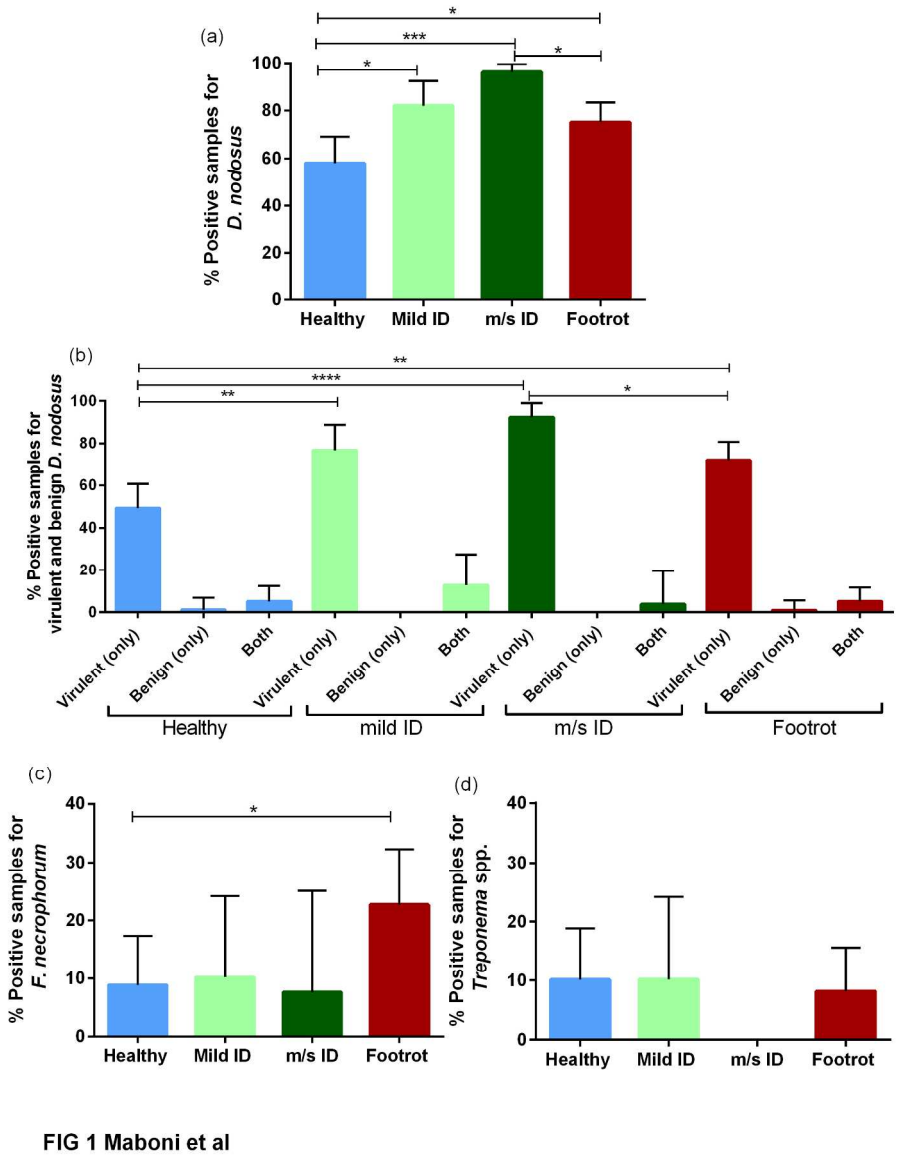

FIG 1 Maboni et al

FIG 1: Bacterial prevalence in the ovine interdigital skin from healthy, interdigital dermatitis and footrot feet biopsies. Prevalence and confidence intervals (CI 95%) are shown for total Dichelobacter nodosus (a); virulent and benign D. nodosus (b); Fusobacterium necrophorum (c); Treponema spp. (d). Mild ID (interdigital dermatitis score 1); m/s ID (moderate to severe ID scores 2, 3 and 4). Data were analysed by Fisher's Exact Test. *P ≤0.05, **P ≤0.01, ***P ≤0.001, ****P ≤0.0001
220x268mm (300 x 300 DPI)

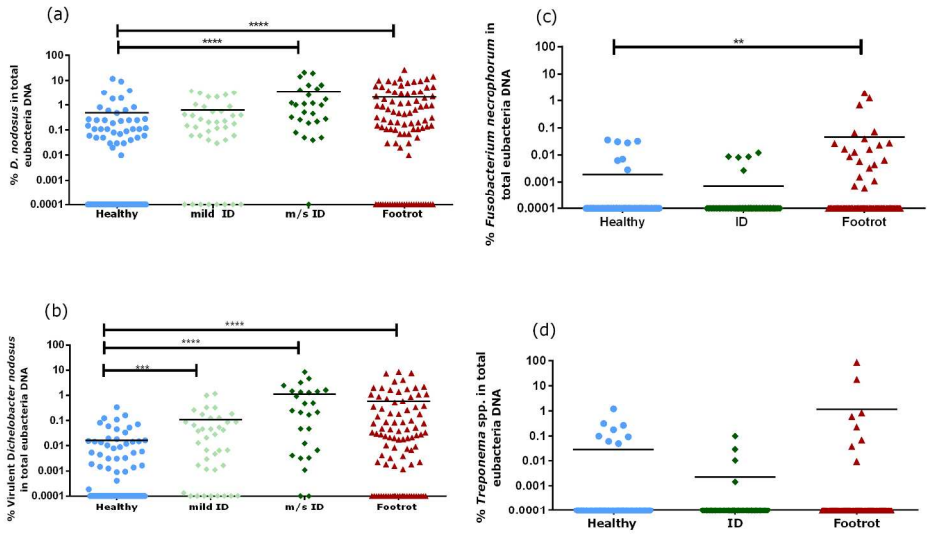

FIG 2 Maboni et al

FIG 2: Bacterial load in the ovine interdigital skin from healthy, interdigital dermatitis and footrot feet biopsies. Load of total Dichelobacter nodosus (a), virulent D. nodosus (b), F. necrophorum (c) and Treponema spp. (d) as percentage of total eubacterial DNA. Due to very low numbers of positive samples, mild ID and msID have been pooled together as ID for F. necrophorum (c), and for Treponema spp. (d). Healthy= 79 samples; mild ID= 39 samples; m/s ID= 26 samples; footrot= 97 samples. Mean is indicated by a black horizontal line. Data were analysed by Dunn's multiple comparisons test. *p ≤0.05, **P ≤0.01, ***P ≤0.001,****P ≤0.0001. Number 0.001 (y axis): results below of the detection limit. mild ID score 1; ms ID: moderate to severe ID scores 2, 3 and 4
213x245mm (300 x 300 DPI)

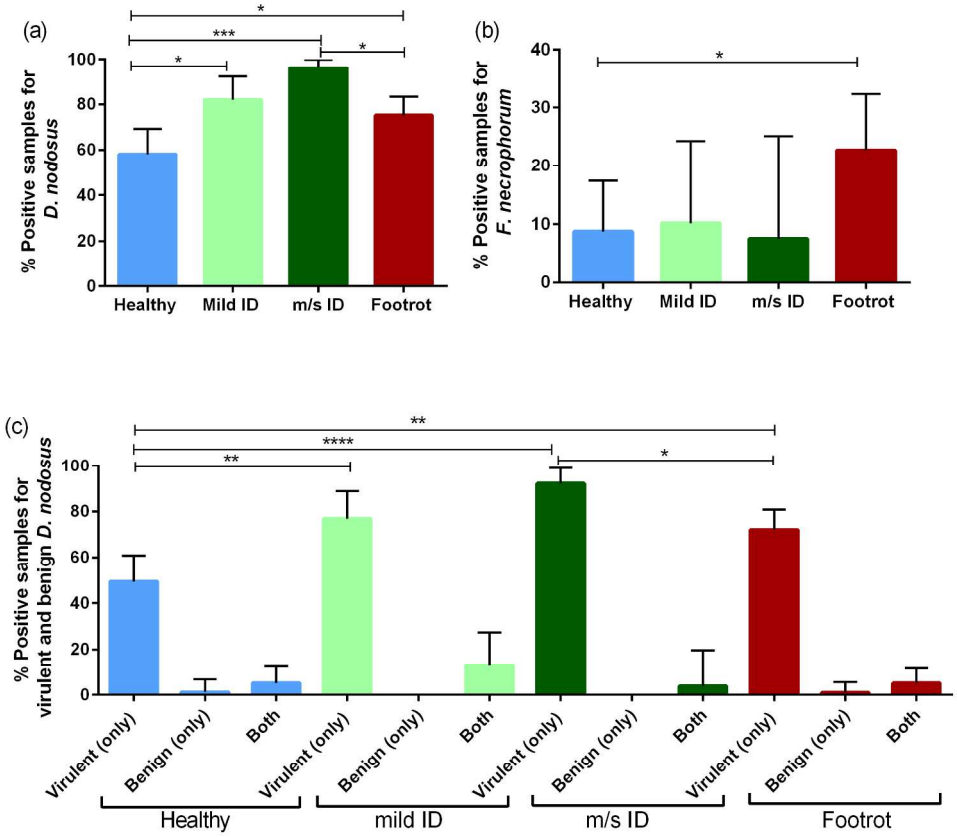

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FIG 1: Bacterial prevalence in the ovine interdigital skin from healthy, interdigital dermatitis and footrot feet biopsies. Prevalence of total Dichelobacter nodosus (a); Prevalence of Fusobacterium necrophorum (b); Prevalence of virulent and benign D. nodosus (c). Mild ID (interdigital dermatitis score 1); m/s ID (moderate to severe ID scores 2, 3 and 4). Data were analysed by Fisher's Exact Test. *P ≤0.05, **P ≤0.01, ***P ≤0.001, ****P ≤0.0001.
220x241mm (300 x 300 DPI)