

UNITED KINGDOM · CHINA · MALAYSIA

# Bacterial colonisation and localisation in ovine

# interdigital dermatitis and footrot

By

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### Abstract

Ovine footrot is the major cause of lameness in sheep within the UK and an economic and welfare concern for sheep farmers globally. It is characterised by under-running of the hoof-horn and preceded by interdigital dermatitis (ID). Under-running footrot is attributed to the host immune response, which is provoked primarily by Dichelobacter nodosus in addition to other bacteria pathogens. However, the precise role of these other bacteria is yet unknown. Therefore, we hypothesise that bacterial invasion and colonisation of the ovine interdigital skin contributes to a strong host inflammatory response leading to the characteristic histopathology observed. In this context, this study examined host inflammatory response [inflammatory cell infiltration, pro-inflammatory cytokines (IL-1 $\beta$ )], histopathological lesions and virulent *D. nodosus* abundance in healthy, ID and footrot conditions in an attempt to gain further insights into the pathogenesis of this important disease. To investigate this hypothesis, two studies were designed: (i) to grade histological lesions in different clinical conditions and (ii) to determine bacterial localisation in post-slaughtered interdigital skin biopsies from the abattoir. Standardised histology lesion grading systems were developed and applied using histochemical techniques (haematoxylin and eosin (H&E), periodic acid Schiff PAS). Bacterial localisation was determined in serial horizontal sections across skin depths combining histology (transverse cryosections + H&E) and qPCR technique for the quantification of bacterial DNA. Furthermore, parallel data of IL-1ß expression and virulent *D. nodosus* load obtained from a different study were compared to histology lesions. Key findings

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were as follows: (i) histological lesions (cell ballooning, parakeratosis, epidermal micro-abscesses and inflammatory cell infiltration) were similar in all clinical conditions, (ii) increased inflammatory cell infiltration score corresponded significantly with high levels of IL-1B expression (p < 0.05) in footrot, and virulent *D. nodosus* load (p < 0.001) across all clinical conditions, (iii) across different skin depths, eubacteria localisation was consistent, D. nodosus localisation was highly variable while *F. necrophorum* was localised in deeper sections of healthy feet. In addition, eubacteria load was significantly higher (p=0.0002) in the epidermis near the skin surface ( $\leq 200 \mu m$ ) of footrot disease samples when compared to healthy samples. Eubacteria components may play contributory roles in footrot pathogenesis based on their localisation in interdigital skin. In conclusion, contrary to previous notion that the severity of disease condition was dictated by progressive pathology, data in this study showed no appreciable difference in the levels of histological lesions and inflammatory response between healthy and diseased (ID, footrot) conditions. Histological lesions and the bacterial components of the skin including the virulent *D. nodosus* contribute to the local inflammatory response which probably drives the progression of footrot disease.

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## Dedication

I dedicate this thesis to the 'I AM THAT I AM' and 'THE ALPHA and OMEGA', the source of my all.

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## List of Abbreviations

| 16S rRNA       | 16S ribosomal Ribonucleic acid      |
|----------------|-------------------------------------|
| APCs           | Antigen presenting cells            |
| Вр             | Base pairs                          |
| BM             | Basement membrane                   |
| BDD            | Bovine digital dermatitis           |
| BHI            | Brain heart infusion                |
| CV             | Coefficient of variation            |
| CODD           | Contagious ovine digital dermatitis |
| R              | Correlation coefficient             |
| Ср             | Cycle crossing point                |
| <sup>0</sup> C | Degree Celsius                      |
| DCs            | Dendritic cells                     |
| DNA            | Deoxyribonucleic acid               |
| DEJ            | Dermal-epidermal junction           |
| Derm           | Dermis                              |
| DPX            | Dibutyl phthalate and xylene        |
| dsDNA          | Double stranded DNA                 |
| Eff            | Efficiency                          |
| Epid           | Epidermis                           |
| FOV            | Field of view                       |
| FISH           | Fluorescence in-situ Hybridization  |
| G              | Grams                               |
| H&E            | Haematoxylin and eosin              |
| h              | Hours                               |
| IMS            | Industrial methylated spirit        |
| ICAM1          | Intercellular adhesion molecule 1   |
| ID             | Interdigital dermatitis             |
| IL             | Interleukin                         |
| IL-1β          | Interleukin 1-beta                  |
| LCs            | Langerhans cells                    |
| LAS            | Leica application suite             |
| LOD            | Limit of detection                  |
| LOQ            | Limit of quantification             |
| LPS            | Lipopolysaccharide                  |
| MT             | Massons trichrome                   |
| Max            | Maximum                             |
| Med            | Median                              |
| μl             | Microlitre                          |
| μm             | Micrometre                          |
| μm²            | Micrometre square                   |
| mM             | Millimolar                          |
| mg             | Milligrams                          |
| ml             | Millilitres                         |

| Mm   | Millimetres                             |
|------|---|
| Min  | Minutes                                 |
| G    | Nanograms                               |
| NTC  | Non-template control                    |
| n    | Numbers                                 |
| OCT  | Optimum cutting temperature compound    |
| PAMP | Pathogen associated molecular pattern   |
| PPRR | Pathogen pattern recognition receptors  |
| %    | Percentages                             |
| PAS  | Periodic acid Schiff                    |
| pg   | Picograms                               |
| PCR  | Polymerase Chain Reaction               |
| qPCR | Quantitative PCR                        |
| rpm  | Revolution per minute                   |
| r    | Rank correlation coefficient (Spearman) |
| RNA  | Ribosomal ribonucleic acid              |
| SD   | Standard deviation                      |
| TIFF | Tagged Image File Format                |
| Таq  | Thermus aquaticus                       |
| TLRs | Toll-like receptors                     |
| TE   | Tris EDTA                               |
| TAS  | Trypticase-arginine-serine              |
| TNF  | Tumour Necrosis Factor                  |
| v/v  | Volume/volume                           |
| v/w  | Weight/volume                           |

### **Chapter 1 Main Introduction**

### **1.1. Literature review**

Disorders affecting the ovine feet are mainly divided into contagious and non-contagious causes. In both cases, sheep mobility is impaired leading to lameness. Examples of non-contagious causes of ovine lameness include granuloma and abscesses of the toe of the foot, pedal joint abscess, white line disease and hyperplasia (Winter *et al.*, 2004; Angell *et al.*, 2015). The majority of ovine lameness are attributed to contagious causes due to contagious ovine digital dermatitis and ovine footrot (Egerton, 2008). This review will be focused on ovine footrot disease with a brief mention of contagious ovine digital dermatitis.

### 1.1.1 Contagious ovine digital dermatitis (CODD)

It is a painful and contagious infection of the ovine feet which was first observed in the United Kingdom (UK) in 1997 (Harwood *et al.*, 1997) and has currently affected 50 per cent farms in the UK and Republic Ireland (Sayers *et al.*, 2009; Angell *et al.*, 2014; Winter *et al.*, 2015b). Similarly, CODD has been reported in Denmark (Rasmussen *et al.*, 2012).

CODD is aetiologically linked to treponemes with three phylogroups particularly implicated: *Treponema medium/Treponema vincentii*-like, *Treponema phagedenis*-like and *Treponema pedis* (Dhawi *et al.*, 2005; Sullivan *et al.*, 2015).

Clinically, CODD is initiated as small ulcers or lesions at the coronary band which progressively spread beneath the horn leading to the separation of dorsal hoof and lameness (Angell *et al.*, 2015).

CODD is significantly different from ovine footrot in a number ways including, clinical appearance, failure of conventional management therapies of footrot disease in CODD, and the aetiological association of *Treponema* in CODD rather than *Dichelobacter nodosus* in ovine footrot (Naylor *et al.*, 1998; Davies, Naylor & Martin, 1999; Lewis *et al.*, 2001).

#### **1.1.2 Ovine footrot**

Footrot is an infectious, necrotic and degenerative disease of ovine feet primarily caused by the Gram negative anaerobe, *Dichelobacter nodosus* (Beveridge, 1941). It accounts for 80-90% of lameness amongst sheep flocks in the UK (Grogono-Thomas & Johnston, 1997; Kaler & Green, 2008).

Footrot has a worldwide distribution and has been reported in major sheepproducing countries such as Australia, New Zealand and the UK (Beveridge, 1941; Zhou & Hickford, 2001; Green & George, 2008). In addition, it has been reported in the USA (Gradin, Sonn & Petrovska, 1993), Canada (Olson *et al.*, 1998), Nepal (Egerton *et al.*, 2002), India (Farooq *et al.*, 2010), Spain (Lacombe-Antoneli, Píriz & Vadillo, 2006) and Scandinavia (König, Nyman & de Verdier, 2011; Gilhuus *et al.*, 2014). Reports have also indicated cases of footrot in Brazil (Aguiar *et al.*, 2011). Although a world-wide disease, ovine footrot is more common in warm, moist weather conditions (Cross, 1978; Cross & Parker, 1981) with low temperature of about 10°C (Graham & Egerton, 1968) and less common in arid and semiarid conditions (Barber, 1979). In the UK, prevalence of ovine footrot is currently estimated to be 3.1% (Winter *et al.*, 2015a). Losses associated with lameness mainly due to footrot cause significant economic and welfare challenges to sheep farmers and veterinarians world-over (Goddard *et al.*,

2006). The effects of lameness on the health and welfare of animals is multiple and may include reduced mobility, feed intake and body condition scores. Consequently, fertility and conception are impaired leading to low birth rates (Wassink *et al.*, 2010). Lameness has also been reported to cause death due to dehydration, necrosis and/or systemic infection resulting from sternal recumbency on the soil (Egerton, Yong & Riffkin, 1989; Marshall *et al.*, 1991). These cumulative effects result in substantial financial losses to the farmers. The cost of footrot in terms of management and production losses has been estimated around £24-80 million pounds per annum in the United Kingdom (Nieuwhof & Bishop, 2005; Wassink *et al.*, 2010). While in New South Wales (NSW), Australia, the associated cost was considered to be Aus \$24.6 million per annum prior to a state-wide footrot eradication programme; which reduced flock prevalence of virulent footrot from 8% in 1988 to 0.54% in 2005 (Dhungyel, Lehmann & Whittington, 2008).

### 1.1.3 Footrot lesion description and clinical signs

A typically healthy ovine interdigital skin is dry with fine hairs (Figure 1.1). On the other hand, the intermediate phase of interdigital dermatitis (ID) appears moist, pinkish/reddish with greyish pasty 'scum' while the endstage footrot is characterised by under-running (separation of hoof-horn; Figure 1.1)(Beveridge, 1941; Green & George, 2008).

Animals with ID may show signs of lameness (Winter *et al.*, 2004), and if untreated could progress to detachment of the hoof-skin junction (underrunning)(Beveridge, 1941; Bennett & Hickford, 2011) spreading to the underlying sole and heel areas. Under-running lesions may continue to the axial and abaxial portions of the foot (Figure 1.1)(Abbott & Lewis, 2005).

The under-running is believed to be due to the intense inflammatory response of the host leading to tissue necrosis and sloughing (Egerton, Roberts & Parsonson, 1969).



**Figure 1-1: Ovine foot anatomy and signs of ovine footrot. A**. Anatomy of ovine foot (EBLEX Ltd. Sheep BRP manual 7, **B**. Lameness in sheep (Hagedon, 2007, **C**. Healthy interdigital skin of foot, **D**. reddened interdigital skin (ID), **E**. Under-running lesion of footrot. Images C and E: Dr Adam Blanchard, SVMS, Image D: (Angell, 2015).

# **1.1.4** Bacterial aetiology and pathogenesis of interdigital dermatitis (ID) and footrot

Historically, the aetiopathogenesis of footrot has been associated with a number of bacteria which include *D. nodosus*, *F. necrophorum* and spirochaetes (Mohler & Washburn, 1904; Beveridge, 1941; Egerton, Roberts & Parsonson, 1969). However, following infection experiment of the ovine feet, Beveridge (1941) concluded that *D. nodosus*, a Gram negative obligate anaerobe was required for footrot to be established, but

that severity of the infection is enhanced in the presence of other pathogens such as *F. necrophorum* and spirochaetes. Whilst the debate on the pathogenesis of ovine footrot continued, further studies suggested that in interdigital dermatitis (ID), an intermediate phase of footrot disease, *F. necrophorum* essentially precedes *D. nodosus* invasion (Roberts & Egerton, 1969). For decades, it was assumed that ID and footrot were two distinct disease conditions (Grogono-Thomas & Johnston, 1997; Winter *et al.*, 2004).

Later findings from swabs and biopsies samples by researchers in the UK revealed significantly increased *D. nodosus* burden in ID and footrot stages of the disease in comparison to healthy condition (Moore et al., 2005; Calvo-Bado et al., 2011; Witcomb et al., 2014, 2015; Maboni et al., 2016). In addition, findings by Witcomb et al. (2014) using swabs and Maboni and colleagues (2016) using biopsies demonstrated higher load of D. nodosus in moderate-to-severe ID when compared to footrot and similarly showed higher load of F. necrophorum in footrot when compared to ID (Witcomb et al., 2014; Maboni et al., 2016). These volumes of evidence further confirmed *D. nodosus* as the primary aetiology of footrot with suggestions that F. necrophorum may probably be playing an opportunistic role in the pathogenesis of the disease. Furthermore, these studies may also indicate that in the UK, ID and footrot are part of the same disease but at different phases of progression. However, not all cases of ID progress to footrot, depending on factors such as environmental conditions, animal susceptibility as well as *D. nodosus* virulence (Graham & Egerton, 1968; Stewart et al., 1986; Kennan et al., 2010).

Aside from *D. nodosus* and *F. necrophorum*, over 27 bacterial genera (*Bacteroides, Corynebacteria, Prevotella and Porphyromonas* etc.) have been associated with footrot ecology (Beveridge, 1941; Egerton, Roberts & Parsonson, 1969; Calvo-Bado *et al.*, 2011; Maboni *et al.*, 2017). In this context, further review in this section will focus on *D. nodosus*, *F. necrophorum* and the microbiome of the ovine footrot.

### 1.1.4.1 Dichelobacter nodosus

*D. nodosus* is a Gram negative, non-spore forming obligate anaerobic bacillus. It is slightly bent or straight with knob-like ends (Beveridge, 1941). *D. nodosus* belongs to the order *Cardiobacteriales* (γ-proteobacteria) and family *Cardiobacteriaceae* with a known genomic size of 1.4 Mbp (1,389, 350 bp) containing 1299 putative genes (Myers *et al.*, 2007). It has been reported to survive in aerobic environment for over 10 days (Myers *et al.*, 2007), suggesting it is aerotolerant and not an obligate anaerobe as previously thought.

### D. nodosus virulence

Understanding the biology of microbial organisms in their animal host is fundamental to understanding disease pathogenesis. The interaction of bacterial organisms with host tissues is mediated by a number of factors, which are generally termed virulence factors.

In *D. nodosus*, virulence is mediated mainly by the possession of fimbriae (esp. type-IV), secretion of extracellular proteases (elastase, collagenase, gelatinase and caseinase)(Kennan *et al.*, 2001) and outer membrane proteins (Myers *et al.*, 2007). In addition, virulence associated genes including *vap* (virulence associated proteins), *vrl* (virulence related locus)

and *intA* have been mentioned (Katz *et al.*, 1991; Rood *et al.*, 1996; Cheetham *et al.*, 2006).

The immunogenic potentials as well as agglutination reactions of *D. nodosus* fimbriae have been explored for the classification of the bacteria into serogroups (Claxton *et al.*, 1989). Currently there are ten fimbrial serogroups (A-I, M) consisting of 19 serotypes. Of the ten serogroups, nine (A-I) have been reported in the UK (Ghimire *et al.*, 1998; Moore *et al.*, 2005). Fimbriae in *D. nodosus* are responsible for its twitching motility as well as protease secretion (Kennan *et al.*, 2001).

The ability of *D. nodosus* to successfully colonise interdigital skin and hoof matrix is majorly due to its secretion of subtilisin-like serine proteases. These proteases play a role in the digestion of skin and hoof constituents (keratin, elastin and collagen), and on this basis differentiates *D. nodosus* into two phenotypes: virulence and benign types (Kennan *et al.*, 2010).

Three proteases which include the acidic proteases, AprV2 and AprV5, as well as the basic protease, BprV have been associated with virulence in *D. nodosus*. These proteases digest elastin and are thermostable (Riffkin *et al.*, 1995). In particular, AprV2 protease has been demonstrated to be essential in *D. nodosus* for the initiation of footrot (Kennan *et al.*, 2010). On the other hand, the possession of AprB2, AprB5 and BprB proteases confer benign status on *D. nodosus* due to their inability to digest elastin and thermolabile nature (Riffkin *et al.*, 1995). The difference between AprV2 protease and its benign form AprB2 is the single point amino acid change at position 92 (Tyr92Arg)(Riffkin *et al.*, 2010).

A conserved bimodal global distribution of *D. nodosus* population has been reported to correspond with virulent (*aprV2*) and benign (*aprB2*) strains (Kennan *et al.*, 2014). Sequencing study has shown that the *aprV2* and *aprB2* distinction of *D. nodosus* is consistent with the severity of clinical disease (Stäuble *et al.*, 2014b). However, the possession of *aprV2* or *aprB2* genes does not always correspond to severity of disease. Indeed, Frosth and colleagues identified positive AprB2 strains of *D. nodosus* (benign) in ovine footrot lesions in Sweden (Frosth *et al.*, 2015) while Stäuble *et al* also detected AprV2 *D. nodosus* (virulent) strains in feet without clinical lesions of footrot (Stäuble *et al.*, 2014a).

### 1.1.4.2 Fusobacterium necrophorum

The genus *Fusobacterium* consists of rods with fusiform morphology. Species in this genus are Gram negative, non-spore forming, pleomorphic and obligately anaerobic, belonging to the family *Bacteriodaceaea*. *Fusobacterium necrophorum, F. nucleatum, F. naviforme, F. necrogenes, F. ulcerans, F. varium, F. russi, F. prausnitzii and F. periodonticum* are the most investigated species of this genus.

*Fusobacterium necrophorum*, a commensal of the ovine gut, is a common inhabitant of soil contaminated with faeces. It is sub-divided into subspecies *necrophorum* and *funduliforme* (Tan, Nagaraja & Chengappa, 1996) and was classified as an animal pathogen in the late 1800s, reviewed by (Langworth, 1977). *F. necrophorum* has been associated with ovine footrot, and its role in the disease is still unclear. Two current hypothesis are being debated: i. *F. necrophorum* establishes ID and subsequently allows colonisation by *D. nodosus* which then initiates the development of footrot (Egerton, Roberts & Parsonson, 1969), and ii. *F. necrophorum* 

involvement is secondary and opportunistic following the establishment of footrot, thereby encouraging persistence of the disease (Beveridge, 1941). Recent molecular studies employing swabs and biopsies across different clinical conditions of the ovine feet showed increased load and prevalence of *F. necrophorum* only after the establishment of under-running footrot (Witcomb *et al.*, 2014, 2015; Frosth *et al.*, 2015; Maboni *et al.*, 2016). These studies have skewed evidence towards the hypothesis that *F. necrophorum* is probably opportunistic and plays a secondary role in the persistence of footrot disease condition.

*F. necrophorum* is a versatile opportunistic organism which has been associated with necrotic diseases of different body parts and organs such of the feet (ovine footrot, bovine foot abscess), liver, mammary gland and the oropharynx of both animals and humans (Nagaraja *et al.*, 2005). Also, it has been isolated from sheep (Egerton & Roberts, 1971), goats (Bennett *et al.*, 2009), cattle (Sun *et al.*, 2011), pigs (Zhou, Dobbinson & Hickford, 2010) and man (Falkler, Enwonwu & Idigbe, 1999).

Aside from ovine footrot, it is commonly associated with animal diseases like bovine hepatic abscess, bovine mastitis and necrotic laryngitis (calf diphtheria), ruminant footrot and abscess as well as mandibular abscesses in antelopes and marsupials (Tan, Nagaraja & Chengappa, 1996; Nagaraja *et al.*, 2005). In humans, *F. necrophorum* is associated with Lemierre's syndrome (Riordan, 2007).

### F. necrophorum virulence

*F. necrophorum* is a versatile pathogen with potential to form synergy with other pathogens in different hosts to initiate disease processes. It is known

to produce different endo and exo-toxins, which facilitate virulence (Nagaraja *et al.*, 2005).

Unlike *D. nodosus* where mixed strains (up to seven) may be found on an infected hoof (Zhou & Hickford, 2000, 2001), only one strain variant of *F. necrophorum* has been identified on an infected hoof sample (Zhou, Bennett & Hickford, 2009). *F. necrophorum* produces haemagglutinin, leads to platelet aggregation, which results in thrombus formation and entrenchment of micro-anaerobic environment] (Forrester *et al.*, 1985), dermonecrotic toxin and haemolysin (initiate reduction in oxygen tension and tissue destruction seen in infections). *F. necrophorum* also produces substances such as volatile sulphur compounds and proteolytic enzymes like phosphatase B that are also involved in tissue destruction (Fifis, Costopoulos & Vaughan, 1996).

Of the virulence factors described, leukotoxin is considered the most significant virulence factor (Tan, Nagaraja & Chengappa, 1996). Leukotoxins are toxic to liver and ruminal epithelial cells as well as cells of the immune (neutrophils and macrophages) system; this can become valuable in the modulation of immune response (Tan, Nagaraja & Chengappa, 1996; Narayanan *et al.*, 2002).

### 1.1.4.3 Microbiome of healthy and footrot affected ovine feet

One major step to determine the contributions of bacteria aside *D. nodosus* and *F. necrophorum* in the pathogenesis of footrot is to describe and compare bacterial diversity in healthy and footrot affected interdigital skin. The ovine interdigital skin is a heavily contaminated, polymicrobial ecosystem which may shape disease outcomes including footrot. Katitch (1979) proposed a polymicrobial pathogenesis of ovine footrot to involve other soil bacteria (*Clostridium perfringens, Actinomyces pyogenes* and other cocci) in addition to *D. nodosus* (Katitch, 1979), reviewed by (Duran *et al.*, 1990)(Table 1.1). Although no data currently exist to indicate whether these other bacteria play a role in footrot pathogenesis, similar anaerobic microbiota in polymicrobial conditions like periodontitis and abscesses have been shown to contribute to disease process due to interaction between the host and bacteria such as *Bacteroides fragilis*, *Porphyromonas gingivalis* and *Fusobacterium nucleatum* (Otto *et al.*, 2002; Nishihara & Koseki, 2004).

The bacterial ecology of ovine healthy feet and footrot lesion is varied and may be dependent on factors such as, environment, including types of soil, weather conditions, proximity to other farms of mixed animal breeds and species; susceptibility of breeds to footrot (Emery, Stewart & Clark, 1984; Skerman & Moorhouse, 1987); and management practices such as quarantining of infected animals, hoof trimming and disposal of trimmed portions (Howell-Jones *et al.*, 2005; Green *et al.*, 2007). Also, inappropriate administration of antimicrobials such as tetracycline can induce changes in the microbiome of the foot (Kaler *et al.*, 2010a) thereby resulting in resistant strains of bacterial species in footrot (Lorenzo *et al.*, 2012).

Earlier studies on ovine interdigital skin ecology were based on phenotypic characterisation (morphology and biochemical reactions). This allowed the identification of bacteria species including *Staphylococcus spp.*, *Streptococcus spp.*, *Corynebacterium pyogenes, D. nodosus* and *F. necrophorum* (Beveridge, 1941; Roberts & Egerton, 1969; Egerton,

Roberts & Parsonson, 1969). However, fastidious organisms like *Treponema* (spirochetes), although cultivable (Walker *et al.*, 1995), are difficult to culture and thereby examined by wet-mount microscopy of lesions (Beveridge, 1941; Egerton, Roberts & Parsonson, 1969). This clearly limits the identification of difficult-to-culture obligate anaerobes or unculturable bacteria in healthy and footrot microbiota.

The introduction of molecular-based identification techniques such as the 16S rRNA and next generation sequencing (NGS) techniques in pathogen detection have improved discrimination of bacterial phenotypes and population in healthy interdigital skin and footrot ecology. These techniques identify the well-conserved and variable regions in the bacteria genome and therefore make excellent identification tools (La Fontaine, Egerton & Rood, 1993; Calvo-Bado *et al.*, 2011; Maboni *et al.*, 2017). In this context, two studies; Calvo-Bado *et al* and Maboni and colleagues have provided the most comprehensive coverage of the microbiome of healthy and footrot affected ovine interdigital skin (Calvo-Bado *et al.*, 2011; Maboni *et al.*, 2017).

The first study utilised 16S rRNA (Sanger sequencing clone libraries and pyrosequencing) to compare the bacterial ecology of healthy, ID and footrot conditions. Greater proportion of *Micrococccus*, *Peptostreptococcus* and *Corynebacterium* were associated with healthy feet; *Corynebacterium* with ID and *Streptococcus*, *Facklamia* and *Abiotrophia* where predominant footrot. In general, members of 3 phyla (Actinobacteria, Firmicutes and Proteobacteria) and 27 genera were identified (Calvo-Bado *et al.*, 2011). Although this study provided a baseline data to advance our knowledge of footrot microbial community, the generalizability of the study is limited by

the low number (15) of sample animals (eight healthy, five ID and two footrot) and limited geographical area (South West of England) used in the study when compared to United Kingdom and world sheep population. Furthermore, samples from four feet of each animal were pooled together, thereby making it difficult to determine the microbiota of individual foot. The second study on the other hand, reported Corynebacteria, Psychrobacter and Acinetobacter as the most abundant genera in the healthy interdigital skin while *Mycoplasma*, *Corynebacteria*, *Psychrobacter* and Treponema as the most abundant in footrot (Maboni et al., 2017). Maboni et al found little difference in the microbiome of healthy and low footrot inflammation samples, but reported different dominant bacteria in healthy high inflammation (Acinetobacter, Corynebacterium, Flavobacterium) and footrot high inflammation (Treponema, Mycoplasma, Porphyromonas)(Maboni et al., 2017). Hence, suggesting the possible contribution of other bacteria organisms in inflammatory response of ovine feet.

The two studies differ in that Maboni and colleagues investigated: (i) a greater number of foot samples (40 healthy, 36 footrot), (ii) Maboni *et al* examined individual foot compared to pooled feet per sheep investigated in the previous study, and (iii) a more recent sequencing technology (Illumina Miseq) was used by Maboni and colleagues compared to the pyrosequencing employed in the previous study.

Together, the two studies have provided greater insights into our knowledge of microbial ecology of the ovine feet in different clinical conditions.

### Table 1-1: Microbial organisms associated with footrot lesions

| Organism   | Method of detection   | Sample type                  | Animal  | References                   |
|--|---|------------------------------|---------|------------------------------|
|  |   |                              | species |                              |
| <i>Treponema spp., Leptospira spp.,</i><br><i>Corynebacterium spp., Fusobacterium</i><br><i>necrophorum, D. nodosus</i>  | Culture   | Swab                         | ovine   | (Beveridge, 1941)            |
| Corynebacterium pyogenes,<br>Fusobacterium necrophorum   | Culture   | Necrotic<br>tissues/exudates | Ovine   | Parsonson et al., 1967       |
| Peptostreptococcus, Megasphaera,<br>Clostridium, propionibacterium,<br>Eubacterium, Leptotrichia,<br>Acidaminococcus, Peptococcus,<br>Tissierella, Woniella, Veionella,<br>Fusobacterium necrophorum, D.<br>nodosus                  | Culture   | Swab                         | Caprine | (Piriz Duran S et al., 1990) |
| Treponema, Arcanobacterium,<br>Staphylococcus, Facklamia<br>Streptococcus, Abiotrophia,<br>Peptostreptococcus, Corynebacterium,<br>Macrococcus, Escherichia, Citrobacter,<br>Acinetobacter, Fusobacterium<br>necrophorum, D. nodosus | 16S rRNA (Sanger<br>sequencing clone libraries<br>and pyrosequencing) | Biopsy                       | Ovine   | (Calvo-Bado et al., 2011)    |
| <i>Mycoplasma, Corynebacterium,<br/>Psychrobacter,<br/>Treponema, Porphyromonas,<br/>Fusobacterium necrophorum, D.<br/>nodosus</i>   | 16S rRNA (Illumina sequencing library)                                | Biopsy                       | Ovine   | (Maboni et al., 2017)        |

### 1.1.5 Bacterial detection and identification

### 1.1.5.1 Culture based methods

*D. nodosus* is an anaerobic organism with special requirements for growth such as 5-10% CO<sub>2</sub> (Beveridge, 1941). Cultivating the organism is often challenging due to the growth of contaminants, which compete with *D. nodosus* for available nutrients and thereby inhibit its growth. However, increasing the proportion of agar (4%) in culture medium limits the spread of contaminants (Skerman, 1975). Culture media reportedly used in the effective cultivation of *D. nodosus* include hoof agar (Thomas, 1958), Eugon agar (Gradin & Schmitz, 1977) and trypticase-arginine-serine (TAS) agar (Skerman, 1975).

The colony morphology of *D. nodosus* on solid media has been described as "raised, convex colonies of 0.5 to 1mm diameter with a translucent centre etched into the agar and a flatter ground glass periphery with fimbriate edges" in hoof agar (Egerton & Parsonson, 1966).

#### Histochemical staining

In histopathology investigations of tissues, the most commonly used staining technique is haematoxylin and eosin (H&E). Although it can detect a number of microbial organisms and allow background information, it is not a specialized stain (Woods & Walker, 1996; Powers, 1998). Microbespecific histochemical stains commonly used include in-tissue Gram stain as well as Giemsa (Beveridge, 1941; Humberstone, 1963; Engbaek, Johansen & Jensen, 1979; Bottone, 1988). However, the sensitivity of these stains is subject to microbial load in the tissue and the examiner's expertise. In footrot investigations, in-tissue Gram stain (Roberts & Egerton, 1969) and Giemsa (Beveridge, 1941) have been used to examine skin-hoof interface for bacterial presence (Figure 1.2). These staining techniques are economical, reproducible and easy to perform. However, Giemsa stain lacks a counterstain thereby affecting its ability to delineate other background structures (Madan *et al.*, 1988).



Figure 1-2: Gram negative rod *D. nodosus* with clubshaped terminals. Source: Karolina Enlund (SLU) <u>www.vetbact.se</u>

### 1.1.5.2 Molecular techniques

Application of molecular methods has improved identification of bacteria including *D. nodosus*. Molecular techniques allow rapid detection of bacteria and thereby eliminate the cumbersome processes involved in identification of bacteria through cultural identification methods (Rasmussen *et al.*, 1994).

### 1.1.5.2.1 Polymerase Chain reaction (PCR)

Polymerase chain reaction (PCR) is a molecular technique used for the amplification of desired DNA segments, using complementary primers to initiate DNA polymerisation (Nguyen, Kahn & Lu, 1994). It has been used in the detection of *D. nodosus* in footrot lesions by targeting and amplifying genes such as *fimA* (Liu & Webber, 1995; John *et al.*, 1999) and *16S rRNA* (La Fontaine, Egerton & Rood, 1993; Zakaria *et al.*, 1998). The variability in the sequences of the *fimA* gene (Mattick *et al.*, 1984) probably reflects immunogenicity and may be responsible for the ability of the test to detect different sero-groups of *D. nodosus* (Cagatay & Hickford, 2005).

PCR is inexpensive, sensitive and saves time. It can detect *D. nodosus* from footrot lesions, cultured agar plates and in swab samples. However, the presence of humic acid in the soil interferes with its sensitivity by inhibiting *Taq* polymerase (Tsai & Olson, 1992). One way to prevent this is by use of high pressure incorporated cycling technology in the DNA extraction process (Marshall *et al.*, 2013). Additional approach is the treatment of samples with DNA-polymerase buffer system which tolerates high impurity load (Rådström, Knutsson & Wolffs, 2004; Hedman *et al.*, 2013).

### 1.1.5.2.2 Quantitative PCR (qPCR)

This is an advance variant of PCR which can additionally quantify nucleic acids aside from detection. It is highly sensitive, specific and beneficial in the determination of bacterial load. This is especially useful in studies where bacterial abundance is desired in different biological samples or over a period of time in different clinical conditions. Specifically, Witcomb *et al* employed this technique in a longitudinal study to show that *F. necrophorum* significantly increase in abundance, following the establishment of footrot in ovine feet. Thereby, providing further evidence of the opportunistic nature of this organism in footrot disease (Witcomb *et*
*al.*, 2014). Also, qPCR is applicable to a variety of sample types including tissues, swabs and soil (Calvo-Bado *et al.*, 2011; Muzafar *et al.*, 2016; Maboni *et al.*, 2016), targeting a number of genes which include the RNA polymerase sigma-70 factor gene (*rpoD*) (Calvo-Bado *et al.*, 2011; Witcomb *et al.*, 2014) and the more conserved *16S rRNA* gene (Frosth *et al.*, 2012; Maboni *et al.*, 2016). Targeting the *16S rRNA* gene has the advantage that it exists in three copies in the *D. nodosus* genome thereby improving the potential for detection in comparison to the *rpoD* gene which exists as a single gene (Frosth *et al.*, 2012).

In a study on ovine pedomics, *D. nodosus* was quantifiable in 25 out of 60 healthy feet using *rpoD* gene as the target (Calvo-Bado *et al.*, 2011). In another study comparing specificity of *D. nodosus* detection techniques, real time PCR technique targeting 16S rRNA gene showed 100% specificity for ten *D. nodosus* strains and exclusivity for 45 non-target bacterial strains as well as two non-target fungal strains tested (Frosth *et al.*, 2012). The high sensitivity observed in this 16S assay may be attributed to the three copies of the gene present in *D. nodosus* genome and also improves the detection potential of this assay.

Despite its efficiency and repeatability, one limitation of qPCR is its inability to discriminate viable from non-viable bacteria cells, which may therefore result in over quantification of bacterial load (Castillo *et al.*, 2006; Pathak *et al.*, 2012). To improve the qPCR detection with regards to viability of organisms, RNA based assays are frequently designed since RNA rapidly degrades in comparison to DNA following bacterial death (Pai *et al.*, 2000; Kobayashi *et al.*, 2009). Also, propidium monoazide (PMA), a DNA intercalator commonly used in microscopic viewing of cell viability

penetrates dead cells and prevents PCR amplification while allowing amplification from viable cells (Nocker & Camper, 2009; Kobayashi *et al.*, 2010). The success of these techniques is dependent on the sample conditions. Amplicon size has been reported to pose problems due to low DNA intercalation binding sites (Gedalanga & Olson, 2009; Nocker, Sossa & Camper, 2007; Contreras *et al.*, 2011).

# 1.1.5.2.3 Fluorescence in-situ hybridisation (FISH) probe

Fluorescence *in-situ* hybridisation is a non-culture based molecular technique that uses ribosomal ribonucleic acid (rRNA) targeted oligonucleotide probe to identify and quantify microbes *in situ* (DeLong, Wickham & Pace, 1989; Amann, Ludwig & Schleifer, 1995). The probes are labelled with fluorescent dyes to identify organisms in the family.

This technique has proved useful in recent investigation of *in situ* detection and localisation of bacteria where *D. nodosus* was significantly higher in ID and footrot conditions in comparison to *F. necrophorum* counts which was higher in footrot (Witcomb *et al.*, 2015).

Furthermore, FISH may provide additional information on the physiological status of bacterial cells since there is a positive correlation between rRNA content and bacterial rate of growth (Kemp, Lee & Laroche, 1993).

# **1.1.5.3** Pathogenesis of ID and footrot

Footrot is initiated by abrasion in the epidermal layer of healthy ovine interdigital skin. This may be prompted by mechanical trauma from stones, rocks, grasses as well as prolong maceration due to high moisture and low temperature (Graham & Egerton, 1968) . The abraded foot is exposed to faecal-contaminated soil, creating the mixed microbiota of the infection. Bacterial interaction and multiplication in the abraded skin result in interdigital dermatitis (ID), in which case the epidermal layer becomes reddened, inflamed, damaged and begins to break apart. The continuous activities of bacteria and their metabolic products cause necrosis of skin tissues, thereby creating an anaerobic micro-environment (Beveridge, 1941; Graham & Egerton, 1968). These conditions permit *D. nodosus* invasion and the resultant inflammatory response by the host. Influx of neutrophils and other immune-competent cells (lymphocytes, plasma cells and macrophages) as a response by the host against bacterial activities cause inflammation and pressure to develop in the hoof horn capsule, resulting in the detachment of the hoof matrix from the underlying soft tissues. However, not all sheep exposed to *D. nodosus* develop footrot; only in less than 50% cases have separation of hoof horn been reported (Wassink *et al.*, 2010). This could probably be due to virulence of the infecting *D. nodosus* strain.

# 1.1.6 Footrot transmission and epidemiology

Requirements for the successful transmission of any infectious disease include the interactions between causative agents, susceptible host and favourable environmental condition. A number of these factors aside from the presence of virulent strains of *D. nodosus* include disruption of intact healthy interdigital skin which may facilitate invasion by *D. nodosus* and other invasive organisms including *F. necrophorum* (Egerton, Roberts & Parsonson, 1969). Also, poor hoof conformation (Kaler *et al.*, 2010b) and breed susceptibility to infection have been reported to contribute to the outcome of infection (Emery, Stewart & Clark, 1984). Other factors include

persistent exposure of sheep feet to moist and damp environment which encourages feet maceration, *D. nodosus* survival and transmission (Graham & Egerton, 1968). Also, the lush pasture obtainable in the UK due to long months of rainfall encourages farmers to increase stocking density through introduction of new sheep in the farm. Both practices increasing the chances of *D. nodosus* transmission among sheep (Wassink *et al.*, 2004; Grøneng *et al.*, 2014). Additionally, *D. nodosus* may be transmitted through co-grazing sheep with other susceptible species such as cattle, goats etc (Knappe-Poindecker *et al.*, 2014a). Soil, pasture and bedding materials have also been implicated in footrot transmission (Beveridge, 1941). Indeed, *D. nodosus* has been demonstrated to survive for 40 days in the soil at 5°C (Muzafar *et al.*, 2016) and has been shown to be transmitted between the feet of Ewes, new-born lambs in a few hours post birth (Muzafar *et al.*, 2015).

# **1.1.7 Management and control**

Although footrot is reported as the major cause of lameness in the United Kingdom (UK)(Grogono-Thomas & Johnston, 1997), there are no known non-transmission periods and there are suggestions that transmission occurs in at least some parts, all year round (Hosie, 2004), thereby making eradication difficult.

Since eradication of footrot is not presently very feasible in the UK, most efforts are directed at reducing prevalence of footrot. Currently an industry agreed five-point plan is advocated (Clements & Stoye, 2014): (i) culling (repeatedly lame animals and/with misshapen feet), (ii) quarantine (incoming animals), (iii) treatment (catch, inspect and treat infected animals promptly and avoid trimming feet), (iv) avoid on-farm propagation of infection (decrease opportunities for sheep to sheep spread of infection through the ground), (v) vaccination (improve immunity in the flock and vaccinate to protect at high-risk periods).

# 1.1.7.1 Culling repeatedly lame animals

Culling of repeatedly lame animals is a potential control measure to mitigate footrot infection and re-infection in UK farms (Abbott & Lewis, 2005). 93% respondent farmers in a same survey expressed a negative opinion on the practicality of the policy. Indeed, less than 30% of UK sheep farmers cull lame animals due to ID/footrot (Wassink *et al.*, 2010). A recent survey of farms in the UK found no correlation between culling and reduced prevalence of lameness in sheep (Winter *et al.*, 2015a).

# 1.1.7.2 Quarantining newly purchased animals

To reduce and/or prevent footrot outbreaks in farms, one important precautionary measure is to ensure in-coming animals or additions to the flocks are quarantined until certified free of infection before introduction into farm stock. Animal quarantining has been successfully used in Australia as part of footrot eradication programme protocol and also linked to low prevalence of footrot in the UK (Wassink *et al.*, 2003b; Winter *et al.*, 2015a; Mills *et al.*, 2012).

In a postal survey in England and Wales (1999-2000), 7% and 51% of farmers who frequently isolated newly purchased and lame animals, respectively, recorded lower prevalence of lameness (Wassink *et al.*, 2003a). This is expected as re-infection and introduction of new serotypes

of pathogens are avoided. Nevertheless, the low success of isolating newly purchased animals was disappointing.

# 1.1.7.3 Treatment of clinically infected animals

Different formulations of antibiotics including Parenteral antibiotics (Penicillin, Streptomycin, Lincomycin, Spectinomycin, Oxytetracycline and Erythromycin) and topical (Oxytetracycline) have shown high efficacy in the presence of high bacterial load and severe infection, with approximately 90% success rate in the treatment of footrot lesions in clinical trials (Egerton, Parsonson & Graham, 1968; Venning, Curtis & Egerton, 1990; Grogono-Thomas *et al.*, 1994; Ware, Scrivener & Vizard, 1994; Jordan *et al.*, 1996; Laven, 2012; Green & George, 2008).

Parental antibiotics are not impeded by necrotic materials in footrot lesions during drug delivery to infected tissues (Jordan *et al.*, 1996) but are rapidly metabolised and excreted and therefore provide no long-lasting protection to re-infection (Abbott, 2000). Also, animals treated cannot be sold for human consumption within the drug withdrawal period (Jordan *et al.*, 1996).

On the other hand, the advantages of topical antibiotics include reduced systemic toxicity, reduced incidence of bacterial resistance as well as attainment of high antimicrobial concentration in the site of infection. They are more efficient when used without prior foot trimming (Kaler *et al.*, 2010a), thereby preventing bacterial shedding and contamination of the environment (Green *et al.*, 2007).

Therapeutic trimming/paring is carried out to remove loose or abnormal hoof tissues on diseased feet. Until the early 2000's, it was regarded as

both a preventive and therapeutic measure against footrot in the UK (Morgan, 1987; Wassink *et al.*, 2003a; Kaler, Wassink & Green, 2009). However, recent studies have shown therapeutic foot trimming to be detrimental; animals with trimmed hoof take longer to recover from lameness even after resolution of footrot lesions. This is because of the slow resolution of hoof horn when damaged (Kaler *et al.*, 2010b; Wassink *et al.*, 2010). Furthermore, a correlation between trimming and high incidence of footrot has been observed (Green *et al.*, 2007).

Another topical treatment regime used for the management of footrot (Morgan, 1987) is foot bathing. Formulations commonly used as footbathing solutions are formalin (3%-5% w/v) and zinc sulphate (10%-20% w/v)(Abbott & Lewis, 2005). As topical agents, their effect is limited to the epidermis of the interdigital skin, thereby preventing further invasion by bacterial aetiology. Generally, the protective effect of footbath formulations (especially formalin and zinc sulphate) is short-lived following application (Moorhouse, Skerman & Green, 1983).

# 1.1.7.4 Flock vaccination

Vaccines are used for both prophylactic and therapeutic purposes in disease management and control. They are administered to elicit protective antibody titres against pathogen antigenic components. In footrot, vaccines are targeted against fimbrial antigens of 10 serogroups consisting of 19 serotypes in *D. nodosus* (Egerton, 1973; Claxton, Ribeiro & Egerton, 1983; Day, Thorley & Beesley, 1985; Ghimire *et al.*, 1998). This is because cross protection between serogroups is low or non-existant and concurrent multiple serogroup infection within a flock is common (Egerton, Morgan &

Burrell, 1972; Claxton, 1985). Hence, the need for multivalent vaccine made of multiple serotypes.

Currently, there is only one formalin-killed, oil-based adjuvant multivalent vaccine (footvax, MSD, UK) licensed in the UK. This vaccine contains nine *D. nodosus* serotypes (A, B1, B2, C, D, E, F, G and H). In a study in the UK, the efficacy of the multivalent vaccine (footvax) to protect against footrot infection is reported to be 62% for a duration of five months during periods of outbreaks and high-challenge (wet conditions)(Duncan *et al.*, 2012).

In multivalent vaccines, studies suggest that antigenic competition among various serotypes result in lower immune response to individual component serotype when compared to similar serotypes in monovalent vaccine (Schwartzkoff *et al.*, 1993a; Raadsma *et al.*, 1994; Hunt *et al.*, 1995). Nonetheless, multi-component vaccines can provide variable degree of protection when administered to infected flocks (Dhungyel, Hunter & Whittington, 2014). Generally, immunity conferred by vaccination is short lived and will require a second or subsequent dose(s) (6-24 weeks) after the first administration depending on the severity of infection (Schwartzkoff *et al.*, 1993b).

# 1.2 Skin anatomy and immune response

# 1.2.1 Structure of the skin

The skin is a barrier protecting the body from the external environment and infectious agents (Marks, 2004; Lee, Jeong & Ahn, 2006). It consists of two anatomically related layers: the dermis and epidermis (Figure 1.3).

The outer epidermis consists of keratinized stratified epithelium that undergoes constant mitotic cell division for renewal (Burr & Penzer, 2005). The thickness of the epidermis is roughly kept in constant proportion by the sloughing-off of its most superficial layer through a process called desquamation. The predominant cells of the epidermis are keratinocytes. These cells are clustered at different layers of the epidermis is sub-divided into four layers: stratum basale (innermost), stratum spinosum, stratum granulosum and stratum corneum (outermost) (Figure 1.3)(Nestle *et al.*, 2009).



**Figure 1-3: Histological photomicrograph of The skin showing dermis and epidermis:** Epidermis is sub-divided into; SC stratum corneum, SGr stratum granulosum, SSp stratum spinosum, SB, Stratum basale. BM basement membrane separates the dermis and epidermis (Davenport *et al.*, 2014).

Keratinocytes are produced by the continuous mitotic activities of the single layer of cuboidal-columnar epithelial cells in the stratum basale, followed by progressive migration and maturation to the superficial layers of the epidermis (Burr & Penzer, 2005). At the stratum spinosum, which is located immediately above the stratum basale, keratinocytes differentiate into flattened polyhedral cells. Cells in this layer shrink slightly except at their point of attachment to adjacent cells, which then present as spiny projections during histopathology tissue processing. Hence, they are also termed spiny or prickle cells (Thibodeau & Patton, 2007). As keratinocytes migrate further through the overlying layer, stratum granulosum, they undergo programmed cell death (apoptosis), which involves fragmentation of DNA and loss of nuclei. Cells in this layer are typified by the presence of dark staining kerato-hyaline granules, which convert tonofilaments in the cells to keratin (Pringle & Penzer, 2002).

The stratum corneum is the most superficial layer of the epidermis containing cells saturated with keratin and characterised by loss of nuclei depicting cell death. Dead keratinocytes also called corneocytes, are shed off as squames. Although fewer in numbers, other cells in the epidermis are Merkel cells, Langerhans cells and melanocytes: they are responsible for sensory perception; immune surveillance and antigen presentation; and melanin production, respectively (Nestle *et al.*, 2009).

The epidermis is separated from the dermis by a thin basement membrane, lining two irregular, finger-like protrusions: dermal papillae, projecting underneath the epidermis and a corresponding epidermal ridges (rete ridges) projecting into the lower dermis (Figure 1.3).

The dermis is a framework of dense connective tissues, which confers rigidity, support and thickness to the skin. It is the deeper layer of the skin and tightly connected to the overlying epidermis by the basement membrane. It mainly consists of elastic and collagen fibres synthesised by flat, spindle-shaped cells with oval nuclei called fibroblasts, shown in figure 1.4 (Kupper & Fuhlbrigge, 2004). Aside from their role in wound healing

and protein synthesis in the extracellular matrix, fibroblasts also contribute to keratinocyte proliferation (El Ghalbzouri et al., 2004). Furthermore, fibroblasts are also involved in inflammatory response as initiators and modulators of pro-inflammatory activation such as cytokine secretion (Baglole et al., 2006; Flavell et al., 2008). Fibroblasts are also associated with the production of pro-inflammatory cytokines such as inlterleukin-1beta  $(1L-1\beta)$  and tumour necrosis factor alpha (TNF-a) in inflamed tissues (Smith et al., 1997). Indeed a recent study on the response of ovine dermal fibroblasts on heat-killed extracts of D. nodosus and F. necrophorum showed significant expression of IL-1 $\beta$  and TNFa (Davenport *et al.*, 2014). The dermis also accommodates specialised immune cell types like T and B-lymphocytes, neutrophils, natural killer cells and macrophages (Figure 1.4). These immune cells migrate into the dermis through a network of closely related blood vessels (Braverman, 1989) and lymphatic vessels when stimulated by pro-inflammatory cytokines (Huggenberger & Detmar, 2011).



# Figure 1-4: Schematic diagram of the skin showing immune response cells:

Keratinocytes respond to stimuli by cytokine production (e.g IL-1 $\beta$ , TNF), which activates dermal dendritic cells (DCs). Innate immune response in plasmacytoid (pDCs) are activated due to stress signal from keratinocytes, release of interferon-a (IFNa) which activate dermal DC. Fibroblast and natural killer T (NKT) cells produce TNF and contribute to local response. DCs promote clonal expansion of skin memory CD4<sup>+</sup> or CD8<sup>+</sup> T cells which can stimulate keratinocytes and fibroblast to amplify response (Kupper and Fuhlbrigge, 2004).

The release of inflammatory mediators (TNF- $\alpha$ , IL- $\beta$ , IL- $\beta$ ) by primary skin cells (fibroblasts) and the activation of other resident cells (Langerhans cells, dermal dendritic cells and masts cells) with immune functions trigger a cascade of activities. Activated LCs and DCs migrate to lymph nodes draining the region of infection, where they present antigens to memory T-cells. Cytokines secreted in response to skin injury act on endothelial tissues to activate the expression of adhesins (P-selectin, E-selectin and intercellular adhesion molecule 1, ICAM1) through the nuclear factor-kB  $(NF- \kappa B)$  pathway, this results in the recruitment and diapedesis of immune cells (macrophages, neutrophils, lymphocytes) into the dermis (Figure 1.5)(Kupper & Fuhlbrigge, 2004). Also, dermal lymphatic vessels transport infiltrated leucocytes and antigen presenting cells (APCs) from inflammatory sites to lymphoid organs, thereby enhancing specificity of immune response.



**Figure 1-5: Innate immune mechanisms in the skin** Image adapted (Kupper and Fuhlbrigge, 2004)

# **1.2.2 Immune functions of the skin**

Epidermal cells (keratinocytes) are the first to encounter insults when skin integrity is breached. Hence, they participate in the stimulation of innate immune response. First, they can express antimicrobial peptides that produce bacteriostatic/bactericidal actions against bacteria cells (Harder *et al.*, 1997; Braff *et al.*, 2005). Second, they express pathogen pattern recognition receptors (PPRs) such as Toll-like receptors TLRs). TLRs are also expressed on sentinel cells (macrophages, dendritic cells) and they recognise conserved molecules on pathogens collectively termed `pathogen

associated molecular pattern (PAMP)'. TLRs 1-10 have been identified in sheep with a range of bacterial ligands such as lipoproteins, lipoteichoic acids and lipopolysaccharides (Chang *et al.*, 2009; Mills, 2011; Regan *et al.*, 2013).

When activated, TLRs are thought to recruit adapter proteins (MYD88, Trap, Trif and Tram) from cell cytoplasm, which mediate the complex process of signalling through MyD88-dependent and TRIF-dependent pathways (Yamamoto *et al.*, 2002, 2003; Shigeoka *et al.*, 2007), resulting in the expression of cytokines such as IL-1, IL-6, tumour necrosis factor (TNF)(Albanesi *et al.*, 2005; Chang *et al.*, 2009), and recruitment of neutrophils, which is necessary in early innate defence against bacterial pathogens (Miller & Cho, 2011).

Once the innate immune system is activated, immune competent cells influenced by the various cytokines and chemokines, migrate to the draining lymph nodes through lymphatic vessels for antigen presentation to B- and T-cells (Kupper & Fuhlbrigge, 2004). Furthermore, cytokines and chemokines which serve as chemo-attractants facilitate the aggregation of immuno-competent cells to the site of injury (Borish & Steinke, 2003; Commins, Borish & Steinke, 2010). These cytokine and chemokine molecules (e.g IL-1, IL-6, IL-8, TNF-a and CCL5) attract and activate phagocytic neutrophils, lymphocytes, macrophages, Langerhans, dendritic cells macrophages and T-cells to the bacterial invasion sites (Pivarcsi, Nagy & Kemeny, 2005; Gröne, 2002).

In the dermis, fibroblast and dendritic cells contribute to the inflammatory response by producing TNF-a, IL-1 $\beta$  and IL-6 and promoting clonal expansion of T-cells, respectively (Kupper & Fuhlbrigge, 2004).

In the context of ovine footrot, the migration of immune-competent cells into the interdigital skin (especially the epidermis) results in the inflammation of the ovine foot (interdigital dermatitis) characterised by swelling, pain and redness (Roberts & Egerton, 1969; Egerton, Roberts & Parsonson, 1969). However, the resulting pressure on the hoof capsule due to increasing aggregation of immune cells in the epidermis has been hypothesised as the cause of under-running in footrot (Egerton, Roberts & Parsonson, 1969).

T-lymphocytes are migrating immune competent cells constituting 90% of the perivascular areas in the dermis. They are made up of CD4<sup>+</sup> T helper cells, CD8<sup>+</sup> cytotoxic T cells and T- suppressor cells. T-cells increase and migrate into the epidermis during inflammation to destroy invading pathogens (Salmon, Armstrong & Ansel, 1994).

# 1.3 Histology of interdigital dermatitis and footrot

#### **1.3.1** Histology of clinically healthy ovine feet

The clinically healthy ovine foot has been histologically linked with mild but active chronic pododermatitis mixed neutrophilic and lymphocytic exocytosis (Davenport *et al.*, 2014; Angell *et al.*, 2015).

# **1.3.2** Histopathology of interdigital dermatitis in ovine feet

Gross examination of ID lesions present as moist, swollen and erythematous (Parsonson, Egerton & Roberts, 1967). These descriptions indicate increased blood flow to the dermis and the subsequent cellular hyperplasia caused by infiltration of the epidermis by migrating leucocytes (neutrophils, lymphocytes and plasma cells) in response to bacterial invasion. De-granulating neutrophils (Figure 1.6) along with clusters of rod

shaped bacteria cells have also been described in the epidermis (Parsonson, Egerton & Roberts, 1967; Davenport *et al.*, 2014).



Figure 1-6: **H&E Histology photomicrograph ovine ID in interdigital skin:** Superficial mild to moderate dermatitis, exocytosis and granulated neutrophils (arrows), mag=100x. Source: (Davenport *et al.*, 2014).

# 1.3.3 Histopathology of footrot in ovine feet

Footrot lesion is defined by the presence of inflammation, necrotic tissues and the separation of the hoof from the underlying soft tissues along the hoof-horn interface (Beveridge, 1941; Deane & Jensen, 1955; Winter *et al.*, 2004). Histologically, leucocyte migration to the epidermis is similar to ID. Abscesses (degenerating neutrophils, cellular debris and plasma proteins), epidermal cell ballooning and oedema have been reported (Davenport *et al.*, 2014)(Figure 1.7).



**Figure 1-7: H&E photomicrographs of ovine footrot in interdigital skin: A:** FR, marked dermatitis, oedema (arrow), congested vessels (\*), **B:** FR epidermal degeneration (ballooning with nuclear pyknosis) ( $\Rightarrow$ ) and large amounts of degenerating neutrophils (pus) (\*) underneath the stratum basale. Source: (Davenport *et al.*, 2014). Mag=50x (A), 100x (B).

Although Davenport and colleagues described the migration of inflammatory cells (Davenport *et al.*, 2014), it was qualitative and did not measure degree of severity of migrating cells and other cellular lesions. The implication of this is that description of lesions is subjective. Furthermore, the study did not examine possible effects of inflammatory cell migration on skin components such as the basement membrane. Generally, skin tissue integrity throughout the disease condition is still poorly understood. Studies to evaluate more suitable protocols for better tissue image quality and quantification of cellular changes could result in more detailed analysis of the disease process, repeatability and standardisation of lesion description.

# **1.3.4 Significance of histopathology investigations in ovine** footrot

Histopathology descriptions of footrot lesions can form the basis of developing a histology scoring system, which, when combined with the clinical conditions of ovine feet, may show differences between healthy, ID and footrot. A histology scoring system already exists in bovine digital dermatitis (BDD), which combines clinical classification and histological classification of features in BDD lesions into mild, moderate and extensive (Rasmussen et al., 2012). Histological similarities exist between the features of BDD and ovine footrot e.g. micro-abscesses accompanied by neutrophils and mononuclear cells in hyperkeratotic epidermis (Bassett et al., 1990). Similar to ovine footrot, bovine digital dermatitis is proliferative and erosive (Laing & Egerton, 1978). Furthermore, aetiopathogenesis of bovine digital dermatitis (BDD) is polymicrobial and involves similar microbial biota to ovine footrot, such as *D. nodosus* (Greenough, 2007), Fusobacterium necrophorum (Schroeder et al., 2003) and Treponema (Yano et al., 2010). However, the two diseases differ in aetiology. While ovine footrot is primarily caused by *D. nodosus*, the definitive aetiology of BDD is yet unclear. It is however linked to *Treponema* (Beveridge, 1941; Evans et al., 2008; Knappe-Poindecker et al., 2013).

# 1.4 Development of a systematic histopathology scoring system

Histopathology scoring is a means by which biological systems are standardised for the purpose of data generation and involves assigning numerical values to identifiable biological parameters. A good scoring system is: (i) definable (ii) reproducible, and (iii) produce meaningful outputs (Crissman *et al.*, 2004).

To develop histopathologic scoring system certain basic benchmarks are required: (i) concealment of sample identities, (ii) detailed observation of samples, (iii) differential changes in lesions (parameters), (iv) scoring criteria/descriptions and (v) consistency in interpretation of parameters (Gibson-Corley, Olivier & Meyerholz, 2013).

To eliminate unconscious bias, sample identities are concealed from the examiner by comprehensive or grouped techniques, as reviewed by (Gibson-Corley, Olivier & Meyerholz, 2013). While comprehensive concealment involves masking individual samples with little background knowledge of the sample, grouped concealment involves categorisation of samples into groups in which the observer is aware of grouping pattern.

Also in histopathologic scoring, detailed sample examination is required to identify and differentiate lesion occurrences in samples from changes due to mechanical effects of treatment action (Shackelford *et al.*, 2002). Criteria for definition of lesions should be clear and devoid of elusive terms like minimal, moderate, or severe, which are highly subjective. To achieve consistency in scoring, single examiners are employed (Cross, 1998; Crissman *et al.*, 2004). However, it is also acceptable to have a review at the end of the study by the same examiner or a different examiner to validate results.

# 1.5 Data measurement scales in histopathology scoring

There are various ways to measure data depending on the study type and objectives of the study. Stevens (1977) described four types of data scales namely nominal, ordinal, interval and ratio (Stevens, 1977). Nominal data scale defines the presence or absence of a parameter e.g presence or absence of abscess in the skin. Ordinal method allocate parameters (lesions) based on their progressive severity into predefined categories. Ratio scale involves deriving data with a true zero point such as the morphometric measurement of anatomic structures of organisms (e.g length of the epidermis in the skin). The interval scale generate data from within a range with true points values e.g measurement of volume using a 100  $\mu$ l micropipette (0-100  $\mu$ l).

Nominal and ordinal scales are termed qualitative because they estimate parameters without a true quantitative reflection of the parameter. On the other hand, ratio and interval scale produce data that are exact measurement of parameters, hence, they are quantitative.

# **1.5.1 Incidence method**

Incidence method involves recording occurrences of a parameter (lesion) in tissue groups (Holland & Holland, 2011) as they occur in an observational study. Related methods are those of the binomial system (presence or absence of lesion) and affected percentage of tissues.

# 1.5.2 Ordinal method

This is a common method used in allocating lesions into predetermined categories (e.g 0-4) in the ascending order of severity of the lesion (Stevens, 1977). Ordinal scoring is done by defining criteria for each category represented by ascending numerical numbers, based on their progressive severity. Each examined tissue is then allocated to a category it best fits.

Different approaches are possible in the ordinal scoring with each having its merits and demerits. Some investigators aggregate multiple lesion types or different anatomical sections of the tissue as ordinal category (Gibson-Corley, Olivier & Meyerholz, 2013). Alternatively, others score different lesions or anatomical sites of tissues separately. The former are

called 'lumpers while the latter 'spliters'. Although lumping is less labourious, more efficient and fast when tissues have no distinguishable distinction, in comparison, splitter technique is more labourious but lesionspecific and repeatable (Cross, 1998).

Another ordinal approach is to decide on specific number of random fields within the microscopic view for each tissue, score each of the fields and then take the average of all random fields score for the tissue. In this instance, the median is the more appropriate measure of central tendency rather than the mean, which is better suited for the interval and ratio data (Gibson-Corley, Olivier & Meyerholz, 2013).

# **1.6 Summary**

Despite the significant improvement in our knowledge of ovine footrot, the disease still remains a burden in the UK in the context of management cost and welfare. Although the primary aetiology has been identified as *D. nodosus*, the role of other bacterial components as well as exact pathogenesis of the disease is still unclear. Histological examination has shown lesions including cell ballooning, inflammatory cells infiltration and micro-abscesses in footrot condition, however, severity of these lesions have not been matched with clinical status of ovine foot. Therefore, our understanding of the changes occuring during the disease process and the host immune responses to bacterial localisation and load is limited. Furthermore, there is the need to develop a systematic histopathological scoring system that can be used to correlate host responses with clinical disease severity and localisation of bacterial infection.

# 1.7 Hypothesis and aims of the study

# 1.7.1 Hypothesis

The tissue damage in interdigital dermatitis (ID) and footrot allows the invasion and colonisation of the epidermis and, to an extent, of the dermis by a range of bacteria in addition to *D. nodosus*, the causative agent of footrot. We hypothesise that, various invasive bacteria contribute to eliciting a strong inflammatory host response leading to the histopathology seen.

# 1.7.2 Aims

1. To develop a robust histopathological scoring system that can be used to correlate host responses with disease state.

2. To determine correlation between severity of histological lesions and disease state

3. To determine the relationship between pro-inflammatory cytokine (IL-1β), virulent *D. nodosus* load and inflammatory cell infiltration.

4. To determine the localisation and abundance of bacteria including *D. nodosus*, *F. necrophorum* and eubacteria in progressive layers of the ovine interdigital skin in clinically healthy and footrot interdigital skin.

# **Chapter 2 : Materials and Methods**

Two distinct but related studies were undertaken in this project. The first study explored micro-lesions in ovine interdigital skin to determine whether variation exists in three clinical conditions (healthy, interdigital dermatitis and footrot). Methods explored in the first study are captured in sections 2.1 to 2.4. In study two, bacterial localisation in successive ovine interdigital skin layers were investigated across two clinical conditions (healthy and footrot) and the methods are captured in section 2.5. Data and statistical analysis of both studies are contained in section 2.6.

# 2.1. Samples and experimental design

All samples were collected from Melton abattoir, Leicestershire, UK, between October and December 2013 for the first study and 2<sup>nd</sup> March, 2016 for the second study. Samples for the first study were collected and processed by other investigators prior to the commencement of this investigation with full ethical permissions.

The study design was divided into two: i) identification and grading of histology lesions and ii) bacterial colonisation and localisation in skin layers. Sample sets in different sections vary and are, therefore, presented in the result sections.

In both studies, ovine feet were collected post-slaughter at the abattoir, categorised according to clinical conditions into healthy, interdigital dermatitis (ID) and footrot (Parsonson, Egerton & Roberts, 1967)(Table 2.1). Feet were scored for conformation and/or clinical presentation (ID,

footrot lesions) by two scorers independently in order to reduce scorer bias.

In the first study, samples were collected from all four feet of the same animals in some cases while in others less than four feet were sampled since feet had to be followed in the abattoir line during carcass processing. Sampled feet were cleaned and disinfected with 70% ethanol (Fisher Scientific, UK) prior to biopsy collection using 6mm disposable sterile biopsy punches (National Veterinary Services). In the second study, samples were collected at one foot per animal. To decontaminate the ovine skin feet surface, soil debris and mud were removed using clean tap water and, washed with disinfectant (chlorhexidine gluconate 4% w/v (40 mg/ml) and wiped with 70% ethanol (Fisher Scientific, UK). Each foot was dried with a clean paper towel before biopsy collection.

To collect biopsies, 6 mm disposable sterile biopsy punches (National Veterinary Services) were used to collect biopsy samples from the interdigital space of healthy and footrot infected feet. Biopsies were briefly immersed in 70% ethanol (Fisher Scientific, UK) to reduce surface bacteria and inhibit multiplication. Biopsies were then submerged into 3 ml of sterile 30% sucrose (150 g sucrose dissolved in 500ml sterile phosphate buffer solution) overnight to cryo-protect (reduce water content and prevent ice crystal formation during snap freezing) biopsy tissues.

Since the samples in both studies were collected from the abattoir, the sample history was unknown.

| Score type                         | Area of the foot                       | score | Description  |
|------------------------------------|--|-------|--|
| Conformation                       | Sole and heel/wall of each digit       | 0     | Undamaged sole and heel area   |
|                                    |  | 1     | Mildly damaged sole and heel (<25%)  |
|                                    |  | 2     | Moderately damaged/misshapen sole and/or heel area of digit (>25%)   |
|                                    |  | 3     | Severely damaged/misshapen sole and/or heel area of the digit (>75%)   |
| Interdigital<br>dermatitis<br>(ID) | Interdigital space                     | 0     | Clean interdigital region with no<br>dermatitis (scald) lesions or fetid<br>smell                                      |
|                                    |  | 1     | Slight ID, irritation of the skin but dry  |
|                                    |  | 2     | Slight interdigital dermatitis with a fetid smell (<5% affected)   |
|                                    |  | 3     | Moderate interdigital dermatitis with a fetid smell (5 to 25% affected)  |
|                                    |  | 4     | Severe interdigital dermatitis with a fetid smell (>25% affected)  |
| Footrot                            | Sole and<br>heel/Wall of each<br>digit | 0     | No underrunning of the heel and sole area  |
|                                    |  | 1     | Active footrot lesion with a degree of<br>under-running of the heel and/or sole<br>area of the digit (<50%)            |
|                                    |  | 2     | Active footrot lesion with a marked degree of under-running of the heel and/or sole area of the digit (>50% and <100%) |
|                                    |  | 3     | Active footrot lesion with complete<br>under-running of heel and/or sole<br>(100%)                                     |

 Table 2-1: Ovine foot scoring scheme (Parsonson, Egerton & Roberts, 1967)

# 2.2 Histology

# 2.2.1 Fixation, embedding and sectioning

# 2.2.1.1 Fixation, paraffin embedding and sectioning

Formalin fixed biopsies were provided embedded in wax overnight in a histology cassette. Post fixation they had been dehydrated through a series of graded ethanol and xylene and embedded in paraffin wax. To expel air bubbles observed in some embedded samples, they were melted and reembedded ensuring absence of air bubbles. Also, care was taken to ensure appropriate orientation of samples to allow the identification of epidermaldermal interface during sectioning.

In order to section tissue blocks for the current investigation, paraffin wax embedded tissues for the trial study were soaked in 10% v/v ammoniated water. This protocol was developed and adapted from the previous study (Davenport *et al.*, 2014). Embedded tissues were trimmed, and 6 µm thick sections were cut from each block with a microtome (Leica RM2255, Germany). Serial sections were floated in a 37°C water bath (to remove folds) and mounted on polysilinated microscope glass slides (Thermo-Scientific, Menzel Gläser Polysine®, Germany). A total of 30-40 slides from each sample, with each containing 3-4 sections were collected and dried at room temperature overnight. Slides were stored at room temperature until ready for histochemical staining. To save time due to the difficulty in sectioning through biopsies, mainly consisting of hoof/soft tissue interface, subsequent sectioning of tissues was carried out by Aziza Alibhai, a technician in the anatomy unit of the School of Veterinary Medicine and Science, University of Nottingham.

# 2.2.1.2 Fixation, OCT embedding and cryo-sectioning

Samples briefly incubated in 70% ethanol and overnight 30% sucrose solution were embedded as follows: OCT compound (VWR International, Belgium) was dispensed into 2/3<sup>rd</sup> volume of plastic embedding mould (cylindrical handle of cut Pastettes) at room temperature and allowed to solidify (serving as a base for biopsy to be placed). Biopsies were carefully placed on the solidified OCT ensuring that appropriate orientation of each biopsy was achieved (dermis facing the bottom of the mould). Additional OCT was dispensed into the mould until the biopsy was submerged and then the mould was immediately transferred into a beaker of isopentane (2-Methylbutane) (Sigma-Aldrich, St. Luis, USA) which has been precooled in liquid nitrogen until the biopsy was snap frozen. Each frozen biopsy was removed from liquid nitrogen and transferred into pre-labelled cellophane bag and preserved in a -80 °C freezer until ready to be sectioned.

Alternating thick (40µm) and thin (9 µm) transverse sections were cut beginning from the dermal layer across biopsies on the cryostat machine (Shandon, UK) with sample chamber and cryobar temperature set at -20°C. The cryostat blade was cleaned with 70% ethanol before each thick section was cut to prevent bacteria transfer between sections. Thick sections intended for DNA extraction to determine bacterial abundance were preserved in 0.5 ml RNAlater<sup>®</sup> (Sigma-Aldrich, St. Luis, USA) at room temperature and incubated overnight before DNA extraction. Corresponding alternate thin sections intended for histology analysis were mounted on polysilinated microscope glass slides (Thermo-Scientific,

Menzel Gläser Polysine®, Germany). Sections were cut from the dermal layers towards the epidermis (to prevent the transfer of bacteria from the contaminated epidermal surface to the supposedly sterile dermis).

Two thin sections were placed on each slide and then preserved in -80°C freezer prior to histological staining. Lost sections during sectioning were noted to enable accurate determination of tissue depths.

Slides were later removed from the -80°C freezer and allowed to warm at room temperature for 30 minutes before histochemical staining (haematoxylin and eosin, H&E).

# 2.2.2 Haematoxylin and Eosin (H&E) Staining

H&E is a histopathological staining technique consisting of two dyes (haematoxylin and eosin). Haematoxylin is a basic dye which causes acidic structures in the cell/tissue such as cell nuclei and keratohylin granules, to change to blue, while the counter stain eosin is an acidic dye which stains eosinophilic structures like extracellular proteins with different shades of red, pink and orange. This staining technique allows for the visualisation of tissue and cellular changes, hence, it was used in this study.

In the H&E staining protocol for the first study, tissue sections were dewaxed in xylene (Fisher Scientific, UK) for 5 minutes and then rehydrated in graded ethanol (Fisher Scientific, UK), from absolute ethanol (100%) through dilutions (mixed with distilled water) of 90% and finally 70%, respectively for 5 minutes each. Following rehydration, tissue sections were rinsed in distilled water for 5 minutes and immersed in haematoxylin (Fisher Scientific, UK) for 2.5 minutes to highlight cell nuclei. Thereafter, tissue sections were washed in tap water for 3 minutes, differentiated in

1% v/v industrial methylated spirit (IMS, acid alcohol) (Fisher Scientific, UK) to remove unbound stains and then immersed in 10% v/v ammoniated water to enhance the blueing effect of haematoxylin. For both steps, slides were immersed for 15 seconds each. Representative slides from the sample set were guickly examined to confirm whether the haematoxylin staining was adequate on a brightfield setting using a Ceti compound microscope (Labpro, UK) set at 10x magnification. Section slides were rinsed in distilled water for 1 minute, counter-stained in eosin (Fisher Scientific, UK) for 4 minutes, and then rinsed in distilled water (dH<sub>2</sub>O) for 5 minutes before they were dehydrated through ascending grades of ethanol dilutions (distilled water): 70%, 90%, and 100%, for 5 minutes each. Finally, the tissue sections were cleared (removal of excess alcohol in tissue) in xylene for an additional 5 minutes and mounted with dibutyl phthalate (DPX; Fisher Scientific, UK) and glass coverslips (Fisher Scientific, UK). Following the optimisation of the H&E staining technique, slides for the preliminary study were stained and analysed while subsequent staining was carried out by the technician (Aziza Alibhai) in the School of Veterinary Medicine and Science, University of Nottingham.

Similar H&E staining protocols for the paraffin sections in the first study were adopted for the cryosections in the second study. However, rehydration steps in graded concentrations of ethanol were excluded since they are frozen sections. Slide sections were stained in a humidifying chamber placed horizontally to prevent tissue sections from floating away during staining procedure.

# 2.2.3 Periodic Acid Schiff (PAS) staining

PAS is a staining technique that aids in the detection of aldehyde groups produced when glycoproteins in the basement membrane is oxidized. This membrane separates the epidermis and dermis in the skin. The technique accentuates the aldehyde groups produced, due to the oxidation of some carbohydrate and glycogen in the basement membrane when treated with periodic acid (Sheehan and Hrapchak, 1980). Further treatment of oxidised tissues with Schiff reagent (basic fuchsin) results in the production of a red/pink colour.

To visualise the basement membrane in sample tissues, section slides were dewaxed and re-hydrated as described above (H&E protocol section 2.2.2). Following rehydration and rinsing of slide sections in distilled water (5 minutes), sections were immersed in a periodic acid solution for 5 minutes, washed in running water for 3 minutes and then rinsed in distilled water for 5 minutes. Sections were incubated in Schiff's reagent for 15 minutes, washed and rinsed in running and distilled water for 3 and 5 minutes respectively. Furthermore, slides were immersed in haematoxylin for 3 minutes, washed for 3 minutes in tap running water and rinsed in distilled water for 5 minutes. Sections were then dehydrated through ascending concentrations of graded ethanol: 70%, 90%, and 100%, respectively for 5 minutes each. Sections were cleared (through the removal of excess alcohol in tissue) in xylene for an additional five minutes and mounted with dibutyl phthalate (DPX; Fisher Scientific, UK) and glass coverslips (Fisher Scientific, UK).

# 2.2.4 Masson's trichrome staining

To examine the dermal collagen abundance, Masson's trichrome (MT) staining technique was used since it differentiates collagen and other structures in the dermal matrix. A commercial MT kit (Polysciences, USA) was used and manufacturer's instructions were followed with few modifications.

To demonstrate dermal collagen location, slides were de-waxed in xylene (Fisher Scientific, UK) for 5 minutes and then rehydrated in decreasing grades of ethanol: 100%, 90% and 70% each for 5 minutes. Rehydrated sections were incubated in Bouin's solution for 1 hour at 60°C in an oven (Thermo-Scientific, UK) to enhance optimal staining by linking dye molecules to cellular features (mordant). Slide sections were rinsed in running water for 5 minutes to remove the excess yellow colour of mordant. To accentuate cell nuclei, section slides were treated with fresh mixture of Weigert's iron haematoxylin (A and B) solutions at a ratio of 1:1 for 2 minutes and then washed in running water for 5 minutes. To highlight acidophilic features like erythrocytes, cytoplasm and collagen, section slides were incubated in 15% Biebrich scarlet-acid fuchsin dye for 25 seconds and rinsed in distilled water for 5 minutes. Section slides were further treated with 5% phosphotungstic acid for 5 minutes as a dual purpose agent: i) as a Biebrich scarlet-acid fuchsin selective mordant, which binds haematein and enhances selective staining of fibrin and connective tissues; ii) as a decolouriser which allows diffusion of Biebrich scarlet-acid fuchsin out of collagen tissues. Sections were then incubated in light-green dye solution for 25 minutes to stain collagen green. The sections were then rinsed in distilled water for 5 minutes and differentiated

in 1% acetic acid solution for 1 minute to remove excess colour. Acetic acid was rinsed off in distilled water for 5 minutes prior to mounting. To mount the slides, stained slides were dehydrated through ascending grades of ethanol: 70%, 90%, and 100% respectively for 5 minutes each. Finally, slide sections were cleared (excess water was removed from the sections) in xylene for an additional 5 minutes and mounted with glass coverslips using dibutyl phthalate and xylene (DPX) (Fisher Scientific, UK).

#### 2.3 Microscopy and image acquisition

In the first study, photomicrographs of five non-overlapping areas containing histological lesions each were captured from the epidermis and dermis of the skin. The inclusion criterion for image capture from sample slides was the availability of sufficient tissue matrix (>75% of the field of view (FOV)) which could yield five non-overlapping images from tissue sections. Subsequently, lesions of interest were identified and graded using grading systems developed for the purpose of this study.

In the second study, photomicrographs of serial cryosections on slides were captured across different layers of the skin. In both studies, images were captured with a bright-field microscope (Leica DM500B, Germany) at 40x (first study) and 5x (second study) magnification followed by processing on the accompanying Leica Application Suite (LAS) image capture software (Leica, Germany) fitted on the microscope.

# 2.4 Analysis of histology photomicrographs (lesions, epidermal and dermal tissues)

In order to quantify areas of ballooned cells and dermal collagen, Image Pro 6.3 (Media Cybernetics, USA) software was used to determine parameters of interest (cell ballooning and dermal collagen).

# 2.4.1 Quantification of ballooned cells in the epidermis

To determine the area of ballooned cells per cross sectional area in the epidermis, photomicrographs were taken, saved in a Tagged Image File Format (TIFF) and uploaded into the analysis software Image Pro 6.3 (Media Cybernetics, USA). Each image was calibrated for the measurement of ballooned cells area in absolute values ( $\mu$ m<sup>2</sup>) (see Image-Pro protocol in Appendix 1). Ballooned cells were identified and manually selected by using the polygon icon on the software to trace around each ballooned cell or group of cells.

Once all ballooned cells in the FOV were traced and measured, acquired data were exported into an Excel worksheet and the percentage area of ballooned cells per photomicrograph calculated (total area of ballooned cell(s)/total area of epidermal cross section x 100).

# 2.4.2 Determination of collagen abundance in the dermis

Similar to the previous description, MT stained photomicrographs of the dermis were uploaded into Image Pro 6.3 (Media Cybernetics, USA). To delineate the pixels in the photomicrographs, limits were set for parameters such as "perimeter", "Area" and "Per Area" by trial and error and a colour (red) on the software was selected to represent collagen (green) tissues.

Once green coloured-tissue fibres on the photomicrograph were recognised by the software, corresponding data representing coloured area is generated and exported to the Excel worksheet and percentage area of dermal collagen per photomicrograph was calculated (per area proportion x 100).

# 2.4.3 Estimation of epidermal and dermal tissues on

# photomicrographs of frozen sections

In order to estimate the proportions of epidermal and dermal tissues on photomicrographs, Image Pro 6.3 software (Media Cybernetics, USA) was used to create different proportions of epidermal/dermal tissue templates. Estimates of tissues were then made based on these templates.

To create epidermal/dermal tissue proportion estimates, photomicrographs saved in a Tagged Image File Format (TIFF) were uploaded into the analysis software. Each image was calibrated for the measurement of epidermal/dermal area in absolute values ( $\mu$ m<sup>2</sup>). To delineate the pixels in the photomicrographs, limits were set for "Area" with colour (red) on the software selected to represent dermal tissues and yellow to represent epidermal tissues.

Once red or yellow coloured-tissue fibres on the photomicrograph were recognised by the software, corresponding data representing each of the tissue area was generated. Data were exported to the Excel worksheet and a percentage area of epidermal and dermal estimate per photomicrograph was calculated [(Area estimate of selected tissue/total tissue estimate) x 100].

#### 2.5 DNA extraction and quantification

DNA extraction was carried out as previously published (Maboni *et al.*, 2016). Firstly, 2 ml micro-centrifuge tube containing thick sections (40 µm) and RNAlater was centrifuged and supernatant (RNAlater) decanted. Tissue sections were washed in 50 µl sterile distilled water (once) and digested in 180 µl buffer ATL (tissue lysis buffer) and 20 µl proteinase K (QIAGEN, Hilden, Germany) for 2 h at 56 °C in a bioshaker (Bioshake IQ, WildTech). Further DNA extraction procedures were carried out as described in the QIAamp cador®kit (QIAGEN, Hilden, Germany) manufacturer manual. Genomic DNA was eluted in 60 µl elution buffer and DNA concentration measured using dsDNA HS-assay kit on qubit 3 fluorometer (Invitrogen, USA).

The qubit genomic DNA quantification involved initial constitution of fresh working solution which was made up of: 200  $\mu$ l of Qubit dsDNA buffer (HS) and 1  $\mu$ l of dye per sample. The mixed working solution components were briefly vortexed in a sterile falcon tube at room temperature ready to be used. To calibrate the qubit equipment, two Qubit assay tubes (1 and 2) were set up and 190  $\mu$ l of prepared working solution was dispensed into each tube. 10  $\mu$ l standard 1 and standard 2 reagents (provided in Qubit kit) were added into tubes 1 and 2 respectively and incubated for 5 minutes at room temperature before they are measured on the Qubit machine. To measure individual sample concentrations, 198  $\mu$ l of working solution was added.

The mixture is incubated for 5 minutes in ice and then measured on the Qubit fluorimeter machine.

#### 2.5.1 Selected bacterial DNA for standard curve preparation

To construct standard curves for bacteria quantification, DNA from *D. nodosus* (provided by Marianne Gilhuus) and *F. necrophorum* (Dr Laura Green, University of Warwick, UK) were used as standards. In addition, *Streptococcus uberis* was used (provided by Dr Sharon Egan, University of Nottingham) for total eubacteria quantification.

To extract DNA from *S. uberis*, a loopful of pure *S. uberis* broth culture was transferred into 10 ml Brain Heart Infusion (BHI) liquid media in a sterile falcon tube and incubated at 37°C overnight (18-24 h). Culture was dispensed into 1.5 ml Eppendorf tubes and cells harvested by centrifugation (12000 rpm for 5 mins at room temperature). Supernatant was discarded and bacteria cell pellets were washed by re-suspension in 0.5 ml TE buffer [10 mM Tris, 5 mM EDTA (pH 7.8)] and centrifuged (12000 rpm for 5 mins at room temperature). Supernatant was removed and bacteria pellets further re-suspended in freshly prepared cell wall disruption buffer containing 60 µl of mutanolysin (5000 units/ml, Sigma-Aldrich, UK, final concentration of 100  $\mu$ g/ml) and 25  $\mu$ l lysozyme (10 mg/ml, Sigma-Aldrich, UK). The mixture was incubated at 37 °C for 30 minutes. To lyse bacteria cells, 20 µl SDS (20% w/v in 50 mM Tris, 20 mM EDTA at pH 7.8) in addition to 3  $\mu$ l proteinase k (20 mg/ml, QIAGEN, Hilden, Germany) were added followed by 1h incubation at 37°C. Further DNA extraction steps were achieved with QIAamp cador®kit (QIAGEN, Hilden, Germany) according to manufacturer's instruction.
#### 2.5.2 Real time PCR quantification of specific bacteria DNA

Target genes, primers and probes used in this study have been previously published (Table 2.2). All samples were quantified in triplicates using PCRLightcycler 480 (Roche Applied Science, Penzberg, Germany).

To construct standard curves for bacteria quantification, 10-fold serial dilutions of *S. uberis* (2 - 0.00002 ng/µl), *D. nodosus* (1 - 0.00001 ng/µl) and *F. necrophorum* (1 - 0.00001 ng/µl) DNA were performed for eubacteria, *D. nodosus* and *F. necrophorum* assays, respectively.

PCR reaction volumes for eubacteria and *D. nodosus* were 20 µl (Strub *et al.*, 2007; Frosth *et al.*, 2012) while *F. necrophorum* was 25 ul (Frosth *et al.*, 2015). Reaction constituents and conditions are shown (Tables 2.3, 2.4).

Data were analysed using the Light-Cycler<sup>®</sup>480 software (Roche Applied Science, UK). The cycle crossing point (Cp) is the point at which fluorescence was first detected above base level by the light-Cycler machine. Data were exported into the Light-Cycler<sup>®</sup>480 software (Roche Applied Science, UK) and programs settings adjusted accordingly. To achieve a common linearity, all sample sections in one qPCR plate run were treated as one experiment. The software automatically generates the Cp and concentration values of target bacteria DNA in each sample section. Data was exported into Microsoft Excel 2013 software and mean of each sample section replicates calculated.

Furthermore, log linear regression analysis of Cp values, standard deviation and coefficient of variation were computed for each concentration of bacteria standard. Efficiency of assays were determined for each qPCR experiment on ten-fold serial dilution of bacteria DNA (standard) used.

Efficiency was determined by using the slope in the equation obtained in the linear regression of Cp against concentration. Determination of the limit of quantification was based on high efficiency, low coefficient of variation and acceptable Cp value.

| Bacteria<br>assay     | Target<br>gene | Function | Sequence 5`-3`                               | Amplicon<br>length (bp)  | Sequence position   | Reference                     |
|-----------------------|----------------|----------|--|--|---|-------------------------------|
| Eubacteria            | 16S rRNA       | Forward  | TCCTACGGGAGGCAGCAGT                          | 466  | 333-351   | (Strub <i>et al.</i> , 2007)  |
|                       |                | Reverse  | GGACTACCAGGGTATCTAATCCTGTT                   |  | 774-799   |                               |
|                       |                | Probe    | 6-FAM-CCAGCAGCCGCGGTA-MGB                    |  | 511-525   |                               |
| D. nodosus            | 16S rRNA       | Forward  | CGGGGTTATGTAGCTTGCTATG                       | 84 1337453-<br>1337474<br>1277483-<br>1277504<br>929056-929077 |   | (Frosth <i>et al.</i> , 2012) |
|                       |                | Reverse  | TACGTTGTCCCCCACCATAA                         |  | 1337391-<br>1337410<br>1277421-<br>1277440<br>928994-929013     |                               |
|                       |                | Probe    | FAM-TGGCGGACGGGTGAGTAATATATAG<br>GAATC-TAMRA |  | 1337415-<br>1337444<br>1277445-<br>1277474<br>929018-<br>929047 |                               |
| F.<br>necrophoru<br>m | gyrβ           | Forward  | AGGATTGCATGGAGTAGGAA                         | 306  | 27-46   | (Frosth <i>et al.</i> , 2015) |
|                       |                | Reverse  | CCTATTTCATTTCGACAATCCA                       |  | 311-332   |                               |
|                       |                | Probe    | FAM-TCTACTTTGGAGGTTGGAGAAACAAC-<br>TAMRA     |  | 160-185   |                               |

#### Table 2-2: Quantitative PCR (qPCR) primers and probe for target genes

Eubacteria primers designed were from sequence homology from several bacterial genera (Strub *et al.*, 2007). Hence, the regions of identity within the 16S rDNA were manually assessed, with *E. coli* (KY007585.1) having the largest amplicon length and therefore, presented as representative member of the group in the table. Three copies of 16S rRNA gene (3 operons) exist in the *D. nodosus* genome, hence the triplicate sequences for it primers and probe.

| Master                 | Eubacteria                 | D. nodosus                 | F. necrophorum             |
|------------------------|----------------------------|----------------------------|----------------------------|
|                        | Vol/reaction(µl)<br>(conc) | Vol/reaction(µl)<br>(conc) | Vol/reaction(µl)<br>(conc) |
| 2xTaqMan master<br>mix | 10                         | 10                         | 12.5                       |
| Primer F               | 1 (6 µM)                   | 1 (8 µM)                   | 1 (10 µM)                  |
| Primer R               | 1 (6 µM)                   | 1 (8 µM)                   | 1 (10 µM)                  |
| Probe                  | 1 (3 µM)                   | 1 (3 µM)                   | 0.25 (10 µM)               |
| BSA                    | -                          | -                          | 0.125(0.1 mg/ml)           |
| PCR grade water        | 2                          | 4                          | 7.625                      |
| Template DNA           | 5                          | 3                          | 2.5                        |
| Total reaction volume  | 20                         | 20                         | 25                         |

Table 2-3: PCR assay reactions and conditions

Table 2-4: Real time PCR reaction conditions

| Reaction conditions | Cycle     | Temp ⁰C | Time mins<br>(secs) |
|---------------------|-----------|---------|---------------------|
| Eubacteria          | Hot start | 50      | 2                   |
|                     | 1         | 95      | 10                  |
|                     | 40        | 95      | 15 secs             |
|                     | 1         | 60      | 60                  |
| D. nodosus          | 1         | 95      | 10                  |
|                     | 45        | 95      | 15 sec              |
|                     | 1         | 60      | 2                   |
| F. necrophorum      | 1         | 95      | 10                  |
|                     | 55        | 95      | 30 secs             |
|                     | 1         | 60      | 2                   |

# 2.6. Statistical and data analysis

All statistical analyses were performed on Graphpad Prism version 6 for windows (Graphpad software, USA) and Microsoft Excel.

# 2.6.1 Statistical and data analysis for histological lesions study

Resulting data are presented as frequencies, mean and percentages. Categorical data within and between clinical conditions and histological lesions were compared using Fisher exact and Chi-square analysis since these analyses involve two nominal variables (clinical conditions versus histological lesions). Analysis of continuous data that were normally distributed was by the student t-test (mean of 2 groups) or Kruskal wallis as well as analysis of variance (ANOVA, one-way) followed by Tukey's posthoc multiple comparison test. Analysis was taken as significant when p < 0.05.

#### 2.6.2 Statistical and data analysis for bacterial localisation study

Resulting data are presented as frequencies, mean and percentages. Analysis of continuous data that were normally distributed was by analysis of variance (ANOVA, 2-way) followed by Sidak's post-hoc multiple comparison test. Analysis was taken as significant when p < 0.05. Eubacterial load, skin depth and number of hair follicles/sectional area were compared using Spearman rank correlation since these data were not normally distributed.

To be able to compare bacteria localisation and load across same depth in different samples, bin groups of skin depth (range 0-200  $\mu$ m) were created. Bacteria averages from corresponding sections of skin depth were allocated into each appropriate bin and standard number of bins for all samples analysed. Bacteria prevalence and load analysis were based on same bins across each samples in different clinical conditions.

# Chapter 3 : Development of scoring system for histopathological lesions in interdigital dermatitis and footrot

# **3.1 Introduction**

Footrot is a disease typified by histological changes in tissue and cellular morphology. Most studies employing microscopy have described characteristic histopathological lesions in footrot to include microabscesses, congestions and inflammatory cell infiltration (Thomas, 1962; Knappe-Poindecker *et al.*, 2014b; Davenport *et al.*, 2014). However, other lesions including cell ballooning, parakeratosis, and haemorrhages have also been mentioned (Thomas, 1962).

To delineate cellular morphology, tissue sections are processed and stained. The choice of applicable staining technique is dependent on the tissue/cellular structure of interest, and may include techniques such as haematoxylin and eosin (H&E), Periodic acid Schiff (PAS) as well as Masson's trichrome (MT) which can impact contrasting colours on different tissue features, thereby enhancing lesion identification. These techniques have been used throughout this chapter.

Despite being over a century old, the haematoxylin and eosin (H&E) staining technique is still the most widely used in histopathology for investigating cellular integrity during disease pathogenesis (Cook, 1997). H&E results in a blue colour being visualised on cell nuclei whilst cytoplasm and extracellular matrix stain varying intensities of pink (Fischer *et al.*, 2008). The basement membrane separating the epidermis from the dermis of the skin is stained pink with periodic acid Schiff (PAS) while the Masson's

trichrome stains collagen fibres green/blue depending on the counter stain used (Wulff, Hafer & Cheles, 2004).

Although stains accentuate tissue structures and aid identification, they may be inconsistent in different tissue conditions (Kuru, 2014). Hence, optimisation is required for differing tissue types in order to obtain appropriate staining effects.

Tissue staining allows for adequate characterisation of morphological changes in tissues (lesions) and may serve as basis for the development histological grading systems to assess severity of change.

Histopathology scoring is an expression incorporating the standardisation of biological systems. It defines biological systems for the purpose of generating data and comparison between researchers. It involves assigning numerical values to identifiable biological parameters such as: parakeratosis, haemorrhage and presence of immune competent cells (neutrophils, lymphocytes and macrophages) in tissues. A good scoring system is: (i) definable (ii) reproducible, and (iii) produce meaningful outputs (Crissman *et al.*, 2004).

Currently, there is lack of information on the quantitative assessment of histology lesions in the different stages of ovine footrot as obtained in bovine digital dermatitis, a similar disease of the bovine feet (Döpfer, Holzhauer & Boven, 2012).

In the context above, this study aimed to obtain information through literature searches on various histology lesions of ovine footrot and other similar diseases of the ovine and bovine feet. Based on the identified lesions, grading system were then designed to enable quantification histological lesions observed in ovine footrot (Table 3.1).

This preliminary study examined the following:

- Optimisation of assays for the standardization of three staining techniques (H&E, PAS and MT) against interdigital skin tissues for optimum contrast and identification of tissue and cellular morphology.
- Assessment of morphological changes observable in tissue sections from clinically healthy, interdigital dermatitis (ID) and footrot conditions.
- iii. Development of semi-quantitative histopathological scoring systems to measure lesions including inflammatory cell infiltration in clinically healthy, interdigital dermatitis and footrot disease conditions.
- iv. Demonstration of the reproducibility of the semi-quantitative histopathological scoring system in section (iii) for subsequent application in a larger sample set.

# Table 3-1: Footrot histopathology scoring template

| S/N | Parameter  | Characteristics   | Scoring scale   | Mag. | Staining<br>technique | References   |
|-----|--|---|---|------|-----------------------|--|
| 1   | Parakeratosis  | Retained nuclei remnant in stratum corneum  | Yes/No  | 400X | H&E                   | (Rasmussen <i>et al.</i> , 2012)                                 |
| 2.  | Abscesses  | Aggregation of inflammatory cells +<br>cellular debris + with/without bacterial<br>presence + fibrotic tissue walling   | Yes/No  | 400X | H&E                   | (Davenport <i>et al.</i> , 2014)                                 |
| 3.  | Congestion   | Increased red blood cells in vessels  | Yes/No  | 400X | H&E                   | (Mendes <i>et al.</i> , 2013;<br>Davenport <i>et al.</i> , 2014) |
| 4.  | Haemorrhage  | Red blood cells outside blood vessels   | Yes/No  | 400X | H&E                   | (Mendes <i>et al.</i> , 2013)                                    |
| 5.  | Cell<br>ballooning   | Hydropic swelling of keratinocytes in the epidermal layer of the skin   | Yes/No  | 400X | H&E                   | (Rasmussen <i>et al.</i> , 2012)                                 |
| 6.  | Inflammatory<br>cells<br>infiltration<br>into the<br>epidermis<br>and dermis | Neutrophils= deep blue multi-lobed nuclei<br>attached by strands<br>Macrophages= deep blue bean/horse shoe<br>shaped nuclei<br>Lymphocytes= Large deep blue nuclei with<br>scanty cytoplasm | <ul> <li>0= no inflammatory cells infiltration</li> <li>1= occasional individual inflammatory<br/>cells within the field of view (FOV)</li> <li>2= focal infiltration of inflammatory cells<br/>(2 or more cells in a focus)</li> <li>3= coalescing inflammatory cells in the<br/>FOV (individual foci of cells cannot be<br/>distinguished)</li> <li>4= diffuse infiltration of inflammatory<br/>cells across the field of view</li> </ul> | 400X | H&E                   | (Mendes <i>et al.</i> , 2013;<br>Davenport <i>et al.</i> , 2014) |
| 7   | Collagen<br>abundance  | Green collagen fibres in the dermis   | Absolute determination of proportion of collagen abundance  | 400X | MT                    | Not available  |
| 8   | Basement<br>membrane<br>integrity (BM)                                       | Disruptions in the BM   | 0= absent<br>1= focal<br>2= multiple  | 400X | PAS                   | (Faleiros, Nuovo &<br>Belknap, 2009)                             |

# 3.2 Optimisation of staining techniques

All staining techniques were first optimised on biopsy tissues to ensure appropriate clarity of images. In the H&E staining, time taken to appropriately stain the cell nuclei (blue) in haematoxylin was 2.5 minutes while increasing the blueing effect in ammoniated water was achieved at 15 seconds. Counter staining of cell cytoplasm by eosin (pink/red) was 4 minutes. This optimisation protocol produced clear and distinct cellular features in tissues (Figure 3.1) and was incorporated into the staining protocol.



**Figure 3-1: H&E stained photomicrographs of ovine interdigital skin. A:** Pre-optimised photomicrograph showing poor colour contrast of epidermal and dermal architecture. **B:** post-optimised photomicrograph showing contrasting features of the epidermis and dermis. Scale bars=200 µm, mag=50x.

Periodic acid Schiff (PAS kit) manufacturer's protocol time for tissue incubation in periodic acid, Schiff's reagent and Harris haematoxylin were slightly modified for optimum effect in this study. Tissue immersion time in periodic acid solution, Schiff's reagent and Harris haematoxylin in PAS technique were 5, 15, and 2.5 minutes respectively. This modified protocol enabled a clearer visualisation of the basement membrane with distinct boundary in the skin tissues (Figure 3.2).



Figure 3-2: PAS stained photomicrographs of ovine interdigital skin showing basement membrane.

Optimisation of Masson's trichrome included length of incubation (20, 25 minutes) in the light green stain and was performed on two sets (slides 3 and 5) of tissue slides. Biebrich Scarlet-Acid Fuchsin was kept at a constant concentration of 15% throughout the optimisation process. Although both time points in this assay showed good collagen presentation (Figure 3.3), the 20-minute immersion time point showed a lighter green presentation of collagen which may become fainter due to variations in tissue uptake of the dye while the 25-minute time point appeared perfectly optimised in colour intensity. Hence, the 25-minute time point for light green dye immersion as well as other modifications

**A**) Poorly contrasted basement membrane (arrow) before modification of PAS protocol. **B**) Clearly identifiable basement membrane (arrow) after modification of protocol. Scale bar=50  $\mu$ m, mag=400x.

of the manufacturer's protocol was incorporated into the final Masson's trichrome stain protocol.



Figure 3-3: Optimisation of light green immersion time in collagen staining protocol.

Photomicrographs A and C are sections from healthy tissues while B and D are sections from footrot tissue. Pairs of photomicrographs AB and CD show Collagen staining (green tissues) following incubation in Light Green solution at 20min (AB) and 25 min (CD). Scale bar=50  $\mu$ m, mag=400x.

# 3.3 Exclusion criteria for the analyses of ovine interdigital skin

Following the staining and image capture from the 17 trial study samples,

exclusion criteria were set to define the quality of tissue images acceptable

for further analysis. Hence, tissue images were excluded from the study if

there was: (i) excessive dermal interruptions of the epidermis due to the

sectioning angle of tissue and (ii) insufficient quantity of dermal and epidermal tissue that could yield five non-overlapping images.

Following the exclusion of non-qualifying tissues, 14 tissues were analysed for parakeratosis, cell ballooning, inflammatory cell infiltration, and basement membrane disruption. It was observed that some dermal tissue sections were severely fragmented during Massons trichrome staining for collagen and were therefore not suitable for further analysis. Therefore, 10 samples were analysed for collagen abundance.

#### 3.4 Study design and sample set

This study was a preliminary observational study designed to develop scoring system with which to investigate histopathological lesions in ovine feet across different clinical conditions (healthy, interdigital dermatitis and footrot).

A total number of 17 biopsy samples including healthy (n=3), ID (n=4)and footrot (n=10) were selected for analysis from a set of samples collected from an abattoir in a previous study. Clinical conditions of biopsies were concealed from the observer so as to exclude observer bias. Biopsy samples were collected post-slaughter, fixed and preserved in paraffin for a previous study. To investigate these biopsies, they were sectioned (6µm) and representative slides stained with H&E, PAS and MT.

For each biopsy, 3 out of 30 slides were selected per staining technique in 15 out of the 17 trial tissues: H&E (slides 6, 16, 26), PAS (slides 3, 13, 23) and MT (slides 4, 14 and 24) (Table 3.3). For two biopsies (MM054 and MM059), only two representative sample slides were selected in some

cases because slides from these tissues were either less than 26 or insufficiently stained.

The selection of every 10<sup>th</sup> slide for staining ensured an average of 240-300µm distance between each slide, considering that 4 to 5 sections of 6µm thickness were placed on each slide. This method was considered appropriate because it reduces bias and ensures adequate representation of each tissue with approximately equal distances between each selected slide that does not allow overlapping of the same tissue/cells so therefore double counting was eliminated.

For the H&E and PAS techniques, 14 out of the 17 biopsy samples were investigated while 3 samples (MM054, MM056 and MM059) were excluded for not conforming to set criteria (section 3.3). The total number of remaining samples were 14 with clinical conditions as follows: healthy (n=3), ID (n=4) and footrot (n=7). Similarly, 10 [(healthy (n=1), ID (n=1), footrot (n=8)] out of the 17 biopsy samples were investigated using MT. Seven samples (MM: 008, 024, 031, 056, 074, 077, 092) were excluded for not meeting set criteria as well as fragmentation of dermal tissues during the staining process.

#### 3.5 Description of histological lesions in footrot

The stratum corneum (outermost layer) of the epidermis was intact in some samples but partially fragmented and lost in others leading to the exposure of underlying sub-corneal layer. Furthermore, retained nuclear remnants in the stratum corneum (parakeratosis) mixed with micro-abscesses and neutrophils were observed in some cases (Figure 3.4 A-B). In other instances, micro-abscesses and neutrophils were also observed in other skin layers especially in the superficial epidermis (stratum granulosum and spinosum; Figure 3.4 C-D). Also observable in some tissues was hypergranulosis (marked presence of keratin granules in the stratum granulosis layer; Figure 3.4 E). This is an indication of tissue response to disturbances of growth and cellular degenerative changes.

Extending from the superficial to mid-epidermis (stratum granulosum and spinosum), keratinocyte ballooning was observed which were sometimes seen to coalesce into forming cavities with associated neutrophils. In addition, inflammatory cells were sometimes observed localised across the dermis and epidermis (Figure 3.5 A-C). Haemorrhages and congestion were also observed in some samples (Figure 3.5 D-E).

In one sample (mm081), a deep intradermal foreign body granuloma (keratin) surrounded by fibrous and inflammatory cells' including activated neutrophils, lymphocytes and macrophages was observed (Figure 3.5 F).



Figure 3-4: H&E stained photomicrographs showing variable tissue changes in the epidermis of ovine interdigital skin.

A) Normal stratum corneum (orthokeratotic), B) Parakeratosis (square) and neutrophils accumulation in the stratum corneum (circle), C) Micro-abscesses in the stratum corneum (rectangle), D) Micro-abscesses in the stratum spinosum (square),
 E) Marked keratohylin granules in the stratum granulosum (hypergranulosis, arrows). Scale bars= 50 μm, mag=400x.



Figure 3-5: H&E photomicrographs of ovine interdigital skin with different lesions.

**A**) Ballooned cells (arrows), **B**) Coalescing ballooning cells (circle) forming cavities with inflammatory cells invasion (arrows). **C**) Inflammatory cells exocytosis in the dermis, **D**) Congestion of dermal vessels (arrows), **E**) Severe spread of red blood cells in tissues (haemorrhage), **F**) Granuloma (keratin) in the dermis surrounded by fibrotic tissues and inflammatory cells (neutrophils, activated neutrophils, macrophages and lymphocytes). Scale bars= 50 µm (mag=400x, A-E), 100 µm (mag=100x, F).

# 3.6 Grading histological lesions in interdigital skin

In order to compare and investigate key histological lesions across different clinical conditions, grading systems were developed and trialled as part of this study as follows:

Parakeratosis was defined as the retention of nuclear remnants in the stratum corneum (Brady, 2004). Grading was based on a nominal data scale which was defined as presence or absence of parakeratosis (Figure 3.6). Similarly, micro-abscesses were classified on a nominal scale as present or absent (Figure 3.7).

Areas of observed balloon cells were individually or collectively measured in absolute terms (ratio scale), and the averages determined using the Image Pro software 6.3 (Media Cybernetics, USA).

Congestion was defined as identifiable presence of dilated blood capillaries with visible red blood cells (erythrocytes)(Van Der Sluijs *et al.*, 2013). Observed blood cells outside capillaries in tissues (haemorrhage) were also noted. These parameters (congestions and haemorrhages) were graded on a nominal scale as present or absent (Figure 3.8).



Figure 3-6: H&E photomicrographs of parakeratosis patterns in ovine interdigital skin

**A**) Normal stratum corneum with no nuclear remnants (orthokeratosis), **B**) Focal accumulation of nuclear remnants (Parakeratosis; rectangles), **C**) Diffuse accumulation of nuclear remnants (parakeratosis). Scale bars=  $50 \mu m$ , mag=400x.



Figure 3-7: H&E photomicrographs of micro-abscesses in ovine interdigital skin.

**A**) Intra-corneal micro-abscesses, **B**) Sub-corneal micro abscesses), **C**) Dermal micro abscesses. Scale bars =  $50 \mu m$ , mag=400x.



Figure 3-8: H&E Photomicrographs of congested blood vessels cells and haemorrhages in the ovine interdigital skin.

**A**) Congested vessels in the dermis (rectangles), and **B**) Haemorrhages with red blood cells spread across entire skin tissues in the field of view (arrows). Scale bars= 50  $\mu$ m, mag=400x.

# 3.6.1 Inflammatory cell infiltration

To grade inflammatory cell infiltration in this study, a five ordinal scale grading system (scores 0 to 4) of progressive histological severity of inflammatory cell infiltration was developed. Tissues were scored based on visual examination of histological images obtained from the epidermis, dermal-epidermal junction and the dermis as follows (Figures 3.9; 3.10; 3.11):

- Score 0: No inflammatory cell infiltration in the field of view (FOV)
- Score 1: Occasional individual inflammatory cells within FOV
- Score 2: Focal infiltration of inflammatory cells (2 or more cells in a focus)
- Score 3: Coalescing inflammatory cells in the FOV (individual foci of cells cannot be distinguished)
- Score 4: Diffuse infiltration of inflammatory cells within FOV





**A** (score 0):No inflammatory cell infiltration in the epidermis, **B** (score 1): Occasional individual inflammatory cells within FOV in the epidermis (square), **C** (score 2): Focal infiltration of inflammatory cells (2 or more cells in a focus) in the epidermis (circles), **D** (score 3): Coalescing inflammatory cells in the epidermis (individual foci of cells cannot be distinguished), **E** (score 4): Diffuse infiltration of inflammatory cells across the field of view in the epidermis. Scale bars= 50  $\mu$ m, mag=400x.



Figure 3-10: H&E photomicrograph descriptors of inflammatory cell infiltration scores in the dermal-epidermal junction (DEJ) of ovine interdigital Skin.

**A** (score 0): No inflammatory cells in DEJ (Spindle shaped fibroblasts and epidermal basal cells (inset)), **B** (score 1): Occasional individual inflammatory cells within FOV in the DEJ (rectangle), **C** (score 2): Focal infiltration of inflammatory cells (2 or more cells in a focus) across DEJ (oval). **D** (score 3): Coalescing inflammatory cells across DEJ (individual foci of cells cannot be distinguished). Scale bars= 50  $\mu$ m, mag=400x.





**A** (score 0): No inflammatory cells in the dermis, **B** (score 1): Occasional individual inflammatory cells within FOV in the dermis (squares), **C** (score 2): Focal infiltration of inflammatory cells (2 or more in a focus) in the dermis (squares), **D** (score 3): Coalescing inflammatory cells in the dermis (individual foci of cells cannot be distinguished, **E** (score 4):Diffuse infiltration of inflammatory cells across the field of view (FOV) in the dermis. Scale bars= 50  $\mu$ m, mag=400x.

# 3.6.2 Basement membrane disruption (BMD)

To determine variations in basement membrane disruptions across different clinical conditions, photomicrographs of PAS stained sections were examined to identify discontinuity in the basement membrane of the dermal-epidermal junction (DEJ). Basement membrane integrity was scored based on a three ordinal scale criteria developed for this study (Figure 3.12):

Score 0: No BM disruption within FOV

Score 1: Focal/single disruption identified within the FOV

Score 2: Multiple BM disruptions identified within the FOV

# 3.6.3 Dermal collagen abundance

Proportions of dermal collagen (stained green) was measured in absolute terms (ratio) from photomicrographs using Image Pro 6.3 (Media Cybernetics, USA; Figure 3.13).



Figure 3-12: PAS photomicrograph descriptors of basement membrane disruptions in ovine interdigital skin.

**A** (Score 0): No basement membrane disruption identified, **B** (Score 1): Focal basement membrane disruption identified (oval), **C** (score 2): Multiple basement membrane disruption identified. Scale bars=  $50 \ \mu m$ , mag=400x.



Figure 3-13: Masson trichrome stained photomicrographs of dermal collagen in ovine interdigital skin.

**A**) MT stained (green colour) dermal collagen, **B**) Dermal collagen (green colour) is processed and impacted yellow colour for image pro software recognition and measurement. Scale bar=  $50 \ \mu m$ , mag= $400 \times R$ .

# 3.7 Evaluation of histological lesions of ovine interdigital skin

Currently, no study has investigated variations in key histological lesions of footrot as the ovine feet progresses from healthy to footrot condition. In this context, this preliminary study investigated the variations in the occurrence of observable lesions across different clinical conditions (healthy, ID, footrot).

# 3.7.1 Inflammatory cell infiltration

The infiltration of inflammatory cells across the epidermis, dermalepidermal junction (DEJ) and dermis varied in different samples (Table 3.2).

In this study, the maximum score (the most severe score) and the median score (most occurring score) for each tissue were determined for each sample. Aside from grading inflammatory cell infiltration in samples, the two scoring approaches were used to assess the suitability of including dermal-epidermal (DEJ) junction scores in the scoring system.

In the healthy samples, the maximum score approach revealed a similar inflammatory cell infiltration across the different layers (epidermis, DEJ, dermis) with maximum score range of 2-3 (Figure 3.14). However, in the median score approach, inflammatory cell infiltration score appeared higher in the dermis when compared to the epidermis (Figure 3.15).

In the ID clinical condition, 2/4 samples in the maximum score approach showed higher infiltration of inflammatory cells in the dermis, with the peak dermal score being 4 (Figure 3.14). Epidermal infiltration was stable (score 3) except in one sample (MM074) while DEJ inflammatory score varied between score 2 and 3 (Figure 3.14). In contrast, the median score approach showed higher infiltration of cells in the dermis over the epidermis in all tissues examined with the highest dermal score of 4 (Figure 3.15).

In the footrot clinical condition, the maximum score approach revealed no consistent pattern in the inflammatory cell infiltration across different layers. However, the peak inflammatory score of 4 was observed in the epidermis. In the median score approach, the peak inflammatory cell infiltration score (score 3) was observed in epidermis (mm047). Also, the least infiltration score (score 0) was observed in the epidermis. Inflammatory cell infiltration was relatively stable and similar in the DEJ and dermis (score 2) (Figure 3.15).

Generally, inflammatory cell infiltration score was higher in the maximum score approach with peak score of 4 in comparison to the median score approach with peak score of 3. In the healthy samples, the peak score of 3 was recorded for all three layers (epidermis, DEJ, dermis) using the maximum approach. Similarly, using the median approach, score of 2 was the peak value for all three layers. In the ID, the peak score of 4 was noted in the dermis in comparison to the

epidermis and DEJ using the maximum approach. In the median approach, the peak score of 3 was also recorded in the dermis when compared to the epidermis and DEJ. Finally, in footrot samples epidermal scores of 4 (maximum approach) and 3 (median approach) were the peak scores using the maximum when compared to the dermis and DEJ.

| S/N | Tissue | Max    | Max   | Мах    | Med.   | Med.  | Med.   | Range  | Range  | Range  | Clinical   |
|-----|--------|--------|-------|--------|--------|-------|--------|--------|--------|--------|------------|
|     | Code   | score  | score | score  | Score  | score | score  | scores | scores | scores | conditions |
|     |        | (epid) | (DEJ) | (derm) | (epid) | (DEJ) | (derm) | (epid) | (DEJ)  | (derm) |            |
| 1   | MM008  | 3      | 3     | 3      | 2      | 1     | 2      | 0-3    | 0-3    | 2-3    | Н          |
| 2   | MM022  | 3      | 2     | 4      | 2      | 2     | 3      | 0-3    | *      | 2-4    | ID         |
| 3   | MM024  | 3      | 3     | 3      | 0      | 2     | 2      | 0-3    | 1-3    | 2-3    | ID         |
| 4   | MM030  | 3      | 3     | 3      | 1      | 2     | 2      | 0-3    | 0-3    | 2-3    | Н          |
| 5   | MM031  | 3      | 2     | 2      | 1      | 2     | 2      | 0-3    | 0-2    | *      | Н          |
| 6   | MM032  | 4      | 3     | 3      | 2      | 2     | 2      | 0-4    | 1-3    | 0-3    | FR         |
| 7   | MM047  | 4      | 2     | 3      | 3      | 2     | 2      | 0-4    | *      | 2-3    | FR         |
| 8   | MM055  | 2      | 3     | 3      | 1      | 2     | 2      | 0-2    | 1-3    | 2-3    | FR         |
| 9   | MM060  | 3      | 3     | 2      | 1      | 2     | 2      | 0-3    | 1-3    | *      | FR         |
| 10  | MM074  | 2      | 2     | 3      | 1      | 2     | 2      | 0-2    | 1-2    | 2-3    | ID         |
| 11  | MM077  | 3      | 3     | 3      | 1      | 2     | 2      | 0-3    | 1-3    | 2-3    | ID         |
| 12  | MM081  | 1      | 2     | 3      | 0      | 2     | 2      | 0-1    | 0-2    | 2-3    | FR         |
| 13  | MM092  | 2      | 2     | 2      | 0      | 0     | 1      | 0-2    | 0-2    | 0-2    | FR         |
| 14  | MM094  | 1      | 2     | 2      | 0      | 2     | 2      | 0-1    | 1-2    | 1-2    | FR         |

Table 3-2: Maximum and median score values of inflammatory cell infiltration in the skin



Clinical status

#### Figure 3-14: Inflammatory cell infiltration into ovine interdigital space.

Data presented as bars represent maximum scores of inflammatory cell infiltration across dermis, dermal-epidermal junction (DEJ) and epidermis of clinically healthy, ID and footrot biopsy samples of skin/hoof interspace.



Clinical status

#### Figure 3-15: Inflammatory cell infiltration into ovine interdigital space.

Data presented as bars represent median scores of inflammatory cell infiltration across dermis, dermal-epidermal junction (DEJ) and epidermis of clinically healthy, ID and footrot biopsy samples of skin/hoof interspace.

# 3.7.2 Association of ballooned cells to the gross pathology of different clinical conditions

In H&E section, cell ballooning occurs as hydropic swelling of keratinocytes in the epidermal layer of the skin (excluding the superficial stratum corneum). We examined different clinical conditions of ovine feet to determine whether variations existed in total area of ballooned cells. Descriptively, ballooning was observed to be highest in ID, followed by healthy and footrot conditions respectively (Figure 3.16).



**Figure 3-16: Cell ballooning degeneration in different clinical conditions (healthy, ID, footrot) of ovine interdigital skin.** Data are presented as percentage area of ballooned cells in the epidermis of clinically healthy (n=3), ID (n=4) and footrot (n=7) biopsies of skin/hoof interface.

# 3.7.3 Comparing parakeratosis in different clinical conditions

Further investigation of H&E stained sections showed the presence of parakeratosis across all clinical conditions, but it was most observed in ID samples, followed by footrot and least in healthy feet samples (Figure 3.17). Further statistical analysis was not applied due to the limited number of trial samples.



# Figure 3-17: Occurrence of parakeratosis in interdigital skin stratum corneum.

Data are presented as percentage parakeratosis in the stratum corneum of clinically healthy, interdigital dermatitis (ID) and footrot biopsy samples of skin/hoof interface.

#### 3.7.4 Association between basement membrane disruption and

# clinical conditions

Basement membrane disruption was highest in the healthy feet samples with a range of 1-2 and least in interdigital dermatitis with a range of 0-1 in the maximum score model (Figure 3.18 A). In the median score, healthy and footrot samples were in the range of 0-1 (Figure 3. 18 B). The results showed no predictable pattern of basement membrane disruption in

relation to clinical condition (Figure 3.18 A-B). Further statistical analysis was not applied due to the limited number of trial samples.



Figure 3-18: Basement membrane disruption in dermal-epidermal Junction of ovine interdigital skin.

Data are presented as maximum scores of basement membrane disruptions in clinically healthy (n=3), ID (n=4) and footrot (n=7) biopsy samples of skin/hoof interface. A) maximum score, B) median score. Black horizontal line represent the median values of scores.

# 3.7.5 Evaluating dermal collagen abundance in different clinical

# conditions of ovine interdigital skin

Collagen abundance in the dermis was investigated across different clinical conditions (healthy, ID and footrot) using Masson's trichrome staining technique, and was least abundant in ID (35%), slightly higher in footrot (41%) and most abundant in healthy clinical condition (≈50%, Figure 3.19). These results need to be cautiously interpreted for two reasons: (i) only one healthy sample was analysed, (ii) small sample size and uneven representation of different gross clinical conditions were analysed.



**Figure 3-19: Dermal collagen abundance in ovine interdigital skin.** Data are presented as percentage dermal collagen density of clinically healthy (n=1), ID (n=1) and footrot (n=8) biopsy samples of skin/hoof interface.

# 3.7.6 Investigating association between micro-abscesses and

# clinical conditions

Although present in all clinical conditions, micro-abscesses were mostly

observed in healthy feet samples (2/3; 67%), with similar occurrences in

ID (2/4; 50%) and footrot (3/7; 43%), respectively (Figure 3.20).


Figure 3-20: Occurrence of micro-abscesses in ovine interdigital skin.

Data are presented as percentage occurrence of micro-abscesses in the epidermis of clinically healthy (n= 3), ID (n=4) and footrot (n=7) biopsy samples.

# 3.8 Evaluating consistency between interdigital skin inflammatory cell infiltration scores performed by independent observers

To assess the reproducibility of the scoring systems, a postgraduate student and an undergraduate project student were enlisted and trained. The training included interpretation of descriptors of inflammatory cell infiltration, identification of inflammatory cells (neutrophils, lymphocytes, macrophages etc) in the skin as well as identifying other skin cells such as keratinocytes. The training was conducted twice before the commencement of independent scoring.

#### 3.8.1 Inter-observer agreement of inflammatory cell infiltration

#### scores

Of a total of 420 H&E stained images that were taken (210 each for epidermis and dermis), 12 images (ten in epidermis and two in dermis) were excluded from the analysis due to insufficient skin tissues on images (skin tissue less than 75%) and poor clarity of the images for assessment. The most frequently occurring category in the epidermis was score 0 (106/200, 53%) with the least being score 4 (3/200, 1.5%). In the dermis on the other hand, the most scored category was score 2 (154/208, 74%) while the least was score 4 (1/208, 0.5%) (Table 3.3).

There was a low percentage of exact agreement score of inflammatory cell infiltration between the 3 scorers; 40% (79/200) in the epidermis and 14% (30/208) in the dermis respectively (Table 3.3). However, a considerable number of disagreements between observers' scores were within one category difference and had a more limited variation where two observers agreed and one disagreed by 1 category. This one-off category agreement scores occurred in 50/200 and 64/208 images analysed in the epidermis and dermis, respectively (Table 3.4).

|   | Epidermis                              |  |   | Dermis                                 |  |   |  |
|---|--|--|---|--|--|---|--|
| Inflammatory cell<br>infiltration score<br>category | No of images<br>scored by<br>observer1 | No of images<br>agreed by all<br>observers | % agreement of<br>inflammatory cell<br>infiltration score | No of images<br>scored by<br>observer1 | No of images<br>agreed by all<br>observers | % agreement of<br>inflammatory cell<br>infiltration score |  |
| 0   | 106                                    | 62   | 58  | 4                                      | 1  | 25  |  |
| 1   | 34                                     | 5  | 15  | 6                                      | 0  | 0   |  |
| 2   | 39                                     | 3  | 8   | 154                                    | 23   | 15  |  |
| 3   | 18                                     | 6  | 33  | 43                                     | 6  | 14  |  |
| 4   | 3                                      | 3  | 100   | 1                                      | 0  | 0   |  |
| TOTAL   | 200                                    | 79   |   | 208                                    | 30   |   |  |

#### Table 3-3: Exact category inter-observer agreement scores of inflammatory cell infiltration in the skin between observers

Combined (exact agreement + 1 category difference) together, the three observers' scores showed agreement range of 28% to 100% in the epidermis and 0% to 100% in the dermis across the five ordinal score categories. There was 80% (85/106) agreement of the three observers in score-0 category, which is the most assigned category in the epidermis with the least percentage agreement score being category 2 (11/39, 28%). In the dermis, agreement was lower, with the three observers' agreement on the most assigned score (score 2) being 49% (75/154). Generally, agreements were observed to be lowest for inflammatory cell infiltration score categories 1 (15/34, 44%) and 2 (11/39, 28%) for the epidermis and categories 2 (75/154, 49%) and 3 (12/43, 28%) for the dermis (Table 3.4).

|  |  | Epidermis   |  | Dermis                                 |   |  |  |
|--|--|---|--|--|---|--|--|
| Inflammatory<br>cell infiltration<br>score<br>category | No of images<br>scored by<br>Observer1 | Overall no of<br>images agreed<br>by all<br>observers | Overall %<br>Agreement of<br>inflammatory cell<br>infiltration score | No of images<br>scored by<br>observer1 | Overall no of<br>images<br>agreed by all<br>observers | Overall %<br>Agreement of<br>inflammatory cell<br>infiltration score |  |
| 0  | 106                                    | 85  | 80   | 4                                      | 3   | 75   |  |
| 1  | 34                                     | 15  | 44   | 6                                      | 6   | 100  |  |
| 2  | 39                                     | 11  | 28   | 154                                    | 75  | 49   |  |
| 3  | 18                                     | 10  | 56   | 43                                     | 12  | 28   |  |
| 4  | 3                                      | 3   | 100  | 1                                      | 0   | 0  |  |
| TOTAL  | 200                                    | 124   |  | 208                                    | 93  |  |  |

#### Table 3-4: Overall inflammatory cell infiltration agreement scores in the skin between independent observers

Furthermore, observer 2 was noted to score consistently lower than observer 1 in score categories 1 [24/34 (71%)] and score category 2 [24/39 (62%)] in the epidermis. Similarly in the dermis, observer 2 scored lower than observer 1 in score categories 2 [117/154 (76%)] and 3[36/43 (84%); Appendix 2]. These indicate the need for further training in the application of inflammatory cell scoring descriptors especially in the dermis.

#### 3.8.2 Evaluating inflammatory cell infiltration score

#### disagreements between independent observers

To investigate if the source of low scoring by observer 2 in comparison to observer 1 was intrinsic or due to other reasons, observer 2 was further trained by observer 1 on the application of scoring descriptors and identification of inflammatory and non-inflammatory cells in the skin. Following the re-training, observer 2 blindly re-assessed eight images in each of four categories: 1 and 2 in the epidermis; 2 and 3 in the dermis. The eight images per category were selected to reflect: four images of equal/same scores between observers 1 and 2 and the remaining four images were areas where observer 2 scored 1-category lower than observer 1 (Appendix 3). These categories of scores were selected based on observed lower scoring by observer 2 in comparison to observer 1. Agreement of inflammatory cell infiltration scores between observer 1 and 2 increased from 50% to 81% in the epidermis and 50% to 100% in the dermis following re-training and re-scoring of images by observer 2. Similarly, percentage agreement of score categories 1 and 2 in the epidermis improved from 50% each to 88 and 63% respectively. In the

dermis, percentage agreement between observers 1 and 2 in inflammatory cell infiltration score categories 2 and 3 increased from 50% each to 100% (Table 3.5). This clearly shows the effect of in-depth training on image analysis and application of inflammatory cell infiltration descriptors.

| Epidermis  |   |  |   |  |  | Dermis  |  |  |  |   |  |
|--|---|--|---|--|--|---|--|--|--|---|--|
| Inflammat<br>ory cell<br>infiltration<br>score<br>category | No of<br>images<br>scored by<br>observer1 | No of<br>images<br>agreed<br>between<br>observer 1<br>and 2<br>(1 <sup>st</sup> scoring) | %<br>agreement<br>between<br>observer 1<br>and 2 (1 <sup>st</sup><br>scoring) | No of<br>images<br>agreed<br>between<br>observer<br>1 and 2<br>(2 <sup>nd</sup><br>scoring | %<br>agreemen<br>t between<br>observer<br>1 & 2 (2 <sup>nd</sup><br>scoring) | Inflammatory<br>cell<br>infiltration<br>score<br>category | No of<br>images<br>scored<br>by<br>observer<br>1 | No of images<br>agreed<br>between<br>observer 1 & 2<br>(1 <sup>st</sup> scoring) | %<br>agreement<br>between<br>observer<br>1 & 2<br>(1 <sup>st</sup><br>scoring) | No of images<br>between<br>observer<br>1&2<br>(2 <sup>nd</sup> scoring) | %<br>agreemen<br>t between<br>observer<br>1 & 2 (2 <sup>nd</sup><br>scoring) |
| 1  | 8   | 4/8  | 50  | 7/8  | 88   |   |  |  |  |   |  |
| 2  | 8   | 4/8  | 50  | 5/8  | 63   | 2   | 8  | 4/8  | 50   | 8/8   | 100  |
|  |   |  |   |  |  | 3   | 8  | 4/8  | 50   | 8/8   | 100  |
| Total  | 16  | 8  | 50  | 13   | 81   |   | 16   | 8  | 50   | 16  | 100  |

#### Table 3-5: Comparison of variability of inflammatory cell infiltration scores between observer 1 and 2, before and after rescoring by observer 2

#### 3.9 Discussion

Footrot is a clinical disease of ovine interdigital skin which is currently diagnosed by clinical examination, laboratory methods and medical history of affected animals/flock. Histological assessment of footrot biopsy lesions can serve to identify pathognomonic features which may define disease state, thereby supporting clinical diagnosis, an emerging trend which is practiced in the assessment of human skin diseases like psoriasis (Gutierrez *et al.*, 2011). Similar to past investigations, histochemical staining techniques (H&E, PAS and MT) were used to identify tissue/cellular changes.

#### 3.9.1 Optimisation of staining techniques

Optimised staining protocols (H&E, PAS and MT) generated good quality images that allowed clear identification of skin architecture and subsequent interrogation of features such as inflammatory cells, ballooned cells, basement membrane and dermal collagen in different clinical conditions. Haematoxylin and eosin staining have been previously used in the assessment of histopathology lesions in ovine feet for footrot studies (Thomas, 1962; Davenport *et al.*, 2014). Similarly, it has been extensively used in bovine digital dermatitis (BDD)(Döpfer *et al.*, 1997; Knappe-Poindecker *et al.*, 2013; Refaai *et al.*, 2013). Also, optimised PAS stain clearly allowed the visualisation of basement membrane in the epidermaldermal junction. This technique has also been used to investigate bovine epidermal laminae in BDD (Faleiros, Nuovo & Belknap, 2009; Mendes *et*  *al.*, 2013). Finally, modification of Masson's trichrome protocol yielded good images for collagen identification and quantification.

#### 3.9.2 Development of histopathology scoring system

Histopathological lesions observed in the ovine interdigital skin were compared across different ovine skin conditions. The strategy was firstly to identify the core histopathological lesions following which histopathological grading systems were developed. Features such as parakeratosis and micro-abscesses were assessed by the nominal grading approach (present/absent). However, no importance was attached to the pattern of occurrence of these lesions by the grading system.

The grading system used for inflammatory cell infiltration was a semiquantitative ordinal system which is based on weighted maximum and median scores of tissues.

In most tissues examined across clinical conditions, cell ballooning was observed in the superficial epidermis (stratum spinosum) and sometimes with cavitations. These lesion types has been previously described in ovine footrot, bovine digital dermatitis and in other epithelial tissues (Thomas, 1962; Fleming & McGee, 1984; Rasmussen *et al.*, 2012). Although the causes of cell ballooning were not directly examined in this study, cell ballooning in hepatocytes has been associated with disruption of cell microtubules and cell injury (Burt, Mutton & Day, 1998).

Generally, cell ballooning suggests on-going cell pathology (Young, 2011) which may result in necrosis (Ishak, 1976). Necrosis is commonly attributed to factors indicative of acute stress such as microbial infections,

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nutritional deficiencies and chemical toxicity (Fleming & McGee, 1984; Syrjänen *et al.*, 1996).

Ovine interdigital skin is populated by diverse microbial flora (Calvo-Bado *et al.*, 2011; Maboni *et al.*, 2017) and this may explain the variation seen in cell ballooning across different tissues.

As regards parakeratosis, it was severest during ID and least observed in clinically healthy samples. This may be indicative of increased proliferation and differentiation of keratinocytes in response to stimuli (Bovenschen, Seyger & Van De Kerkhof, 2005). Also data on basement membrane disruption suggested that basement membrane disruption was most severe in healthy feet than ID and footrot. This was contrary to our expectations as studies in other species like cattle and horses have linked basement membrane disruption to disease state like laminitis (Pollitt, 1996; Hendry et al., 2003). These results must be cautiously interpreted since only a small sample size was analysed. Furthermore, collagen abundance in the dermis was quantified to determine if it is affected by severity of infection. As the most abundant protein in the extracellular matrix, collagen is rapidly degraded by collagenases (Ghajar, George & Putnam, 2008; Reiser, Adair & Reinheckel, 2010). Bacterial species including those already reported in footrot microbiome such as *Clostridium* perfringens, Porphyromonas spp., and Prevotella spp. have been identified as collagenolytic species (Mayrand et al., 1980; Robertson et al., 1982; Uitto et al., 1988). In addition, mammalian immune cells and fibroblasts involved in immune response to bacterial invasion have also being been reported to elicit collagenolytic effects (Matrisian, 1992).

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A small number of samples were examined in the current trial investigation, therefore, it may not be sufficient to make any justifiable claim on the association between collagen density and the clinical status of samples using the Masson's trichrome (MT) technique. Also, the inability of this staining technique to differentiate collagen types and the time consuming protocols involve in the technique makes it less beneficial in the context of the current study and less feasible in a larger sample size.

#### 3.9.3 Sources of inter-observer variation in scoring of

#### inflammatory cell infiltration into the skin

This study demonstrated the possibility of histologically grading lesions including inflammatory cell infiltration as has been previously done in related diseases like bovine digital dermatitis (Mendes *et al.*, 2013). Grading of pathological lesions is increasingly becoming part of diagnostic evaluation of degenerative diseases (Xia *et al.*, 2012). An important consideration in histopathological grading system is reproducibility. In this study, observer-score agreement recorded were 64% in the epidermis and 59% in the dermis. Generally, variations in agreement scores amongst observers in this type of study is expected and do not invalidate the reliability of the study. In published studies using the Gleason histology grading systems for the diagnosis of prostate cancer, agreement between observers ranged from 10 to 71% (Harada *et al.*, 1976; Rousselet *et al.*, 1986; Di Loreto *et al.*, 1991; Özdamar *et al.*, 1996; Allsbrook *et al.*, 2001; Singh *et al.*, 2011).

The increased level of agreement after further training suggests that initial training was probably inadequate prior to first scoring and may have

contributed significantly to the low agreements in some categories. Hence, further training may have provided additional opportunity for image viewing, improved understanding and application of the scoring descriptors. All these may have contributed to the overall increase in agreement scores observed. A reasonable approach to enhance reproducibility would be to provide multiple trainings on scoring criteria and sufficient materials such as slides clearly depicting inflammatory cell types, dermal fibroblasts and epidermal keratinocytes.

Generally, reproducibility of the scoring of inflammatory cell infiltration may often be imperfect because of imprecise definitions of thresholds for cells foci and/or coalition of foci in the scoring descriptors. To improve reproducibility, scoring descriptors have to be simple and clearly defined.

This is the first attempt at grading and quantifying histological microlesions in footrot as well as developing a scoring system for the grading of severity in inflammatory response during footrot. Inter-observer scoring was good in some categories (0, 4) and improved in others (1, 2, 3). Although 100% agreement was observed in category 4, there is need for cautious interpretation as only three images were scored in this category. This preliminary study was limited by the few number of trial samples, our inability to achieve desired orientation of sections during sectioning and the time intensive nature of processing histopathology tissues. These are all areas that can be improved in future studies especially now that optimisation of techniques has been achieved and automated staining could be considered.

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#### 3.10 Summary

The objectives of this preliminary study were to optimise staining techniques, identify and grade histological lesions in addition to validating inflammatory cell infiltration grading system in ovine feet.

The study optimised and modified three staining techniques (H&E, PAS and MT) for the assessment of histological lesions.

Key findings in the study include: (i) development of grading and quantification systems for histological lesions including parakeratosis, cell ballooning, micro-abscesses, inflammatory cell infiltration and basement membrane disruption; (ii) the study demonstrated the repeatability of histopathological scoring of inflammatory cell infiltration in the ovine interdigital skin during footrot, (iii) the study also observed that histological lesions including inflammatory cell infiltration occur irrespective of the gross clinical condition of the feet.

To further improve the applicability of the grading systems, reviews were made which include slight modifications of some grading systems including parakeratosis, micro-abscesses according to the pattern their occurrence. Inflammatory cell infiltration of the DEJ was excluded since in most cases it was similar to either of epidermal or dermal scores and therefore, provided no additional information. Similarly, collagen abundance was excluded from further analysis since it provides no information on collagen type and it is time consuming considering that the entire study is time dependent.

Finally, parakeratosis, cell ballooning, micro-abscesses, inflammatory cell infiltration, basement membrane disruption in addition to congestion and haemorrhages have been incorporated in the grading scheme for subsequent use in a larger study (Table 3.6).

#### Table 3-6: Final histopathology lesions scoring template of ovine interdigital skin

| S/N | Parameter  | Characteristics  | Scoring system  | Mag. | Staining<br>technique | References   |
|-----|--|--|---|------|-----------------------|--|
| 1   | Parakeratosis  | Retained nuclei remnant in stratum corneum   | 0= absent, 1= focal, 3= diffuse   | 400X | H&E                   | (Rasmussen <i>et al.</i> ,<br>2012)                              |
| 2.  | Abscesses  | Aggregation of inflammatory cells +<br>cellular debris + with/without bacterial<br>presence + fibrotic tissue walling  | Absent, intra-corneal, sub-corneal, dermal  | 400X | H&E                   | (Davenport <i>et al.</i> , 2014)                                 |
| 3.  | Congestion   | Increased red blood cells in vessels   | Yes/No  | 400X | H&E                   | (Mendes <i>et al.</i> , 2013;<br>Davenport <i>et al.</i> , 2014) |
| 4.  | Haemorrhage  | Red blood cells outside blood vessels  | Yes/No  | 400X | H&E                   | (Mendes <i>et al.</i> , 2013)                                    |
| 5.  | Cell<br>ballooning   | Hydropic swelling of keratinocytes in the epidermal layer of the skin  | Yes/No  | 400X | H&E                   | (Rasmussen <i>et al.</i> , 2012)                                 |
| 6.  | Inflammatory<br>cells<br>infiltration<br>into the<br>epidermis<br>and dermis | Neutrophils= deep blue multi- lobed nuclei<br>attached by strands<br>Macrophages= deep blue bean/horse shoe<br>shaped nuclei<br>Lymphocytes= Large deep blue nuclei with<br>scanty cytoplasm | <ul> <li>0= no inflammatory cells infiltration</li> <li>1= Occasional individual inflammatory cells within FOV</li> <li>2= focal infiltration of inflammatory cells (2 or more cells in a focus)</li> <li>3= coalescing inflammatory cells in the FOV (individual foci of cells cannot be distinguished)</li> <li>4= diffuse infiltration of inflammatory cells across the field of view</li> </ul> | 400X | H&E                   | (Mendes <i>et al.</i> , 2013;<br>Davenport <i>et al.</i> , 2014) |
| 7   | Basement<br>membrane<br>integrity (BM)                                       | Disruptions in the BM  | 0= absent<br>1= focal<br>2= multiple  | 400X | PAS                   | (Faleiros, Nuovo &<br>Belknap, 2009)                             |

# Chapter 4 : Histopathological analysis of ovine interdigital skin

#### 4.1 Introduction

The ovine interdigital skin is the focus of initial damage and bacterial colonisation before the establishment of interdigital dermatitis and footrot disease (Beveridge, 1941; Egerton, Roberts & Parsonson, 1969). Footrot is a necrotizing disease which causes extensive destruction of epidermal cells/tissues in the ovine feet leading to pain and lameness (Beveridge, 1941; Thomas, 1962; Egerton, Roberts & Parsonson, 1969). The disease is a result of complex interaction between environment, physical damage, bacteria and the host tissues resulting in various pathologies.

Bacterial components of ovine footrot have been thoroughly investigated and quantified at different stages of the disease to understand the temporal shift in bacterial population dynamics and increase knowledge on the pathogenesis of the disease and distinction between healthy, interdigital dermatitis and footrot (Egerton, Roberts & Parsonson, 1969; Witcomb *et al.*, 2014; Davenport *et al.*, 2014; Maboni *et al.*, 2016). However, histopathological lesions have received less attention and have at best been mentioned qualitatively in most studies (Thomas, 1962; Egerton, Roberts & Parsonson, 1969; Davenport *et al.*, 2014). Parsonson et al. (1967) classified clinical stages of ovine footrot into three different stages based on pathological changes to define healthy, interdigital

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dermatitis (ID) and footrot. However, these three stages have not been linked to histopathology.

Key histologic lesions that are known to occur during footrot include parakeratosis (hyper-proliferation of keratinocytes), cell ballooning [characteristic indicator of cellular response to injury; (Burt, Mutton & Day, 1998)], micro-abscesses (an indicator of necrosis and secondary invasion by pyogenic bacteria), cavities, congestion, haemorrhages and infiltration of inflammatory cells, mainly neutrophils in the epidermis and dermis (Thomas, 1962; Egerton, Roberts & Parsonson, 1969; Davenport *et al.*, 2014). Studies on bovine digital dermatitis have linked histological lesions to define four stages of disease (Refaai *et al.*, 2013) indicating this approach may be of utility to classify footrot infections in greater detail.

The work described in this chapter examines representative biopsy samples from healthy, ID and footrot conditions in ovine feet with a view to gaining insight into their respective histological lesions in respect of clinical conditions.

#### 4.2 Sample set

In this study, 120 samples of ovine interdigital skin biopsies were collected for investigation. Two samples were missing while two other samples (ST037 and ST118) were excluded from further analysis as they conformed to our inclusion criteria: (i) excessive dermal interruptions of the epidermis due to the sectioning angle of tissue and (ii) insufficient quantity of dermal and epidermal tissue that can yield five non-overlapping images. Tissues in samples ST037 were insufficient to provide five non-overlapping images while in ST118 there were dermal interruptions of the epidermal layer.

Finally, a total of 116 samples were analysed and spread across different clinical conditions: 55 healthy, 31 Interdigital dermatitis (ID) and 30 footrot. Out of these, all four legs were sampled in 26 animals (104 biopsies) while three legs were sampled in 4 animals (12 biopsies). The samples were equally spread across same number of front and hind limbs (58 each). Samples were collected in three abattoir visits (21/10/13, 13/12/13 and 16/12/13) and were selected across different clinical conditions in order to determine the occurrence and variation of lesions across different clinical conditions. Sample collection was based on convenience and availability at the time of abattoir visits and hence the variation in the sample number per clinical condition. Clinical conditions (healthy, ID and footrot) of samples were determined by two independent scorers based on the classification of Parsonson et al. (1967). Clinical Identities of samples were blinded from the investigator in order to exclude bias during the analysis.

#### 4.3 Histopathological assessment of lesions in different

#### conditions of the ovine interdigital skin

This study assessed and compared the histological lesions of footrot across different clinical conditions (healthy, interdigital dermatitis (ID) and footrot). Lesions were graded as nominal (micro-abscesses, congestion and haemorrhages), categorical (parakeratosis, basement membrane disruption and inflammatory cell infiltration) and in absolute measures (area of cell ballooning).

The aim of this study was to examine possible association between the severity of histological lesions and different clinical conditions.

#### 4.3.1 Epidermal lesions

Since clinical lesions in footrot are mostly localised in the epidermis, this study analysed lesions in biopsy sample sections on the basis of skin layers (epidermis or dermis) across clinical conditions (healthy, ID, footrot).

There was no difference in parakeratosis observed across the different clinical conditions (Figure 4.1 A) with percentage parakeratosis found as follows: healthy 73% (40/55), ID 87% (27/31) and footrot 77% (23/31). Micro-abscesses were assessed on the basis of location in the epidermis: intra-corneal abscess (stratum corneum) and sub-corneal abscess (stratum granulosum + spinosum). Occurrence of intra-corneal abscess was highest in the healthy 44% (24/55) followed by footrot 33% (10/30) and least in the ID condition 26% (8/31) with no statistical significance. Similarly, sub-corneal abscesses were observed the most in healthy samples 25% (14/55) and closely followed by ID and footrot which were 23% (7/31, 7/30) each. The difference was not significant across clinical conditions (Figure 4.1 C-D).

Since the epidermal ballooning of cells is an indication of host cell response to injurious substances including infectious agents such as *D. nodosus*, *F. necrophorum* (important pathogens of footrot), we compared mean percentage values of ballooned cells area across clinical conditions. There

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was no statistical difference in the mean percentage values of ballooned cells which was highest in the ID samples (4.0%), followed by healthy (3.7%) and least in footrot (3.0%) samples (Figure 4.1 B).

Furthermore, the study explored the infiltration of inflammatory cells into the epidermis to determine if differences exist in the severity of the inflammatory cell infiltration score across clinical conditions. Epidermal inflammatory scores were represented by the maximum score (highest category score/tissue) and median score (most frequent score/tissue) approaches. There were no differences in the median (score 2) of the inflammatory cell infiltration observed in the healthy, ID and footrot samples in the maximum score approach (Figure 4.1 E). Similarly in the median score approach, inflammatory cell infiltration score 1 was the median value in all clinical conditions with no statistical difference (Figure 4.1 F).



#### Figure 4-1: Epidermal histological lesions of ovine interdigital skin.

Data are presented as maximum and median scores as well as percentages which represent occurrences of lesions in stacked bars and scattered plot of clinically healthy (n=55), ID (n=31) and footrot (n=30) biopsy samples of skin/hoof interface. **A**) Parakeratosis, **B**) Cell ballooning, **C**) Intra-corneal micro-abscesses, **D**) Sub-corneal micro-abscess, **E**) Inflammatory score infiltration (maximum score), and **F**) Inflammatory score infiltration (median score). Statistical analysis: Chi square (A,C-F), one-way ANOVA B. The horizontal black line indicates the mean (B), and the median (E, F) score values.

Since micro-abscesses also involve the aggregation of inflammatory cells (viable and dying), samples with and without epidermal micro-abscesses were compared in the context of severity of inflammatory cell infiltration across clinical conditions. Using the maximum score approach, inflammatory cell infiltration was significantly high in healthy samples (p=0.008, Figure 4.2 A) with abscesses and significantly higher (p=0.0004) in ID and footrot samples with abscesses in comparison to samples without (Figure 4.2 C, E). In the median score approach, inflammatory cell infiltration was significantly high in ID (p=0.009, Figure 4.2 D) and footrot (p=0.01, Figure 4.2 F) samples but not significant in the healthy (p=0.071, Figure 4.2 B).

Similarly, percentage values of ballooned cell area was compared to inflammatory cell infiltration in the epidermis to determine if there was a relationship. A positive association between percentage values of ballooned cell area and epidermal inflammatory cell infiltration using the maximum score approach was significant in the healthy (r=0.40, p=0.002) and ID (r=0.49, 0.005) clinical conditions (Figure 4.3 A, C). Using the median approach, association was positive, but weak and not significant in healthy (r=0.19, p=0.17) and ID (r=0.16, p=0.388) samples (Figure 4.3 B, D). Similarly in the footrot samples, there was weak but positive association between percentage values of ballooned cell area and epidermal inflammatory cell infiltration which were not significant in both the maximum score approach (r=0.16, p=0.410) and median score approach (r=0.31, p=0.097) (Figure 4.3 E-F).





Data are presented as maximum and median scores which are represent scattered plot of clinically healthy (n=55), ID (n=31) and footrot (n=30) biopsy samples of skin/hoof interface. **A**) Healthy (maximum score) **B**) Healthy (median score), **C**) ID (maximum score), **D**) ID (median score), **E**) Footrot (maximum score), and **F**) Footrot (median score). Statistical analysis: Mann Whitney test (A-F). The horizontal black line indicates the median score values. Significance is represented by asterisk on a straight line and is designated as; \* =  $p \le 0.05$ , \*\* =  $p \le 0.01$ , \*\*\*



## Figure 4-3: Epidermal cell ballooning compared with severity of inflammatory response across different conditions of ovine interdigital skin.

Data are presented as maximum and median scores which are presented as scattered plot of clinically healthy (n=55), ID (n=31) and footrot (n=30) biopsy samples of skin/hoof interface. A) Healthy (maximum score) B) Healthy (median score), C) ID (maximum score), D) ID (median score), E) Footrot (maximum score), and F) Footrot (median score). Statistical analysis: Spearman rank correlation (A-F). Significance is designated as; \* =  $p \le 0.05$ , \*\* =  $p \le 0.01$ .

#### 4.3.2 Dermal lesions

The dermis is the deeper layer of the skin and contains numerous blood and lymphatic vessels. These vessels serve as a conduit to inflammatory cells to the skin especially during inflammation (Nestle et al., 2009). Therefore, lesions of vascular origin and inflammatory cell infiltration were investigated in this layer of the skin.

All lesions investigated in the dermis (congestion, haemorrhages, dermal abscesses and inflammatory cell infiltration) were present in the three clinical conditions. The inflammatory cell infiltration scores ranged from 2 - 4 with no differences in the median (score 3) of the inflammatory cell infiltration in the healthy, ID and footrot samples in the maximum score approach across different clinical conditions (Figure 4.4 C). However, in the median score approach, the inflammatory cell infiltration scores ranged between scores 1 and 3 in the healthy, 2 - 4 in ID and 2 - 3 in footrot with the median being score 2 across all clinical conditions (Figure 4.4 D).

Although haemorrhage was mostly prevalent in ID (35%), closely followed by healthy (31%) and footrot (30%), there was no significant difference (Figure 4.4 B). However, the occurrence of dermal micro-abscesses appeared the same (10%) in ID and footrot while it was 7% in healthy samples. No significant difference was observed across the different clinical conditions (Figure 4.4 E). In contrast, the occurrence (74%) of congestion was significantly high in ID (p=0.04) when compared to healthy (49%) and significantly higher (p= 0.01) when compared to footrot (40%) clinical conditions respectively (Figure 4.4 A).



#### Figure 4-4: Dermal histological lesions of ovine interdigital skin biopsies.

Data are presented as maximum and median scores as well as percentages of lesion occurrences across healthy (n=55), ID (n=31) and footrot (n=30) interdigital space biopsy samples of: **A**) Congestion, **B**) Haemorrhages, **C**) Inflammatory cell infiltration (maximum score), **D**) Inflammatory cell infiltration (maximum score), **D**) Inflammatory cell infiltration (median score), and **E**) Dermal micro-abscesses. Statistical analysis: Fisher's exact test showed significant difference (p=0.04) between ID and healthy condition and also between ID and footrot condition (p=0.01)(A). Chi square (B-E). Horizontal black line in C and D indicate median values of scores. Significance is represented by asterisk on a straight line and is designated as; \* =  $p \le 0.05$ , \*\* =  $p \le 0.01$ .

#### 4.3.3 Other lesions observed

#### 4.3.3.1 Basement membrane disruption

The basement membrane separates the epidermis from the dermis and anchors the basal keratinocytes of the epidermis. Therefore, we hypothesized that cellular trafficking through this membrane as well as basal keratinocytes necrosis may lead to disruptions in the integrity of the basement membrane. Categories of disruptions (0=no disruption, 1= focal disruption, 2= multiple disruptions) were analysed and compared across clinical conditions to determine if specific disruption patterns were consistent with clinical conditions. Using the maximum score approach, there were no differences (p=0.308) in the BM disruptions observed across clinical conditions (Figure 4.5 A). Similarly, the median score approach showed no difference (p=0.241) in BM disruption pattern across clinical conditions (Figure 4.5 B).



### Figure 4-5: Basement membrane disruption in ovine interdigital skin.

Data are presented as percentages of maximum and minimum scores of lesions in healthy (n=55), ID (n=31) and footrot (n=30) clinical conditions. **A**) Basement membrane disruption (maximum score), **B**. Basement membrane disruption (median score). Statistical analysis: Chi square analysis (A, B).

#### 4.3.3.2 The inflammatory cell infiltration score in different foot

#### conformation is similar across different clinical conditions

Foot integrity/conformation is a measure of the degree of normality and/or deformity of the foot. We hypothesized that a deformed foot may indicate past or on-going disease condition including footrot. Deformity may also predispose the foot to increased microbial invasion and hence, increased inflammatory response. To investigate this hypothesis, parallel foot integrity data (Maboni *et al.*, 2017) which was obtained from the same animals used in the current study, were assessed. These data were compared with inflammatory cell infiltration across different clinical conditions.

Overall, there were no differences in the median values in both the maximum and median score approaches with regards to the severity of epidermal cell infiltration in the healthy (Figure 4.6 A-B), ID (Figure 4.6 C-D) and footrot (Figure 4.6 E-F) conditions.



Figure 4-6: Comparison of foot conformation and epidermal inflammatory cell infiltration in ovine interdigital skin.

Data are presented as foot conformation scores against epidermal inflammatory cell infiltration (maximum and median scores) across healthy (n=55), ID (n=31) and footrot (n=30) conditions. **A**) Healthy (maximum score, p=0.77), **B**) Healthy (median score, p=0.92), **C**) ID (maximum score, p=0.93), **D**) ID (median score, p=0.08), **E**) Footrot (maximum score, p=0.73), **F**) Footrot (median score, p=0.68). Similar inflammatory cell infiltration occurs across different foot conformation. Ovine foot conformation scoring descriptors were defined as: Score **O**- undamaged claw with normal shape. Score **1**- mildly damaged or misshapen claw (<25%). Score **2**- moderately damaged or misshapen claw (>75%). Statistical analysis: Chi square (A-F).

Similarly, in the dermis, severity of inflammatory cell infiltration was variable across different foot conformation in healthy (Figure 4.7 A-B), ID (Figure 4.7 C-D) and footrot (Figure 4.7 E-F) conditions.



Figure 4-7: Comparison of foot conformation and dermal inflammatory cell infiltration in ovine interdigital skin.

Data are presented as foot conformation scores against dermal inflammatory cell infiltration (maximum and median scores) across healthy (n=55), ID (n=31) and footrot (n=30) conditions. **A**) Healthy (maximum score, p=0.54), **B**) Healthy (median score, p=0.58), **C**) ID (maximum score, p=0.64), **D**) ID (median score, p=0.97), **E**) Footrot (maximum score, 0.52), **F**) Footrot (median score, 0.49). Similar inflammatory cell infiltration occurs across different foot conformation. Ovine foot conformation scoring descriptors were defined as: Score 0- undamaged claw with normal shape. Score 1- mildly damaged or misshapen claw (<25%). Score 2- moderately damaged or misshapen claw (>75%). Statistical analysis: Chi square (A-F).

#### 4.4 Discussion

In this study H&E and PAS allowed the characterisation of inflammatory cell infiltrates and histological lesions in healthy, ID and footrot conditions. Histological changes associated with footrot were quantitatively compared across different clinical conditions using newly described grading systems developed in chapter three.

The key findings in this chapter were the occurrence of histological lesions and inflammatory cell infiltrates in all clinical conditions (healthy, ID and footrot). Generally, lesions reportedly associated with footrot in this study were consistent with previous reports (Thomas, 1962; Davenport *et al.*, 2014). To the best of our knowledge, this investigation appears to be the first to quantitatively analyse histopathological lesions in ovine footrot.

#### 4.4.1 Histopathological lesions of footrot in the epidermis

The stratum corneum (SC) constitutes the topmost layer of the epidermis and it is made up of corneocytes which represent the end result of keratinocyte migration and maturation (Pierard *et al.*, 2000). Therefore, disorders in the anatomy of the SC such as the retention of nuclei in the stratum corneum (parakeratosis) may indicate disturbances of epidermal cell development.

Parakeratosis was identified as focal or diffused patches within normal SC (orthokeratosis) irrespective of clinical condition. Extravasated red blood cells were seen in few cases. While parakeratosis appeared more prevalent in the ID clinical condition, the difference was not statistically significant and only suggestive of a higher cell turn-over. Any increased cell turn-over

may be due to cellular response to infectious agents including *D. nodosus* which is also more prevalent during the ID phase (Witcomb *et al.*, 2014). A study on aetiology and pathogenesis of ovine footrot suggested low grade inflammation caused by *Fusobacterium necrophorum* may lead to parakeratosis (Roberts & Egerton, 1969).

Intra-corneal, sub-corneal and dermal abscesses were noted in the stratum corneum, stratum spinosum of the epidermis and the dermis in some tissues. Given that these abscesses were mostly observed to form around degenerating keratinocytes, it is possible that keratinocytes were involved in the formation of these lesions. In this study, micro-abscesses (intra-corneal and sub-corneal) were identified across all different clinical conditions but with no significant difference. A recent study on contagious ovine digital dermatitis (CODD) also reported intra-corneal abscesses (Angell *et al.*, 2015) in ovine feet which means this lesion is not specific to ovine footrot.

Additionally, keratinocytes in some samples were observed at various levels of ballooning degeneration across different clinical conditions and in some instances, ballooned cells coalesced to form cavities with observable neutrophils. This is an indication of cell lysis which may result in necrosis. This observation has been previously reported in footrot (Thomas, 1962; Davenport *et al.*, 2014) with a suggestion that may probably be due to clearance of degenerated cells by neutrophils.

In this study, there was no difference found in the mean area of cell ballooning between healthy, ID and footrot conditions. However, a weak

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positive association between mean cell ballooning area and severity of inflammatory cell infiltrates was noted in the healthy and ID conditions but not in footrot samples. Cell ballooning may be caused by microbial infections, nutritional deficiencies and chemical toxicity (Fleming & McGee, 1984; Syrjänen *et al.*, 1996). Chemotherapeutic agents such as antibiotics may have been previously administered to individual animals or flock as part of management regime on the farm site, and could have contributed to the ballooning observed. Indeed antibiotics such as mafenide acetate has been shown to initiate ballooning degeneration in human keratinocytes (Gibson *et al.*, 2008). Unfortunately, medical history of individual animals sampled was not available since study samples were obtained from abattoir with no access to the medical history of the animals.

It has been proposed that cell ballooning may initiate keratinocyte hyperproliferation (Freedberg *et al.*, 2001) and play a role in the recruitment of immune competent cells into the epidermis (Nickoloff, 1988). Both actions contribute to the epidermal hyperplasia and thickening that has been hypothesised as the cause of under-running lesions in footrot (Egerton, Roberts & Parsonson, 1969). In this study inflammatory cell infiltration in both the epidermis and dermis appeared similar. This finding is different to data from studies on bovine digital where inflammatory infiltrates were highly increased with severity of disease (Döpfer, Holzhauer & Boven, 2012; Refaai *et al.*, 2013).

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The reason for the difference observed in these two studies may probably be due to the low number of samples used in the bovine digital dermatitis study (n=24) in comparison to this study (n=116). Furthermore, samples in this study were collected in wet autumn-winter season (Sabine Tötemeyer, personal communication) which leads to enhanced ovine feet maceration. Skin maceration causes disruption of corneocytes in the stratum corneum (Spears *et al.*, 1999; Minematsu *et al.*, 2011) which results in the initiation of keratinocytes hyper-proliferation (Segre, 2006) and encourages microbial invasion leading to inflammatory response (Mugita *et al.*, 2015). This increased maceration and infection may explain the high prevalence of footrot infection in temperate wet environment.

This study also found a significant increase in the severity of inflammatory cell infiltrates in samples observed with micro-abscesses when compared to samples without abscesses. This observation was irrespective of clinical condition. A number of factors including cell ballooning, presence of micro-abscesses and infectious agents contribute to the aggregation of inflammatory cells in the epidermis (Barker *et al.*, 1991; Borregaard, 2010).

#### 4.4.2 Histopathological lesions of footrot in the dermis

Dermal lesions including inflammatory cell infiltrate, haemorrhage and vascular congestion were observed and appeared similar across all clinical conditions except congestion. The intensity of inflammatory cell infiltration appeared higher in comparison to the epidermis. This was expected as the blood vessels supplying the skin are located in the dermis (Braverman, 1989; Huggenberger & Detmar, 2011). Consequently, inflammatory cells exocytose into the dermis and subsequently migrate to the epidermis.

Congestion was significantly increased in ID when compared to healthy and footrot conditions. The data presented here is in line with observations from hoof of dairy cows laminitis which had increased vascular congestion in diseased hooves (Mendes *et al.*, 2013). Increased congestion indicates enhanced blood flow to the skin and may precede subsequent clinical manifestation of disease (Clarkson *et al.*, 1996). ID is the inflammatory phase of the footrot disease complex typified by peak *D. nodosus* load (Witcomb *et al.*, 2014). Moreover, this phase of the disease is clinically characterised by increased reddening of the skin (erythema) due to vascular congestion.

#### 4.4.3 Other lesions investigated

PAS showed basement membrane (BM) disruption(s) across all clinical conditions but indicated no trend; an observation that suggests BM disruption is independent of clinical condition of the ovine foot. Findings in the current study are inconsistent with Hendry et al. (2003) where there was a significant difference in BM disruption among healthy and ulcerated bovine hooves (Hendry *et al.*, 2003). A possible explanation for the difference in findings may be due to the approach used for detecting disruption. While the current study used PAS staining, that of Hendry and colleagues used immunohistochemistry, a more specific technique to detect laminin and integrins as markers of BM disruption.

BM disruption has been linked with the degradation of collagen type IV, a major constituent of basement membrane which anchors proteins including proteoglycans, enactins and laminins (Liotta *et al.*, 1980; Liotta & Stetler-Stevenson, 1991).

Sources of collagenases include dermal fibroblasts as well as inflammatory cells which can degrade collagen (Busiek *et al.*, 1992). In addition, microbial organisms (*Porphyromonas spp.*, *Prevotella spp.*, and spirochetes like *Treponema spp.*) linked to ovine footrot microbiome have also been reported to express collagenases (Mayrand & Grenier, 1985; Uitto *et al.*, 1988, 1992). Although the cause of basement membrane disruption was not directly investigated, we hypothesize that the effect of inflammatory cell infiltration trafficking may be contributory to the disruption of the basement membrane. It is also possible that some BM disruptions observed may have been artefacts caused by tissue processing procedures since PAS technique may not distinguish artefacts from actual lesions.

This study further compared foot conformation score to corresponding inflammatory cell infiltrates in order to determine if foot condition influenced inflammatory response observed. Poor foot conformation has been reported to increase the risk of development of lameness and footrot (Kaler *et al.*, 2010b). Inflammatory cell infiltrates in this study were independent of foot conformation as well as clinical condition in both the epidermis and dermis.

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#### 4.5 Study limitations

Although this study has successfully provided quantitative information on the histological lesions of footrot, it has certain limitations.

Firstly, the use of post-slaughtered abattoir samples in this study prevented access to the history of sampled animals. Also, with abattoir samples it is difficult to define a healthy status as apparently healthy but lame animals may be incubating sub-clinical infections with limited pathology or previously infected and recovered. Moreover, abattoir samples depicting footrot are natural infections which may have been modified by several other factors not known/considered during investigations. An alternative approach to limit this would have been a longitudinal study in which medical history of the animals is collected and animals monitored. However, ethical considerations will prove challenging. To mitigate this limitation, a large sample set was employed in this study.

Secondly, there was lack of sufficient data with which to make comparison and draw useful conclusions concerning ovine footrot.

Thirdly, semi-quantitative scoring system of histological lesions does not require high grade expertise. However, it is it is limited by its time and labour intensity.

Finally, the use of PAS in the detection of BM disruption is limited as it may not differentiate true disruptions from artefacts due to physical preparation and damage. Thereby leading to inconsistent results. To minimise this effect, multiple PAS stained images (15) were analysed per sample.

### 4.6 Summary and conclusion

This study has quantified histopathological lesions in different clinical conditions. However, it could not match specific lesions to disease stages. Firstly, the occurrence of histopathological lesions including parakeratosis, micro-abscesses, cell ballooning, inflammatory cell infiltrates, haemorrhages and BM disruption are present and similar across different clinical conditions.

Secondly, this study also provided histopathological evidence for the description of erythema (reddening of skin) characteristic of ID stage in footrot disease.

Thirdly, this study has histologically shown that background inflammation (represented by inflammatory cell infiltrates) exists in apparently healthy feet.

For the first time, the results of this study have provided quantitative data on the histopathology lesions of interdigital skin across different gross conditions of the ovine foot. This will serve as background knowledge for the further elucidation of disease pathogenesis.

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# Chapter 5 : Associating histological lesions with proinflammatory cytokine and virulent *Dichelobacter nodosus* abundance

### 5.1 Introduction

The skin acts a screen which shields the body from physical, chemical and microbial damage. However, under certain conditions including mechanical injuries and macerations, the skin integrity undergoes disruption leading to the exposure of sub-epidermal structures. Disruption of ovine interdigital skin has been associated with disease conditions such as ovine footrot (Egerton, Roberts & Parsonson, 1969). Footrot is a degenerative disease of the ovine foot characterised by podo-dermatitis and underrunning of the hoof-horn (Beveridge, 1941). The disease is mediated by the influx of inflammatory cells as a host response to bacterial colonisation (Egerton, Roberts & Parsonson, 1969). Other histological lesions characterised in the ovine interdigital skin include cell ballooning, micro-abscesses, congestion and haemorrhages (Beveridge, 1941; Thomas, 1962; Egerton, Roberts & Parsonson, 1969; Davenport *et al.*, 2014).

Footrot is a polymicrobial disease with several associated bacteria (Calvo-Bado *et al.*, 2011; Maboni *et al.*, 2017). However, the essential bacteria necessary for initiation of the disease is *D. nodosus* (Beveridge, 1941; Witcomb *et al.*, 2014). On the basis of virulence, *D. nodosus* is categorised into two distinct groups (benign and virulent) differentiated by the presence of protease genes: *apr*B2 (benign) and *apr*V2 (virulence)(Kennan *et al.*, 2014). In the UK, the virulent strain is more prevalent amongst flocks (Moore, Wassink & Green, 2005; Maboni *et al.*, 2016).

It has been proposed that the innate immune response is a component in the development of footrot. Histological studies have associated *D. nodosus* with mild-moderate inflammatory cell infiltration (Egerton, Roberts & Parsonson, 1969). In addition, increased IL-1 $\beta$  and CXCL8 expression have been associated with *D. nodosus* in footrot but not in healthy samples (Davenport *et al.*, 2014; Maboni *et al.*, 2017). However, it could not be defined if it was *D. nodosus* that initiated the expression of IL-1 $\beta$  and CXCL8 (Maboni *et al.*, 2017).

IL-1 $\beta$  is one of the primary cytokines produced by keratinocytes in the epidermis of the skin. It is involved in the activation of effector mechanisms that trigger inflammation in the skin (Mizutani, Black & Kupper, 1991; Williams & Kupper, 1996). It has also been associated with the development of pathologic lesions in psoriasis, an immune mediated disease of the human skin (Nickoloff, 1991). In addition to IL-1 $\beta$  there are a range of other pro-inflammatory cytokines that could drive pathology in the skin. One of these is CXCL8 (IL-8) which has been shown to mediate early neutrophil attraction to the sites of inflammation (Hoffmann *et al.*, 2002) and plays a role in the proliferation and migration of keratinocytes (Tuschil *et al.*, 1992; Jiang *et al.*, 2012).

Footrot is an epidermal disease in which most lesions are localised in the epidermis and involve epidermal cells (keratinocytes). The involvement of keratinocytes in the immune response of the skin leads to our hypothesis

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that aside from inflammatory response to infectious agents including *D. nodosus*, histological lesions in footrot may also play contributory roles in the overall inflammatory response observed in footrot.

The aim of the work described in this chapter was to provide further information regarding the role of the inflammatory marker IL-1 $\beta$  and the clinical signs and histopathology. This was achieved by comparing virulent *D. nodosus* load and IL-1 $\beta$  expression against scores for inflammatory cells infiltration and other histological lesions across different clinical conditions.

### 5.2 Sample set

Out of the 116 ovine interdigital skin biopsies analysed for histological lesions in chapter four, a subset of samples [healthy (n=41), ID (n=24), footrot (n=23)] were selected for further analysis. The selection was based on the availability of parallel IL-1 $\beta$  expression and virulent *D. nodosus* load data obtained from the same animals but for a different study in our group (Maboni *et al.*, 2017). These data (IL-1 $\beta$  expression and *D. nodosus* load) were compared to corresponding histopathology lesions (identified in chapter four) to determine if potential relationships exist.

### 5.2.1 IL-1 $\beta$ expression levels increases with severity of

### inflammatory response in ID and footrot

Since IL-1 $\beta$  expression levels and inflammatory cells are components of the innate immune system and serve as markers of inflammation, the expression levels of IL-1 $\beta$  expression was analysed against inflammatory cell infiltration scores in the epidermis and dermis of biopsy samples across different clinical conditions. In the epidermis of healthy samples, expression levels of IL-1 $\beta$  was slightly higher but not significant in inflammatory scores 3 and 4 compared to scores 1 and 2 in the maximum score approach. However, in the median score approach there was no definite pattern (Figure 5.1 A-B). Analysis of ID condition showed significantly higher (p=0.05) levels of IL-1 $\beta$ expression in inflammatory cell infiltration score 4 in comparison to score 1. Notably, increased levels of IL-1 $\beta$  expression corresponded with higher inflammatory scores (Figure 5.1 C), although in the median model, there was no difference (Figure 4.6 D). In footrot, levels of IL-1 $\beta$  expression was significantly higher (p=0.05) in samples with respect to inflammatory score 4 and 3 in comparison to category 1 (Figure 5.1 E-F).



Figure 5-1: IL-1 $\beta$  expression levels significantly increase with severity of epidermal inflammatory response in ID and footrot affected ovine interdigital skin.

Data are presented as relative expression of IL-1 $\beta$  against maximum and median inflammatory scores in healthy (n=41, **A-B**), ID (n=24, **C-D**), and Footrot (n=23, **E-F**). Statistical analysis: Chi square (A-B), t-test (D), one-way ANOVA with Tukey's multiple comparison showed significant difference in C (p=0.05), E (p=0.05) and F (p=0.05. Horizontal black line indicates mean IL-1 $\beta$  expression. Significance is represented by asterisk on a straight line and is defined as; \* =  $p \le 0.05$ . Number of samples with inflammatory cell infiltration score and corresponding IL-1 $\beta$  expression data are represented by n.

In the maximum and median score approaches in the dermis, higher expression levels of IL-1 $\beta$  corresponded with inflammatory cells scores 3 and/or 4 across healthy (Figures 5.2 A- B), ID (Figures 5.2 C-D) and footrot (Figures 5.2 E-F) conditions, but was not significant (p>0.05).



Figure 5-2: IL-1β expression levels slightly increase with severity of dermal inflammatory response in ovine interdigital skin.

Data are presented as relative expression of IL-1 $\beta$  against maximum and median inflammatory scores of healthy (n=41), ID (n=24) and footrot (n=23). **A**) Healthy (maximum score), **B**) Healthy (median score), **C**) ID (maximum score), **D**) ID (median score), **E**) Footrot (maximum score), **F**) Footrot (median score). Statistical analysis: One way ANOVA (A, C, E, F), t-test (B, D). Number of samples with inflammatory cell infiltration score and corresponding IL-1 $\beta$  expression data are represented by n.

Since the relative expression of IL-1 $\beta$  of biopsy samples represent an average for each tissue, we further compared the IL-1 $\beta$  expression with a combined scoring (addition of inflammatory cell infiltration scores of epidermis and dermis per tissue) of epidermis and dermis as a composite unit for each sample.

There were no differences in either the maximum and median score approaches for the expression of IL-1 $\beta$  across the different clinical conditions (Figure 5.3 A-F).





Data are presented as relative expression of IL-1 $\beta$  against summation of maximum and median inflammatory scores of healthy (n=41), ID (n=24) and footrot (n=23) biopsy samples. **A**) Healthy (maximum score), **B**) Healthy (median score), **C**) ID (maximum score), **D**) ID (median score), **E**) Footrot (maximum score), **F**) Footrot (median score). Statistical analysis: One way ANOVA analysis (A-C, E-F), t-test (D). Horizontal black line indicates mean IL-1 $\beta$  expression while n is the number of samples with inflammatory cell infiltration score and corresponding IL-1 $\beta$  expression data.

### 5.2.2 Comparing the basement membrane disruptions and IL-1β expression in different clinical conditions

This comparison was based on the hypothesis that levels of IL-1 $\beta$  expression correlate with inflammatory cell trafficking through the basement membrane and may contribute to the disruption observed in the basement membrane.

In the maximum score approach, the mean IL-1 $\beta$  expression levels decreased with increasing BM disruption (Figure 5.4 A) in the healthy samples but increased with increasing BM disruptions in the ID and footrot (Figure 5.4 C, E). In the median approach, most data point were concentrated at the zero score in all clinical conditions (Figure 5.4 B, D, F).



Figure 5-4: IL-1 $\beta$  expression levels increases slightly with the disruption of basement membrane in ID and footrot affected ovine interdigital skin. Data are presented as relative expression of IL-1 $\beta$  against maximum and median basement membrane integrity scores. **A**, **B** represent healthy (n=41) samples; **C**, **D** are ID (n=24) samples and **E**, **F** are footrot (n=23) samples. Statistical analysis: One way ANOVA (A, C, E), t-test (B, D). Basement membrane scores: 0 (no disruption), 1 (focal disruption), 2 (multiple disruptions). Horizontal black line indicates mean of IL-1 $\beta$  expression. Number of samples with basement membrane scores and corresponding IL-1 $\beta$  expression data are represented by n.

### 5.2.3 Comparing the epidermal cell ballooning with high and low IL-1β expression in healthy and footrot

IL-1 $\beta$  expression values were categorised within 25<sup>th</sup> (low) and 75<sup>th</sup> (high) quartiles in healthy and footrot conditions (Maboni *et al.*, 2017) and compared with area of cell ballooning in the epidermis to determine any association. A slight increase in the ballooned cell area correspond to samples with high IL-1 $\beta$  expression when compared to samples low IL-1 $\beta$  expression. However, there was no statistical difference in the mean ballooned cell area between high and low IL-1 $\beta$  expressing samples in healthy (Figure 5.5 A) and footrot (Figure 5.5 B) samples.





Data are presented as the mean of ballooned cell area against high and low IL-1 $\beta$  expression in different clinical conditions; **A**) Healthy [low (n=10), high (n=15)] and **B**) Footrot [low, high (n=4)]. Statistical analysis: t-test (A). Due to the few samples in the footrot analysis (B), statistical analysis was not conducted. Horizontal black line indicates mean of ballooned cell area. Number of samples with ballooned cell area and corresponding IL-1 $\beta$  expression data are represented by n.

## 5.2.4 IL-1β expression increased in the presence of intra-corneal and sub-corneal micro-abscesses but not dermal micro-abscesses in interdigital skin biopsies

Since micro-abscesses involve aggregation of viable and degenerating neutrophils (inflammatory cells), it was thought that their presence may serve to influence the inflammatory response. Micro-abscesses were observed in ovine interdigital skin sections across different clinical conditions, therefore, the relationship between the levels of IL-1 $\beta$  expression in response to the presence of micro-abscesses (intra-corneal, sub-corneal and dermal) was investigated. In the presence of intra-corneal abscesses, IL-1 $\beta$  expression was significantly higher (*p*=0.003) in healthy samples (Figure 5.6 A) and, in ID samples (*p*= 0.024) (Figure 5.6 D) but was not significant in footrot samples (Figure 5.6 G). Similarly, for the presence of sub-corneal abscesses, IL-1 $\beta$  expression was significantly higher (*p*=0.003, Figure 5.6 H) and ID samples (*p*=0.028, Figure 5.6 E). However, there was no difference in the levels of IL-1 $\beta$  expression with respect to dermal abscesses across different clinical conditions (Figure 5.6 C, F, I).



Figure 5-6: IL-1 $\beta$  expression and occurrence of micro-abscesses in ovine interdigital skin.

Data are presented as scattered plots of healthy (**A**, **B**, **C**); ID (**D**, **E**, **F**) and footrot (**G**, **H**, **I**) samples. Micro-abscesses are represented as: Intra-corneal (A, D, G); Subcorneal (B, E, H) and Dermal (C, F, I) abscesses. Statistical analysis: t-test showed levels of IL-1 $\beta$  expression were significantly higher in A (p=0.003), B (p=0.002), D (p=0.024), E (p=0.028), H (p=0.0031), but no difference in (C, F, G). Horizontal black line indicates mean IL-1 $\beta$  expression while n represents number of samples with corresponding IL-1 $\beta$  expression data. Significance is designated by asterisk on a straight line and is defined as; \* = p≤0.05, \*\* = p≤0.01.

Furthermore, we investigated the relationship between the occurrence of epidermal micro-abscesses (intra- and sub-corneal) against high and low IL-1 $\beta$  expressing biopsy samples. Intra-corneal micro-abscesses were significantly increased (*p*=0.015) in high IL-1 $\beta$  expressing samples in comparison to low expressing samples (Figure 5.7 A) in the healthy condition. In the sub-corneal analysis, there were no micro-abscesses identified in the low IL-1 $\beta$  expressing samples, therefore, statistical analysis was not applied (Figure 5.7 C). Similarly, in comparing occurrence of intra-corneal (Figure 5.7 B) and sub-corneal (Figure 5.7 D) lesions in footrot condition, available samples were limited for any valid statistical analysis.



Figure 5-7: Comparing association between occurrence of epidermal micro-abscesses and levels of IL-1β expression.

Data are presented as bar graphs of occurrences of intra-corneal [**A** (n=2, 11), **B** (n=1, 2)] and sub-corneal abscesses [**C** (n=0, 8), **D** (n=0, 4)] against high and low IL-1 $\beta$  expression in healthy [A (n=10,15), C (n=10, 15)] and footrot [B (n=4, 4), D (n=4, 4)] ovine interdigital biopsies. Statistical analysis: Fisher's Exact test showed significant difference in A (p=0.015), C (p=0.008). B and D were not statistically significant. Significance is represented by asterisk on a straight line and is designated as; \* =  $p \le 0.05$ , \*\* =  $p \le 0.01$ .

### 5.2.5 Levels of IL-1β expression associated with parakeratosis in

### ovine interdigital skin

Since we observed co-localisation of intra-corneal abscesses with parakeratosis in a number of samples, expression levels of IL-1 $\beta$  was analysed in response to the presence of parakeratosis (Figure 5.8). Levels of IL-1 $\beta$  expression were significantly increased (*P*=0.008, Figure 5.8 A) in diffused parakeratosis in comparison to samples without parakeratosis in healthy clinical condition. Although there was an increased level of IL-1 $\beta$  in response to parakeratosis score-2 in ID, it was statistically not significant

(Figure 5. 8 B). In the footrot condition, there was no difference in the levels of IL-1 $\beta$  expression across categories of parakeratosis (Figure 5.8 C).



Figure 5-8: Comparison of IL-1 $\beta$  expression levels and presence of parakeratosis in ovine interdigital skin Data are presented as scattered plots of: **A**). Healthy (n=41), **B**) ID (n=24) and **C**) Footrot (n=23) conditions. Statistical analysis: One-way ANOVA followed by Tukey's multiple comparison test showed significant increase in A (p=0.008) but no difference in (B-C). Horizontal black line indicates mean IL-1 $\beta$  expression while n represents number of samples with parakeratosis and corresponding IL-1 $\beta$  expression data. Significance is designated by asterisk on a straight line and is defined as; \*\* =  $p \le 0.01$ .

IL-1 $\beta$  expression levels and parakeratosis with/or without micro-abscesses in different clinical conditions were also determined. In healthy condition with micro-abscesses, levels of IL-1 $\beta$  expression increased significantly (*p*=0.006) in parakeratosis score 2 when compared to score category-0 (Figure 5.9 A). However, no difference was observed in the absence of micro-abscesses (Figure 5.9 B).

In the ID condition, IL-1 $\beta$  expression level was slightly higher in parakeratosis score2 in comparison to score1 but was not significant (Figure 5.9 C) in the presence and/or absence of abscesses (Figure 5.9 D).

Similarly, in the footrot condition, no difference was observed in the levels of IL-1 $\beta$  expression across parakeratosis scores in the presence of abscesses (Figure 5.9 E) and absence of abscesses (Figure 5.9 F).



Figure 5-9: Comparing occurrence of parakeratosis and IL-1 $\beta$  expression With or without presence of abscesses in different clinical conditions of ovine interdigital skin.

Data are presented as scattered plots of; healthy [**A** (n=22), **B** (n=19)], ID [**C** (n=6), **D** (n=17)] and footrot [**E** (n=9), **F** (n=14)] samples. Statistical test: t-test showed significant difference in A (p=0.006) but no difference in C, One way ANOVA (B, D-F). Horizontal black line indicates mean of IL-1 $\beta$  expression. Numbers of samples with parakeratosis scores with the corresponding IL-1 $\beta$  expression data are represented by n. Significance is represented by asterisk on a straight line and is defined as; \* =  $p \le 0.05$ .

### 5.3 Comparing association between inflammatory cell infiltration and virulent *Dichelobacter nodosus* abundance

Considering that footrot pathology is mediated by the immune response of the host, it was thought necessary to compare *D. nodosus* (causative agent) against inflammatory cell infiltration as a marker of host inflammatory response in order to determine corresponding association.

In the maximum score approach, there was no difference in the mean load of virulent *D. nodosus* and inflammatory cell infiltration scores (Figure 5.10 A) in the epidermis of healthy samples. However, using median scores, virulent *D. nodosus* load was significantly higher (p<0.0001) in inflammatory cell score 3, when compared to scores 1 and 2 (Figure 5.10 B). Similarly in the ID condition, using maximum scores showed no difference in virulent *D. nodosus* load (Figure 5.10 C), while in the median score, virulent *D. nodosus* load was significantly higher in inflammatory cell score 2 when compared to score 1 and 3 (p<0.0001) (Figure 5.10 D). Also, virulent *D. nodosus* load in footrot condition was significantly (p<0.0001) increased using maximum scores at inflammatory score 3 in comparison to scores 1 and 2 respectively (Figure 5.10 E), but there was no difference using median scores (Figure 5.10 F).

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Figure 5-10: High abundance of virulent *D. nodosus* correlates to the severity of inflammatory cell response in the epidermis of ovine interdigital skin.

Data are presented as the abundance of virulent *D. nodosus (log* load) against inflammatory cell infiltration score in the epidermis in two models (maximum and median scores). **A**, **B** are healthy samples (n=30); **C**, **D** are ID samples (n=26) while **E**, **F** are footrot samples (n=18). Statistical analysis: One way ANOVA with Tukey's multiple comparison test showed significant differences (p<0.0001) in B, D-E, but no difference in A, C. There was no difference in t-test analysis (F). Horizontal black line indicates mean of *D. nodosus* load while significance is represented by asterisk on a straight line and is designated as; \* = p≤0.05, \*\* = p≤0.01, \*\*\* = p≤0.001. Number of samples with inflammatory cell infiltration score and virulent *D. nodosus* load data are represented by n.

In the dermis, both scoring approaches (maximum and median) showed variation with no difference in the mean *D. nodosus* load against different inflammatory cell infiltration scores across the different clinical conditions: healthy (Figure 5.11 A-B), ID (Figure 5.11 C-D) and footrot (Figure 5.11 E-F).



Figure 5-11: Abundance of virulent *D. nodosus* inconsistent with inflammatory cell response in the dermis of ovine interdigital skin.

Data are presented as the abundance of virulent *D. nodosus* (*log* load) against dermal inflammatory cell infiltration score in two models (maximum and median scores) in the dermis. **A**, **B** are healthy samples (n=30); **C**, **D** are ID samples (n=26) and **E**, **F** are footrot samples (n=17), respectively. One way ANOVA showed no difference in the mean load of *D. nodosus* in A (p=0.576) and C (p=0.131). Similarly, t-test analysis showed no difference in B (p=0.326), E (p=0.794) and F (p=0.704) respectively. Statistical analysis was not applicable in D since the mean was obtainable only in inflammatory infiltration category 2. Horizontal black line indicates mean of *D. nodosus* load while number of samples with inflammatory cell infiltration score and corresponding virulent *D. nodosus* load data are represented by n.

### 5.4 Discussion

This study analysed possible interactions that occur in the ovine foot between histological lesions, markers of inflammation including proinflammatory cytokine (IL-1 $\beta$ ) and inflammatory cell infiltrates as well as virulent D. nodosus in order to gain further insight into the pathogenesis of ovine footrot.

### 5.4.1 Association between histopathological lesions and proinflammatory cytokine (IL-1β)

This study revealed a significant difference in IL-1 $\beta$  expression levels between epidermal inflammatory cell infiltration score 1 and 4 in ID and footrot feet, but not in healthy feet. This suggests that IL-1 $\beta$  expression level may be directly mirror the severity of inflammatory cell infiltrates and correlate with disease condition (ID, footrot) in the epidermis. Moreover, both IL-1 $\beta$  and inflammatory cell are components of the innate immune response. In addition, IL-1 $\beta$  play a role in the recruitment of inflammatory cells and proliferation of keratinocytes (Maas-Szabowski, Shimotoyodome & Fusenig, 1999; Feldmeyer *et al.*, 2010). It has been proposed that this cell influx into the epidermis may be responsible for the epidermal hyperplasia and the concomitant pressure which initiate the separation of the hoof-horn capsule (Egerton, Roberts & Parsonson, 1969).

Furthermore, IL-1 $\beta$  expression was compared with inflammatory cell infiltration in the dermis and whole biopsy samples (epidermis and dermis). In both analyses, the mean levels of IL-1 $\beta$  expression increased with severity of inflammatory cell infiltrates but did not correlate with the clinical

conditions. This finding correlated with data on the role of CXCL8 (IL-8) which is also known to mediate early neutrophil attraction to the sites of inflammation and plays a role in the proliferation and migration of keratinocytes (Tuschil *et al.*, 1992; Hoffmann *et al.*, 2002; Jiang *et al.*, 2012). Put together, these data suggest that the severity of IL-1 $\beta$  expression levels in the epidermis of diseased feet (ID, footrot) play contributory roles in the epidermal hyperplasia and pressure leading to the separation of the hoof-horn in footrot (Egerton, Roberts & Parsonson, 1969). On the other hand, keratinocytes are key epidermal cells involved in the synthesis of IL-1 $\beta$  (Mizutani, Black & Kupper, 1991).

It has been hypothesised that footrot is mainly an epidermal disease due to the pathology associated with epidermal tissues (Beveridge, 1941; Thomas, 1962; Egerton, Roberts & Parsonson, 1969). This study found a significant increase in IL-1 $\beta$  expression levels of healthy samples with epidermal parakeratosis in comparison to samples without parakeratosis suggesting that cellular hyperplasia is connected with IL-1 $\beta$  expression and may indicate on-going pathological processes. However, ID and footrot samples with parakeratosis showed a slight increase in IL-1 $\beta$  levels but this was not statistically significant when compared to samples without parakeratosis. This may be due to the fewer samples analysed in ID and footrot conditions or the superficial skin sloughing off which characterise these clinical conditions. Furthermore, IL-1 $\beta$  expression levels were significantly increased in healthy samples with diffuse parakeratosis colocalised with micro-abscesses. Co-localisation of parakeratosis and micro-abscesses in the stratum corneum indicate hyperplasia (hyperproliferation) of keratinocytes (Cox & Watson, 1972), which may be caused by the presence of oxygen radicals and proteolytic enzymes released by activated inflammatory cells in the human stratum corneum (Kato *et al.*, 1990, 1991).

IL-1 $\beta$  expression levels were significantly higher in samples with microabscesses in comparison to samples where abscesses were absent. This suggests micro-abscesses as either sources of IL-1 $\beta$  or are driven by IL-1 $\beta$  irrespective of clinical status. In addition, the presence of pyogenic organisms reported in ovine feet (Calvo-Bado *et al.*, 2011; Maboni *et al.*, 2017) may initiate these inflammatory responses. The correlation of IL-1 $\beta$ with micro-abscesses matches observations of higher expression of IL-1 $\beta$ in sheep with pyogranulomas inflammation (Pépin *et al.*, 1997).

The formation of an abscess involves recruitment of neutrophils for the clearance of microbial organisms (Borregaard, 2010). This recruitment of neutrophils in skin abscesses has been linked to the up-regulation of IL-1β expression by cells such as keratinocytes, dendritic cells, macrophages and mast cells (Nakamura *et al.*, 2009; Matsushima *et al.*, 2010; Igyarto *et al.*, 2011). The presence of over 27 bacterial genera including pyogenic species on the skin surface is a possible explanation for the source of organisms in the micro-abscesses in apparently healthy skin.

# 5.4.2 Virulent *Dichelobacter nodosus* (vDN) abundance increases with high inflammatory cell infiltration in the epidermis

This study compared virulent *D. nodosus* load against inflammatory cell infiltration to determine if relationships are dependent on clinical condition or severity of inflammation. Significantly high virulent *D. nodosus* load tend to correspond with increase in the epidermal inflammatory cell infiltration except using median scores where the virulent D. nodosus load was significantly higher in inflammatory cell infiltration score 2 than score 3. This may be explained by the wide variation in the virulent *D. nodosus* load observed in the samples associated with score 3. This study confirms the findings in a recent publication from our group where the authors reported a significant association between high IL-1 $\beta$  expression and high virulent D. nodosus load in footrot samples (Maboni et al., 2017). However, the present study in contrast to the Maboni et al investigation, found a significant link between high IL-1 $\beta$  expression and high virulent *D. nodosus* load in healthy and ID samples. The differences between the two studies may be due to a number of factors: i) the molecular marker (IL-1 $\beta$ ) of inflammatory response used in the Maboni *et al* study is more specific and represented whole skin sample while the histology marker (IL-1 $\beta$ ) of inflammatory response used in the current study is less specific and represented inflammation in the epidermal layer of the skin, (ii) Other sources of inflammatory cell infiltration such as micro-abscesses were observed in the current study and may have contributed to the inflammation observed. Put together, the two studies suggest that

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inflammatory response to virulent *D. nodosus* is dependent on the severity of inflammation rather than the clinical condition of the foot.

In contrast to the epidermis, there was no definite pattern of inflammatory cell infiltration observed in the dermis. Virulent *D. nodosus* is believed to be localised in the superficial epidermal matrix of the skin and may be responsible for the differences observed (Maboni *et al.*, 2016). Further investigation of this speculation will be beneficial.

Findings in this study agree with earlier hypotheses that, (i) footrot disease is mainly an epidermal infection and, (ii) aside from *D. nodosus*, other histological lesions including micro-abscesses, cell ballooning may contribute to the inflammatory response in the ovine foot, (iii) inflammatory response in the ovine foot is dependent on the severity of inflammation rather than clinical condition, (iv) inflammatory cell infiltration into the epidermis contributes to the destruction of epidermal architecture and subsequent separation of the hoof from soft tissues (Thomas, 1962; Egerton, Roberts & Parsonson, 1969).

The ovine interdigital skin is a complex ecosystem with over 27 bacterial genera (Calvo-Bado *et al.*, 2011). Hence, other microbial components of the skin aside from *D. nodosus* may contribute to eliciting the inflammatory cell infiltration observed in this study. We speculate that the localisation of these bacterial components in the skin may provide further information as to their ability to elicit inflammatory response in the ovine interdigital skin.

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### 5.5 Study limitations

A number of limitations in this study needs to be acknowledged. Firstly, the inflammatory cells were not distinguished into various cell types which may have provided further information regarding the type of inflammation processing occurring (acute or chronic). Secondly, only D. nodosus out of over 27 genera reported to inhabit the ovine skin were analysed in the context of inflammatory response. Hence, it is impossible to conclude that D. nodosus is the sole organism responsible for the inflammatory response observed. Notwithstanding these limitations, this study provides useful insight into the inter-relationship between histological lesions, inflammatory response (IL-1 $\beta$ , inflammatory cells infiltrate) and virulent D. nodosus.

### 5.6 Summary and conclusion

Key findings showed that significant increase in the expression levels of IL-1 $\beta$  correlated with; (i) the presence of parakeratosis in healthy samples, (ii) severity of inflammatory cell infiltration in ID and footrot, and (iii) presence of epidermal micro-abscesses in healthy, ID and footrot affected samples. In addition, inflammatory response to virulent *D. nodosus* is dependent on the severity of the inflammatory cell infiltration rather than clinical condition of the foot.

Interestingly, this study showed that higher levels of IL-1 $\beta$  expression mirror increased severity of inflammation in the epidermis but not the dermis; thereby further confirming the hypothesis that footrot is an epidermal disease. Together, it can be hypothesised that inflammatory

response (inflammatory cell infiltration), *D. nodosus* load and histological lesions including micro-abscesses contribute to the pathology seen in footrot disease.
# Chapter 6 : Spatial localisation and abundance of bacteria in ovine interdigital skin

#### 6.1 Introduction

The ovine interdigital skin is a vast ecosystem hosting abundant number of bacteria species. 16S rRNA analysis of samples from ovine interdigital skin revealed about 27 genera of various bacteria (Calvo-Bado *et al.*, 2011). A number of these bacteria are commensals while others have been associated with diseases including ovine footrot. In footrot disease, *D. nodosus* and *F. necrophorum* are the two dominant pathogens discussed in respect of pathogenesis while other bacteria have only been mentioned as part of the polymicrobial community.

Therefore, this study examined bacteria including *D. nodosus* and *F. necrophorum* with regards to their localisation in ovine interdigital skin. Previous studies have investigated these bacteria in the context of aetiology and pathogenesis using whole biopsies (Calvo-Bado *et al.*, 2011; Witcomb *et al.*, 2015; Maboni *et al.*, 2016).

Calvo-Bado *et al.* (2011) examined healthy, interdigital dermatitis and footrot biopsies for *D. nodosus* using conventional, nested and real time PCR approaches. The authors reported the detection of *D. nodosus* in all clinical conditions (healthy, interdigital dermatitis and footrot) by PCR or nested PCR but *D. nodosus* load only in ID samples by targeting the *rpoD* genes using real time PCR. On the other hand, Maboni *et al.* (2016) investigated eubacteria, *D. nodosus* and *F. necrophorum* by targeting 16S

*rRNA* (eubacteria, *D. nodosus*) and *gyrB* (*F. necrophorum*) genes using the real time PCR approach in healthy and diseased interdigital skin samples. The authors found significantly higher *D. nodosus* and *F. necrophorum* load in ID and footrot samples in comparison to healthy samples. Similarly in the first localisation study to examine *D. nodosus* and *F. necrophorum* in ovine skin, Witcomb *et al.* (2015) examined epidermal and dermal layers of ovine interdigital skin sections using fluorescence *in situ* hybridisation (FISH). The authors reported significant load of *D. nodosus* and *F. necrophorum* in the footrot samples in comparison to the healthy feet.

In all of these studies, cognisance was not given to bacterial localisation in successive strata across the depths of ovine interdigital skin biopsies. This information is important as it will further improve our knowledge of the bacterial patho-physiology in footrot disease.

The determination of bacterial load from whole biopsies of ovine interdigital skin provides no information on spatial localisation of these organisms; while examination of vertical sections of ovine interdigital skin for bacteria and other skin features such as hair follicles permits limited 2-dimensional observations (Jahns & Alexeyev, 2014). The advantages of transverse (horizontal) sectioning is that it provides a three-dimensional view of tissue features such as follicles as well as allowing precise determination of tissue depths (Jahns & Alexeyev, 2014).

Recently, the localisation and abundance of bacterial 16S rRNA genes was demonstrated in transverse cryosections across different depths of healthy human skin using real time PCR (Nakatsuji *et al.*, 2013). Notably, this study demonstrated the localisation of total bacteria DNA beyond known depths (3mm) of hair follicles in human skin. The authors concluded that the healthy human skin permits the localisation of certain groups of bacteria in deeper skin matrix.

Since the techniques by Nakatsuji and colleagues allowed for the determination of bacterial identity, load and spatial localisation in the skin, it was deemed appropriate to conduct a similar analysis in the current study.

## 6.2 Study Hypothesis and aim

## 6.2.1 Hypothesis

This study hypothesise that low abundance of eubacteria, *D. nodosus* and *F. necrophorum* will be detected in deep healthy intact interdigital skin layers but abundantly localised in footrot infected skin.

# 6.2.2 Aim and objectives

This study was undertaken to demonstrate the trend of bacterial localisation in different layers of the ovine interdigital skin.

To accomplish this aim the following objectives were set:

- Investigate bacterial localisation and load in successive layers of ovine interdigital skin and depth of bacterial colonisation.
- Assess the association of follicular density and epidermal proportion to bacterial load.

#### 6.3 Study design and sample set

A total of 15 samples of ovine interdigital skin biopsies comprising healthy (10) and footrot (5) were collected immediately post-slaughter for investigation. Each biopsy was sectioned into alternate thin (9  $\mu$ m) and thick (40  $\mu$ m) sections for histology and bacterial counts enumeration, respectively. These sections are then analysed as individual samples.

The suitability of thin biopsy sections (9 µm) for further downstream analysis is based on their orientation as observed by H&E staining. The orientation of 6 healthy samples (H2, H3, H6, H7, H8, H10) out of the 15 samples initially enrolled were observed to be inappropriate and were excluded from further analysis. Finally, only 9 samples [healthy (H1, H4, H5, H9), footrot (F1, F2, F3, F4, F5)] were suitable for downstream analysis.

#### 6.4 Optimisation of methods

#### 6.4.1 Real time PCR assay sensitivity

The abundance of selected bacteria (*D. nodosus*, *F. necrophorum* and eubacteria) in transverse/horizontal sections of biopsies taken from ovine interdigital skin was determined. Data from 18 qPCR runs (2 runs per sample) for each of the three bacteria assays were pooled and standard curves created for analysis (Figure 6.1). *D. nodosus* and *F. necrophorum* were selected since these bacteria are mostly associated with the pathogenesis of ovine footrot and form the basis of the current hypotheses for the initiation and persistence of the disease (Beveridge, 1941; Egerton, Roberts & Parsonson, 1969; Witcomb *et al.*, 2014). As expected, there was

an inverse linear relationship between CP values and log concentration of standard DNA with acceptable correlation coefficients (R<sup>2</sup>). The range values of correlation coefficient (R<sup>2</sup>) was 0.9952-0.9954 while slope values of standard curves was -3.342 to -3.487. The cut-off for the limits of quantification (LOQ) were determined as 29.8, 33.3 and 31.7 cycles for eubacteria, D. nodosus and F. necrophorum respectively. These corresponded to 0.2 pg in eubacteria, and 0.1 pg in each of D. nodosus and F. necrophorum (Figure 6.1, Table 6.1). Criteria for confident quantification was based on acceptable efficiency and minimal coefficient of variation in mean Cp. Coefficient of variation (0.01) was the same for all three bacteria DNA templates while efficiency for limits of quantification was within acceptable range (90%-105%)(Bustin et al., 2010) for eubacteria (90%), D. nodosus (95%) and F. necrophorum (98%) (Table 6.1). Bacteria were considered detected but not quantified in a section when there is consistent signal in 2-3 replicates and mean Cp was above quantification cut-off but lower than mean Cp signals from the negative controls (OCT, NTC). The range of bacterial detection in OCT compound  $(10^{-6} - 8 \times 10^{-5} \text{ ng/}\mu\text{I})$  and non-template control/sterile distilled water (0 -7 x  $10^{-6}$  ng/µl)] indicate low level contamination (Table 6.2). However, these values were lower than the determined limits of quantification (2  $\times$  $10^{-4}$  ng/µl, Table 6.1) - suggesting that contamination load did not influence quantification in the study.



#### Figure 6-1: Determination of limits of quantification in qPCR bacteria assays of ovine interdigital skin.

Log DNA concentration (ng/µl) is plotted against Cp value of qPCR reaction. Standard curve was generated from 18 qPCR assays (2 assays per sample). For every dilution/sample 2-3 replicates were used. Black dash lines represent Cp values indicating limits of quantification in all three assays while black and red lines represent minimum Cp values of OCT and non-tissue control (NTC) respectively. *D. nodosus* and *F. necrophorum* were selected since they are associated with the aetiology and persistence of ovine footrot (Beveridge, 1941; Egerton, Roberts & Parsonson, 1969; Witcomb *et al.*, 2014).

|                | -          |                  |     |      |          |                | -          | Jinouosu         | 5   |      | i i neel opnorali |                |            |                  |     |      |          |  |  |  |  |
|----------------|------------|------------------|-----|------|----------|----------------|------------|------------------|-----|------|-------------------|----------------|------------|------------------|-----|------|----------|--|--|--|--|
| Conc.<br>ng/µl | Mean<br>Cp | Mean Cp<br>range | SD  | Cv   | Eff<br>% | Conc.<br>ng/µl | Mean<br>Cp | Mean Cp<br>range | SD  | Cv   | Eff<br>%          | Conc.<br>ng/µl | Mean<br>Cp | Mean Cp<br>range | SD  | Cv   | Eff<br>% |  |  |  |  |
| 2              | 15.4       | 15.2-15.9        | 0.1 | 0.01 |          | 1              | 19.5       | 19.2-19.7        | 0.2 | 0.01 |                   | 1              | 18.4       | 17.9-18.9        | 0.2 | 0.01 |          |  |  |  |  |
| 0.2            | 18.9       | 18.8-19.2        | 0.2 | 0.01 |          | 0.1            | 23.2       | 22.8-23.6        | 0.4 | 0.02 |                   | 0.1            | 21.8       | 21.5-22.1        | 0.2 | 0.01 |          |  |  |  |  |
| 0.02           | 22.5       | 22.2-22.4        | 0.2 | 0.01 |          | 0.01           | 26.6       | 26.2-26.7        | 0.3 | 0.01 |                   | 0.01           | 25.2       | 25.0-25.6        | 0.3 | 0.01 |          |  |  |  |  |
| 0.002          | 26.2       | 25.9-26.1        | 0.2 | 0.01 | 90       | 0.001          | 30.2       | 29.8-30.5        | 0.3 | 0.01 | 92                | 0.001          | 28.7       | 28.0-29.2        | 0.2 | 0.01 | 95       |  |  |  |  |
| 0.0002         | 29.8       | 28.9-29.8        | 0.4 | 0.01 | 90       | 0.0001         | 33.3       | 32.8-33.6        | 0.4 | 0.01 | 95                | 0.0001         | 31.7       | 31.1-32.6        | 0.5 | 0.01 | 98       |  |  |  |  |
| 0.00002        | 32.5       | 31.3-32.5        | 0.7 | 0.02 | 93       | 0.00001        | 36.2       | 35.9-36.1        | 0.7 | 0.02 | 99                |                |            |                  |     |      |          |  |  |  |  |

 Table 6-1: Cut-off values of Cp for the determination limit of quantification of bacteria qPCR assays

 Fubacteria
 D. nodosus

CV= coefficient of variation, Eff= efficiency, SD= standard deviation, red rectangles= limits of quantification determined based on low CV and good Eff.

| Table 0-2: Negative control values of QPCK assay |
|--|
|--|

| Controls | qPCR assay     | Bacterial Concentration (pg/µl) |         |        |        |        |       |       |        |       |  |  |  |  |  |
|----------|----------------|---------------------------------|---------|--------|--------|--------|-------|-------|--------|-------|--|--|--|--|--|
|          |                | H1                              | H4      | H5     | Н9     | F1     | F2    | F3    | F4     | F5    |  |  |  |  |  |
| ОСТ      | Eubacteria     | 0.08                            | 0.03    | 0.04   | 0.06   | 0.02   | 0.06  | 0.02  | 0.04   | 0.001 |  |  |  |  |  |
|          | D. nodosus     | 0                               | 0       | 0      | 0      | 0      | 0     | 0     | 0      | 0     |  |  |  |  |  |
|          | F. necrophorum | 0                               | 0       | 0      | 0      | 0      | 0     | 0     | 0      | 0     |  |  |  |  |  |
| NTC      | Eubacteria     | 0.001-                          | 0-0.002 | 0.001- | 0.001- | 0.002- | 0.004 | 0.01- | 0.001- | 0     |  |  |  |  |  |
| (water)  |                | 0.003                           |         | 0.007  | 0.003  | 0.003  |       | 0.002 | 0.002  |       |  |  |  |  |  |
|          | D. nodosus     | 0                               | 0       | 0      | 0      | 0      | 0     | 0     | 0      | 0     |  |  |  |  |  |
|          | F. necrophorum | 0                               | 0       | 0      | 0      | 0      | 0     | 0     | 0      | 0     |  |  |  |  |  |

NTC= non template control, OCT= optimum cutting temperature compound, health samples (H1, H4, H5, H9) samples, footrot samples (F1-F5)

### 6.5 Genomic DNA load

To investigate bacterial load in successive transverse skin sections of nine biopsy samples (healthy H1, H4, H5, H9; footrot F1-F5), total DNA was extracted and quantified from serial transverse sections. Also, DNA was extracted from embedding material (OCT compound) without tissues as negative control. DNA was not detected in OCT sections except in sample H1 (54.6 ng/60µl).

The DNA load in both healthy and footrot samples increased from the outermost skin layer and peaked at about 1000  $\mu$ m (bin 5) before decreasing with further depths. Comparing genomic DNA at the same depth across different clinical conditions, the median values of mean DNA load was higher in footrot samples in comparison to healthy samples. However, no significant difference was observed (Figure 6.2).



Figure 6-2: Genomic DNA load in healthy and footrot biopsy samples of ovine interdigital skin

Genomic DNA load from same skin depths were binned into groups (200  $\mu$ m/bin) and same bin from each sample compared. Blue box and whiskers represent healthy samples (H1, H4, H5, H9) while red are footrot samples (F1-F5). The lower box boundary represents 25th percentile, the line inside the box represents the median, and the upper box boundary indicates the 75th percentile. Upper and lower whiskers indicate the 10th and 90th percentiles, respectively.

## 6.6 Bacterial localisation in ovine interdigital skin

To compare bacterial localisation and load across the same depth of skin layers across different samples, 11 bin groups representing skin depths from 0 to 2200  $\mu$ m (range 0-200  $\mu$ m/bin) were created. This allowed comparing bacterial DNA presence and load in the same depths across different samples and clinical conditions.

In footrot samples, *D. nodosus* was quantified across depths of 2200  $\mu$ m (F3 and F5), 600  $\mu$ m (F2), but still detected in sections up to 2200  $\mu$ m. In sample F4 *D. nodosus* was not quantifiable but detected across entire skin depths while it was below the limit of detection in sample F1. In healthy

samples, the levels of *D. nodosus* were too low to be quantifiable but detection was possible across samples. In 2/4 healthy samples (H1 and H5), detection was almost entire across all depths except at 801-1000  $\mu$ m and 1201-1600  $\mu$ m (H1) and 801-1000  $\mu$ m (H5). In the other 2/4 samples (H4 and H9) *D. nodosus* detection was localised between 601-1000  $\mu$ m (H4) and inconsistently at depths of 401-600  $\mu$ m and 1601-1800  $\mu$ m (H9)(Figure 6.3).

*F. necrophorum* in healthy samples was detected in deep skin tissues. It was detected at depths of 601-1000µm and 1401-2200µm (H4), 1201-2200µm (H5) and 601-2200µm (H9). *F. necrophorum* was below the limit of detection in sample H1. In footrot samples, it was quantified in 2/5 samples at depths of 0-2200µm in F1 and 0-1600µm in F3 (detected beyond this depth). In samples (F2, F4, F5), *F. necrophorum* was detected across entire skin depths (0-2200 µm) (Figure 6.3).

Compared across different depths, *D. nodosus* abundance was clearly higher in footrot when compared to healthy samples where *D. nodosus* levels were too low to be quantified (Figure 6.4 A). Similarly, *F. necrophorum* DNA load was higher in footrot samples when compared to healthy samples where levels were too low to be quantified. In addition, *F. necrophorum* was observed deeply localised at depths of 601-2200µm in healthy samples (Figure 6.4 B). Interestingly, *D. nodosus* and *F. necrophorum* levels were quantifiable only in one sample (F3).

Eubacterial DNA was quantified across the entire skin depth ( $0-2200\mu m$ ) in all footrot samples (F1-F5) as well as in 2 healthy samples (H1, H4).

Also, it was quantified to depths 1600 and 1000 $\mu$ m in H5 and H9 while only detected in deeper sections beyond these depths (Figure 6.3).

Since eubacterial DNA load was determined within and across all samples (9) investigated, it was further analysed and compared between clinical conditions. Eubacterial load in healthy samples was relatively similar across the same depths but peaked at depths of 201-400 $\mu$ m (Figure 6.5). In footrot samples eubacterial load progressively decreases from the outermost skin surface to depths of 1000  $\mu$ m. Eubacterial load at the same depths of 1200-2200 $\mu$ m. Comparing eubacteria load at the same depths, median values of eubacteria load in footrot were much higher and significantly (p=0.0002) higher at depths of 0-200 $\mu$ m when compared to healthy samples (Figure 6.5).

| Healthy Samples  |   |    |    |    |    |    |                 |        |        |                       |      | Footrot samples |   |    |              |              |      |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|--|---|----|----|----|----|----|-----------------|--------|--------|-----------------------|------|-----------------|---|----|--------------|--------------|------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
|  |   | Н  | 1  |    |    | ŀ  | 14              |        |        | H5                    |      |                 |   | H9 |              |              |      | I  | -1 |    | F2 |    |    |    |    | F  | 3  |    |    | 4  |    | F5 |    |    |    |    |
| В  | E | Eu | Dn | Fn | в  | Eu | Dn              | Fn     | в      | Eu                    | Dn   | Fn              | в   | Eu | Dn           | Fn           | в    | Eu | Dn | Fn | в  | Eu | Dn | Fn | в  | Eu | Dn | Fn | в  | Eu | Dn | Fn | в  | Eu | Dn | Fn |
|  | 1 |    |    |    | 1  |    |                 |        | 1      |                       |      |                 | 1   |    |              |              | 1    |    |    |    | 1  |    |    |    | 1  |    |    |    | 1  |    |    |    | 1  |    |    |    |
|  | 2 |    |    |    | 2  |    |                 |        | 2      |                       |      |                 | 2   |    |              |              | 2    |    |    |    | 2  |    |    |    | 2  |    |    |    | 2  |    |    |    | 2  |    |    |    |
|  | 3 |    |    |    | 3  |    |                 |        | 3      |                       |      |                 | 3   |    |              |              | 3    |    |    |    | 3  |    |    |    | 3  |    |    |    | 3  |    |    |    | 3  |    |    |    |
|  | 4 |    |    |    | 4  |    |                 |        | 4      |                       |      | П               | 4   |    |              |              | 4    |    |    |    | 4  |    |    |    | 4  |    |    |    | 4  |    |    |    | 4  |    |    |    |
|  | 5 |    |    |    | 5  |    |                 |        | 5      |                       |      |                 | 5   |    |              |              | 5    |    |    |    | 5  |    |    |    | 5  |    |    |    | 5  |    |    |    | 5  |    |    |    |
|  | 6 |    |    |    | 6  |    |                 |        | 6      |                       |      |                 | 6   |    |              |              | 6    |    |    |    | 6  |    |    |    | 6  |    |    |    | 6  |    |    |    | 6  |    |    |    |
|  | 7 |    |    |    | 7  |    |                 |        | 7      |                       |      |                 | 7   |    |              |              | 7    |    |    |    | 7  |    |    |    | 7  |    |    |    | 7  |    |    |    | 7  |    |    |    |
|  | 8 |    |    |    | 8  |    |                 |        | 8      |                       |      |                 | 8   |    |              |              | 8    |    |    |    | 8  |    |    |    | 8  |    |    |    | 8  |    |    |    | 8  |    |    |    |
|  | 9 |    |    |    | 9  |    |                 |        | 9      |                       |      |                 | 9   |    |              |              | 9    |    |    |    | 9  |    |    |    | 9  |    |    |    | 9  |    |    |    | 9  |    |    |    |
| 1  | 0 |    |    |    | 10 |    |                 |        | 10     |                       |      |                 | 10  |    |              |              | 10   |    |    |    | 10 |    |    |    | 10 |    |    |    | 10 |    |    |    | 10 |    |    |    |
| 1  | 1 |    |    |    | 11 |    |                 |        | 11     |                       |      |                 | 11  |    |              |              | 11   |    |    |    | 11 |    |    |    | 11 |    |    |    | 11 |    |    |    | 11 |    |    |    |
| Legend       Lir         B       Binned groups(200µm/bin)       Eu         Eu       Eubacteria quantified       Dr         Dn       D. nodosus quantified       Fn         Fn       F. necrophorum quantified       Dr         Eu       Eubacteria detected       Dn         Dn       D. nodosus detected       Dn |   |    |    |    |    |    | mit c<br>J<br>N | of qua | antifi | catic<br>12<br>6<br>6 | on(p | g/60            | Binned groups           (Depth µm)           1=0-200           2=201-400           3=401-600           4=601-800           5=801-1000           6=1001-1200           7=1201-1400           8=1401-1600           9=1601-1800 |    |              |              |      |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| n lettophorun detected   |   |    |    |    |    |    |                 |        |        |                       |      |                 |   |    | 10=1<br>11=2 | 1801<br>2001 | -200 | 00 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |

# Figure 6-3: Localisation map of eubacteria, *D. nodosus* and *F. necrophorum* at different depth of ovine interdigital skin.

Map shows bacterial localisation and abundance in healthy (H1, H4, H5, H9) and footrot (F1, F2, F3, F4, F5) samples. Columns represent bacteria localisation at different depths ( $\mu$ m) while rows represent bin groups which indicate same depths across different samples.





Data presented as bar represent percentage of bins positive for bacteria per clinical condition: *D. nodosus* (**A**) and *F. necrophorum* (**B**).



Figure 6-5: Eubacterial DNA load in healthy and footrot biopsy samples of ovine interdigital skin.

Eubacteria DNA from same skin depths were binned into groups (200  $\mu$ m/bin) and same bin from each sample compared. The median values of eubacteria mean were compared in each bin group across samples and clinical conditions and analysed using 2-WAY ANOVA test followed by Sidak's multiple comparison (p=0.0002). The blue box and whiskers represent healthy samples (H1, H4, H5, H9) while red are footrot samples (F1-F5). The lower, inside and upper boundaries in the box represent 25th percentile, the median, and the 75th percentile respectively. Upper and lower whiskers indicate the 10th and 90th percentiles. Significance is represented by asterisk on a straight line and is designated as; \*\*\* =  $p \le 0.001$ .

# 6.7 Factors affecting eubacteria localisation in ovine interdigital skin

# The distribution pattern of eubacterial load in ovine interdigital skin samples were investigated with respect to depth of skin sections, number of follicles in skin sections as well as proportion of epidermal and dermal tissues.

Relatively low eubacterial load of range 0.4-19.6 pg/60µl was observed across healthy samples. In comparison, footrot samples eubacterial load was higher in four out of five samples with peak values of 1218 pg/60  $\mu$ l observed in the superficial skin depth ( $\leq$ 500 $\mu$ m; Figure 6.6).

We compared follicular depth to eubacterial load to establish if there was any association. Localisation of eubacterial load did not extend beyond follicular depths (2119-2710  $\mu$ m) in healthy samples while in footrot samples, eubacterial load extended beyond follicular depths (1460-2699  $\mu$ m; Figure 6.6).

Follicular density was also compared to eubacterial load in order to find out if it predicts bacteria localisation and load. In the healthy samples, eubacteria load distribution was variable irrespective of the number of hair follicles in the section. In samples H4, H5 and H9, there were sections with observed follicles, however, eubacteria was below detection limits (Figure 6.7).

In footrot there was inter-sample variation. In samples F1, F2 and F3 there appeared to be a positive association between number of hair follicles and eubacteria load (r= 0.5, 0.7, 0.6). F2 and F3 were such that as the number of hair follicles increase eubacteria load increases. In sample F1, there was a stable eubacteria load localised in sections with number of follicles ranging from 1-20. However, in sections with number of follicles ranging from 30-35, eubacteria load was variably distributed (Figure 6.7). In F4 and F5 it appeared that there was a negative association (r=-0.6, -0.2) such that as number of follicles increased, eubacterial load decreases (Figure 6.7). An interesting observation in this result is the variable bacteria load noticed in sections without follicles across all footrot samples.

Furthermore, proportions of epidermal tissues was compared to eubacterial load. In the healthy samples, eubacterial load was observed to be low, relatively stable and variably distributed across different proportions of the epidermis. In samples H1 and H4, eubacterial load was variably distributed across different proportions of epidermal tissues while in H5 and H9, eubacterial load was mainly localised in sections with epidermal proportions of 50-90% (Figure 6.8). Footrot samples showed high variability with between samples with no overall pattern (Figure 6.8). Notably, peaks of bacterial load in all footrot samples were observed in sections with epidermal tissues  $\geq$ 80% which correspond to the outermost layers of the skin.

In summary, bacterial DNA (*D. nodosus*, *F. necrophorum*, and eubacteria) was detected sub-epidermal in healthy and footrot samples using real time PCR. However, only eubacterial DNA was quantifiable in both clinical conditions. In addition, eubacterial load was higher in footrot when compared to healthy samples. Eubacterial localisation appears to be influenced by hair follicles in the healthy but not footrot samples.



**Figure 6-6: Eubacterial load distribution within ovine interdigital skin.** Eubacterial load presented as circles in individual sections at progressive depths of biopsy samples. Blue (healthy): H1(n=29), H4(n=45), H5(n=42), H9(n=38)) and red (footrot): F1(n=36), F2(n=45), F3(n=n=46), F4(n=40), F5(n=41). Black vertical lines (numbers) represent depth of follicles. Spearman correlation (r) and p-values are represent by numbers.



Figure 6-7: Comparing eubacterial load to follicular density in ovine interdigital skin.

Eubacterial load presented as circles with associated number of follicles in individual sections of biopsy samples. Blue (healthy: H1(n=29), H4(n=45), H5(n=42), H9(n=38) and red (footrot: F1(n=36), F2(n=45), F3(n=46), F4(n=40), F5(n=41). Spearman correlation (r) and p-values are represent by numbers.



Figure 6-8: Comparing eubacterial load to epidermal tissue proportion in ovine interdigital skin.

Eubacterial load presented as circles with associated proportion of epidermal tissues in individual sections of biopsy samples. Blue (healthy): H1 (n=29), H4(n=45), H5(n=42), H9(n=38) and red (footrot): F1(n=36), F2(n=45), F3(n=46), F4(n=40), F5(n=41).

#### 6.8 Discussion

The ovine interdigital skin bacteria flora has undergone rigorous quantitative interrogations in the last decade. However, one area that has not been adequately elucidated is the localisation of bacteria in different layers of the skin. Our study provide descriptive data comparing the prevalence and load of important bacteria in footrot disease with regards to their localisation across interdigital skin in healthy and footrot samples. Important findings in this study are as follows: 1) the bacteria localisation map reveals the unevenness of bacteria prevalence within layers of ovine interdigital skin, 2) localisation of eubacteria load in deep ovine interdigital skin is irrespective of clinical condition, 3) hair follicles seem to be associated with eubacteria localisation in healthy ovine interdigital skin.

#### 6.8.1 Eubacteria localisation

Eubacteria was consistently localised within and across all samples investigated. A low but stable eubacteria load extended deep into layers of healthy ovine interdigital skin whereas in the same depth in footrot samples, eubacteria load was higher. In accordance with the present results, a previous study on serial sections of healthy human skin also demonstrated eubacterial DNA in deep facial and palm skin tissues (5mm)(Nakatsuji *et al.*, 2013). This is similar to the current study in which eubacterial DNA was detected to such low depths as 2710  $\mu$ m (~3mm) in healthy samples. Other previous studies using whole biopsies also reported eubacteria presence in healthy ovine interdigital skin (Calvo-Bado *et al.*, 2011; Witcomb *et al.*, 2015; Maboni *et al.*, 2016). Generally eubacterial load was higher in serial layers of footrot samples across interdigital skin depth when compared to same depth in healthy samples. As expected, eubacterial load was significantly higher in the outermost layer of footrot skin ( $\leq 200\mu$ m) in comparison to the same depth in healthy samples. Other studies on a human skin model have also reported higher bacteria abundance in the superficial layers of human skin using cultural techniques (Röckl & Mueller, 1959; Lange-Asschenfeldt *et al.*, 2011). Detection of bacteria DNA in deep layers of healthy ovine interdigital skin may suggests that the skin is not an impervious barrier as previously thought. Similarly, this hypothesis has been argued in the human skin model (Nakatsuji *et al.*, 2013). The contributions of other bacteria flora of the skin to footrot pathogenesis is unclear. However, these bacterial flora including *D. nodosus* and *F. necrophorum* form the polymicrobial complex description of footrot disease.

Using cultural as well as culture independent techniques, over 27 major bacteria genera have been demonstrated in the ovine feet. Abundant genera in the healthy include (*Macrococcus*, *Peptostreptococcus*, *Corynebacteria*, *Psychrobacter* and *Acinetobacter*) while in the footrot lesions, abundant genera are (*Treponema*, *Porphyromonas spp*., *Prevotella*, *Peptostreptococcus*, Clostridium, *Macrococcus*, *Staphylococcus*, *Corynebacteria*, *Mycoplasma* and *Psychrobacter*)(Beveridge, 1941; Egerton, Roberts & Parsonson, 1969; Calvo-Bado *et al.*, 2011; Maboni *et al.*, 2017).

A number of bacteria species have been cultured from footrot disease which include *Treponema*, *Porphyromonas spp.*, *Prevotella spp.*,

*Peptostreptococcus spp*. and *Clostridium spp*. (Beveridge, 1941; Egerton, Roberts & Parsonson, 1969). In particular the studies by Calvo-Bado *et al.* and Maboni and colleagues highlighted the relative changes in the microbiota of the ovine interdigital skin across clinical conditions (dysbiosis) and further advance the need to examine the contributions of these bacteria organisms (Calvo-Bado *et al.*, 2011; Maboni *et al.*, 2017). Compared to monomicrobial aetiology, ploymicrobial infections tend allow higher bacterial persistence in the site of infection, enhanced severity of infection and increased resistance to antimicrobials (Kesavalu, Holt & Ebersole, 1998; Nagashima, Takao & Maeda, 1999; Mastropaolo *et al.*, 2005; Dalton *et al.*, 2011; Murray *et al.*, 2014).

In addition, it is imperative to unravel the mechanism and conditions that leads to the localisation of bacteria in deep matrix of intact ovine skin.

#### 6.8.2 D. nodosus localisation

As expected, *D. nodosus* localisation was more prevalent in successive layers of skin in footrot samples than in healthy. Although variable, the localisation of *D. nodosus* in two healthy samples cuts across entire skin depth. A recent study reported high prevalence (58%, 46/79) of *D. nodosus* in healthy biopsies (Maboni *et al.*, 2016). It was suggested that *D. nodosus* detected were either localised in the stratum corneum or that probable sub-clinical infection was on-going (Maboni *et al.*, 2016). Similarly, other previous studies have reported variable prevalence rates of *D. nodosus* in healthy samples as follows: 32% (Moore *et al.*, 2005), 50% (Witcomb *et al.*, 2015) and 68% (Witcomb *et al.*, 2014). In the current study, the skin surface was washed and disinfected, thereby

reducing surface bacteria. Furthermore, D. nodosus was detected across entire skin depths in 4/5 footrot samples investigated and quantified across entire skin depth in 2/5 with 1/5 superficially quantified. Previous studies based on Giemsa stain and fluorescent in situ hybridisation (FISH) have suggested D. nodosus was primarily localised in superficial epidermis of ovine interdigital skin (Egerton, Roberts & Parsonson, 1969; Witcomb et al., 2015). However, Witcomb et al. (2015) reported a single cell of D. nodosus in the dermis of footrot infected ovine skin. Considering that FISH is a less sensitive technique in comparison to qPCR used in our study, it is therefore possible that more *D. nodosus* cells may be undetected in the dermis. In addition, qPCR technique is able to amplify small fragments of target DNA, thereby detecting low levels of *D. nodosus* that may be undetectable with fluorescent in-situ hybridisation (FISH). Furthermore, the vertical sectioning approach adopted in the FISH study may have prevented the exposure of large surface area to staining as compared to the current study in which the entire transverse section tissues was available.

Data from the current study must be interpreted with caution since only in few footrot samples was *D. nodosus* quantifiable. This may be due to low *D. nodosus* load since our analysis is based on thin sections (40  $\mu$ m) of biopsy in comparison to other studies which analysed whole biopsies (Calvo-Bado *et al.*, 2011; Maboni *et al.*, 2016). Also competition from other eubacteria flora quantified in these sample sections may have contributed to reduce *D. nodosus* load (Witcomb *et al.*, 2014). Other factors that may reduce *D. nodosus* load in footrot samples include sloughing of superficial

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necrotic tissues rich in *D. nodosus* and increase availability of oxygen (toxic to anaerobic *D. nodosus*) due to separation of hoof from underlying soft tissues (Beveridge, 1941; Roberts & Egerton, 1969; Witcomb *et al.*, 2014). Another explanation for the variation in *D. nodosus* load observed may also be based on how long the community was established. The highest *D. nodosus* load has been reported in interdigital dermatitis in comparison to other clinical conditions (Moore *et al.*, 2005; Witcomb *et al.*, 2014, 2015; Maboni *et al.*, 2016).

Although a low number of samples were quantifiable in this study, it provides baseline information regarding variation in *D. nodosus* load within and across ovine footrot skin samples. It also highlights the need for higher sample estimates to achieve statistical significance with such study design. Using an on-line sample size calculator (http://biomath.info/power/) at 80% power and a significance level alpha of 0.05, 19 samples (healthy and footrot) will be required to achieve a standardized difference (effect size). Finding D. nodosus in the skin does not necessarily result in disease onset. Initiation of disease is largely dependent on the invading phenotype of *D. nodosus* and other factors. Based on virulence, *D. nodosus* is classified into 2 distinct strains: benign *D. nodosus* which is typified by the presence of the protease gene *aprB2* and associated with the benign form of disease while the virulent type which carries the gene *aprV2* is associated with virulent form of disease (Kennan et al., 2014). Some studies in the UK reported 91% (Maboni et al., 2016) and 96% (Moore et al., 2005) virulent D. nodosus prevalence. Put together, the two studies suggest that the virulent strains of *D. nodosus* is predominant in the UK. However, aprV2

and *aprB2* positive isolates do not necessarily correspond to the clinical stage or severity of disease in the UK (Personal communication Blanchard, Davies & Tötemeyer). Although not differentiated in this study, it is assumed that most *D. nodosus* in the current study may also be virulent. Further work need to be done to determine if skin invasion is dependent on strain type.

#### 6.8.3 F. necrophorum localisation

*F. necrophorum* is a commensal found in the digestive system of ruminants (Smith & Thornton, 1997) including the sheep mouth (Bennett et al., 2009). Hence, it is possible for these organisms to spread in the environment. F. necrophorum was found to be localised in deep healthy skin tissues ( $\geq 600 \ \mu m$ ) confirming its invasive ability. This result suggests that *F. necrophorum* persists in deep healthy tissues and under favourable conditions may contribute to the initiation of dermatitis which can subsequently lead to D. nodosus colonisation (Beveridge, 1941; Egerton, Roberts & Parsonson, 1969; Witcomb et al., 2015; Maboni et al., 2016). An earlier study by Egerton and colleagues hypothesised that F. necrophorum is essential for the invasion of the skin by D. nodosus and subsequent development of footrot (Egerton, Roberts & Parsonson, 1969). F. necrophorum has been suggested to be entirely localised in the epidermis (Witcomb et al., 2015; Maboni et al., 2016). This hypothesis is further supported in the context of higher *F. necrophorum* prevalence in swab samples (75%)(Witcomb et al., 2014) compared to whole biopsies (63%)(Maboni et al., 2016). In the current study however, F. necrophorum was consistently localised across skin depths in 4/5 footrot samples suggesting that further study is required for a definitive conclusion to be drawn. However, the higher load and localisation of *F. necrophorum* observed in this study is consistent with earlier studies that have reported increased *F. necrophorum* load and prevalence in footrot when compared to healthy condition in biopsies (Witcomb *et al.*, 2015; Maboni *et al.*, 2016). This result in addition to previous studies further provide evidence in support of the hypothesis that *F. necrophorum* is an opportunistic pathogen with a secondary role in footrot disease (Beveridge, 1941; Witcomb *et al.*, 2014, 2015; Frosth *et al.*, 2015; Maboni *et al.*, 2016). However, the exact role of *F. necrophorum* is still undetermined. It has been shown to play synergistic roles (Brook & Walker, 1986; Tan, Nagaraja & Chengappa, 1996) with other pathogens to enhance disease severity in other polymicrobial diseases such as perirectal abscess (Brook & Frazier, 1997).

#### 6.8.4 Effect of hair follicles on bacteria localisation

Hair follicles have been reported to serve as a bacterial reservoir and hence, possible routes of bacterial entry into healthy skin (Montes & Wilborn, 1970). The analysis of sequential transverse sections in this study showed that eubacterial load corresponds to follicular depth in healthy samples but extended beyond follicular depth in footrot. This suggests that hair follicles may play a role in eubacteria localisation in intact healthy skin. In a similar polymicrobial disease of bovine feet, no association was found between treponemes and hair follicles (Evans *et al.*, 2009).

There was no association between follicular density and eubacteria load. Moreover, there were sections with hair follicles in which bacteria were undetected. Conditions dictating hair follicles colonisation are yet unclear. Lange-Asschenfeldt (2011) reported that about 25% of hair follicles are colonised by bacteria in the superficial stratum corneum of human skin. It is possible that follicle associated eubacteria abundance may be dictated by increased frequency of colonisation of some follicles (Kearney *et al.*, 1984).

#### 6.8.5 Effect of epidermal proportion on eubacteria localisation

There was no association between epidermal tissue proportion and eubacteria load. Previous studies have suggested footrot disease to be an epidermal disease (Egerton, Roberts & Parsonson, 1969). We postulate that depth of skin tissue rather than epidermal tissue proportion is the determinant of bacterial localisation in the healthy skin.

#### 6.9 Study limitations

Although the study has successfully demonstrated that bacteria DNA is localised in the sub-epidermal ovine skin layers, a number of important limitations need to be considered. Data from real time PCR do no differentiate between viable and dead or lysed cells. However, results in this study are justifiable as bacterial localisation was more prevalent in footrot samples. Furthermore, there is need for cautious interpretation due to the small sample size in this study. In particular, *D. nodosus* and *F. necrophorum* load were not further analysed due to low number of sections that were quantified in sample biopsies. This notwithstanding, the study provided background information on bacterial localisation in the footrot lesions. Although *D. nodosus* and *F. necrophorum* were detected in deeper skin layers, the presence of other bacteria in the eubacteria composite

cannot be ignored. Components of the eubacteria flora may reduce *D. nodosus* and *F. necrophorum* load through competition for space and nutrients (Witcomb *et al.*, 2014). Furthermore, caution is required during sample processing (OCT embedding, cryo-sectioning, DNA extraction) to limit additional contamination of samples which may increase bacterial load. Finally, although the technique in this study allows the determination of spatial localisation of bacteria, it does not allow spatial visualisation of bacteria *in-situ*, thereby limiting further analysis with regards to the association of bacterial localisation with skin features such as epidermis, dermis and hair follicles etc.

#### 6.10 Summary and Conclusion

This study set out to determine bacterial localisation in successive ovine skin layers. We demonstrated that *D. nodosus* localisation is variable across healthy skin sections while *F. necrophorum* is localised in deeper healthy tissues in comparison to footrot samples. Eubacterial load is uneven across the skin and dependent on skin depth with the highest load in the topmost layers and decreasing in deeper layers. If tissue invasion is considered a virulence trait, then eubacteria components may be involved in disease pathogenesis. Hair follicles seem to play a role in bacteria localisation in intact ovine skin. However, factors determining follicular colonisation and associated bacteria abundance are unclear.

#### **Chapter 7 General Discussion**

#### 7.1. Thesis discussion overview

This thesis focused on two main aspects to further our current knowledge of ovine footrot. First, semi-quantitative grading and quantification systems of footrot histology lesions in ovine feet were developed, optimised and applied. Prior to this study, mainly qualitative description of histology lesions of ovine footrot were reported (Beveridge, 1941; Thomas, 1962; Egerton, Roberts & Parsonson, 1969; Davenport *et al.*, 2014). Grading and quantification of histology lesions enabled standardisation of data and subsequent comparison of histology lesions across different clinical conditions. In addition, determination of the severity of histological lesions allowed comparison with other parameters such as proinflammatory marker genes (IL-1 $\beta$ ) and virulent *D. nodosus* load (causative agent of footrot).

Second, this thesis adopted the methods of Nakatsuji et al., (2013) which combined histology (cryosectioning and H&E staining) and cultureindependent techniques (qPCR) to explore bacterial localisation and abundance in transverse sections of progressive layers of ovine interdigital skin in healthy and footrot samples. With the exception of the study by Nakatsuji and colleagues in human samples (Nakatsuji *et al.*, 2013), this study appears to be the first to have applied this method to study ovine footrot. Hence, it provided information on the localisation pattern of eubacteria, *D. nodosus* and *F. necrophorum* in successive strata of ovine interdigital skin. Previous studies have mainly examined these bacteria component of the ovine feet at skin surfaces (swabs)(Moore, Wassink &

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Green, 2005; Witcomb *et al.*, 2014; Frosth *et al.*, 2015) and in whole skin (biopsy)(Calvo-Bado *et al.*, 2011; Witcomb *et al.*, 2015; Maboni *et al.*, 2016) levels.

#### 7.2 Ovine interdigital skin disruption

The initiation of ovine footrot has been attributed to disruptions in the integrity of intact healthy ovine interdigital skin followed by bacterial colonisation. To demonstrate the importance of skin disruption in the pathogenesis of footrot, several studies established the infection process by first disrupting the integrity of healthy ovine interdigital skin, followed by bacteria exposure (Beveridge, 1941; Roberts & Egerton, 1969; Egerton, Roberts & Parsonson, 1969; Kennan *et al.*, 2001, 2010). Evidence that skin disruption is required to establish infection in these studies was their inability to establish infection in intact healthy skin.

In the environment, skin disruption may be caused by mechanical factors such as stones, rocks and sharp objects in the farm environment. Additionally, skin disruption may be caused by maceration when the skin is exposed to prolong wet and damp environment. Bacterial invasion of macerated feet by bacterial pathogens in the environment may result in infection and development of lesions (Egerton, Roberts & Parsonson, 1969). This is similar to the formation of skin lesions in the perineal areas of human patients with faecal incontinence caused by skin maceration (Gray *et al.*, 2007; Langemo *et al.*, 2011).

In the UK, most periods of the year are wet and damp therefore encouraging macerations of ovine feet as sheep graze on pasture. The presence of urine and faecal materials in the environment and bedding

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materials create a good condition for microbial colonisation of the macerated interdigital skin (Egerton, Roberts & Parsonson, 1969).

Histologically, lesion formation in faecal incontinence involves two stages: i) maceration of the skin, and ii) skin penetration by faecal pathogens. Excessive exposure of the skin to water disrupts the intercellular lipid architecture of the stratum corneum (epidermis)(Spears *et al.*, 1999; Bouwstra *et al.*, 2008; Minematsu *et al.*, 2011), extending interstitial space and reduces cell attachments in the stratum corneum of the epidermis. These events combine to allow cutaneous penetration of macromolecules and microbial agents (Minematsu *et al.*, 2011; Tan *et al.*, 2010). Host reaction to microbial invasion and penetration of foreign molecules initiate inflammatory response which triggers lesions observed at histology. This is important in the context of the current study to explain observed histological lesions in apparently healthy skin.

#### 7.3 Histological lesions in the ovine interdigital skin

In the current study, histological lesions including parakeratosis, cell ballooning, micro-abscesses and inflammatory cell infiltration and basement membrane disruption were not different in severity between disease states (ID and footrot) and healthy state in the ovine feet. In contrast, *D. nodosus*, *F. necrophorum* and total bacteria load were reportedly higher in the disease states (ID and footrot) when compared to healthy condition from corresponding parallel samples in the study by Maboni and colleagues (Maboni *et al.*, 2016). This may mean that, while bacteria load was defined by clinical condition, histological lesions were independent of clinical conditions. In addition, the findings in the current

study suggest that other factors including bacterial presence contribute to the development of histological lesions. It could be hypothesised that maceration facilitate the influx of microbial agents into the interdigital skin. This probably may have contributed to the observed lesions.

Moreover, similar histological lesions to the current study have been reported in other disease conditions in ruminants such as contagious ovine digital dermatitis (Angell *et al.*, 2015), bovine digital dermatitis (el-Ghoul & Shaheed, 2001; Cruz *et al.*, 2005) as well as severe hoof disease in Roosevelt elk (Han & Mansfield, 2014). Together, these studies provide insights into the non-specificity of histological lesions in ovine footrot that may be common in ungulates. These diseases share similarity with regards to their ploymicrobial aetiological agents.

# 7.4 Factors affecting inflammatory cell infiltration in ovine

#### interdigital skin

Of note in this study is the severity of inflammatory cell infiltration which was found to be similar between diseased (ID and footrot) and healthy samples. Previous studies have reported this observation in healthy ovine feet (Davenport *et al.*, 2014; Angell *et al.*, 2015). Similar investigations in cattle also found inflammatory cell infiltration in apparently healthy hooves (Tarlton *et al.*, 2002; Mendes *et al.*, 2013). However, Tarlton *et al.* attributed the inflammatory response observed to the changes in collagen expression and keratinization of epidermal laminae caused by intensive breeding regimes during the peripartum period. Unfortunately, this could not be verified in the current study given the lack of medical history of sampled animals since samples were collected from the abattoir.

Notwithstanding, however, a range of events including cell ballooning (keratinocyte activation), presence of micro-abscesses as well as bacteria load including virulent *D. nodosus* load were observed to be associated with severity of inflammatory cells infiltration in the skin stroma.

Epidermal cell ballooning is part of host cell response to stimuli including bacterial infection which is preceded by hyper-proliferation to remove pathogen infected area (sloughing) (Edwards, Dymock & Jenkinson, 2003). Ballooned cells (activated keratinocytes) synthesise and release pro-inflammatory cytokines including IL-1 $\beta$  for the recruitment of inflammatory cells (Refaai *et al.*, 2013). In the current study, there was correlation between increased cell ballooning and severity of inflammatory cell infiltration. Similarly, higher levels of IL-1 $\beta$  expression corresponded with increased cell ballooning and diffused parakeratosis. Combined together, these results suggest host immune response to on-going pathological insults which may be due to microbial infections (Fleming & McGee, 1984; Syrjänen *et al.*, 1996).

Further analysis in this study found significant link between the severity of inflammatory cell infiltration and the presence of micro-abscesses across different clinical conditions (Chapter four). Aside from the role played in the recruitment of inflammatory cells, micro-abscesses also reduce the availability of therapeutic drugs to bacteria (Ullberg, 1954; Joiner *et al.*, 1981) in the abscess matrix. This means that some bacteria present in the abscess lesion may evade the effects of topical treatments and footbaths to persist in the tissue. *Corynebacterium pyogenes* has been associated with micro-abscesses in ovine footrot (Gregory, 1939) while other pyogenic bacterial genera such as *Staphylococcus* and *Streptococcus* have also been

reported in ovine feet and footrot lesions (Calvo-Bado *et al.*, 2011; Maboni *et al.*, 2017). Similarly, strains of treponemes isolated from bovine digital dermatitis were shown to induce abscess formation in mice (Elliott, Alt & Zuerner, 2007). *Treponema* have similarly been reported in ovine feet (Beveridge, 1941; Thomas, 1962; Calvo-Bado *et al.*, 2011; Frosth *et al.*, 2015; Maboni *et al.*, 2016).

Further to our analysis in this study, virulent *D. nodosus* load (primary cause of footrot) was significantly associated with severity of inflammatory cell infiltration in all clinical conditions. It is worth noting however that exposure of ovine feet to *D. nodosus* does not always translate to disease establishment (Wassink *et al.*, 2010).

#### 7.5 Bacterial localisation in ovine footrot

Although *D. nodosus* is the primary causative agent of footrot, other bacterial agents are required for bacterial invasion as well as development and persistence of lesions in the skin. In an *in-vivo* study to initiate footrot infection with *D. nodosus* in macerated sheep feet, Roberts and Egerton (1969) reported that *F. necrophorum* was required to induce infection and that the addition of *Corynebacterium pyogenes* in the inoculum produced higher incidence of footrot (Roberts & Egerton, 1969). Over 27 bacterial genera have been reported in healthy ovine feet as well as in footrot lesions (Calvo-Bado *et al.*, 2011; Maboni *et al.*, 2017). Although much of these bacterial organisms may constitute environmental contaminants, it is possible some bacteria play contributory role in driving inflammatory response and other lesion formation. However, these bacteria and their

roles are still not clearly elucidated since current investigations are mainly focused on two pathogens (*D. nodosus* and *F. necrophorum*).

Several bacteria including *Treponema*, *Corynebacterium pyogenes*, *Staphylococcus spp* in addition to *D. nodosus* and *F. necrophorum* have been frequently demonstrated in culture, swabs and biopsy samples of healthy and footrot lesions (Beveridge, 1941; Parsonson, Egerton & Roberts, 1967; Calvo-Bado *et al.*, 2011; Maboni *et al.*, 2017), and were previously thought to be localised in the superficial epidermis of ovine feet (Witcomb *et al.*, 2015; Maboni *et al.*, 2016). On the contrary, studies in this thesis demonstrated the presence of bacterial DNA including *D. nodosus* and *F. necrophorum* across entire depths of ovine interdigital skin. Total bacterial load in footrot samples was significantly higher in the superficial skin ( $\leq$  200 µm) when compared to healthy samples. It is possible that the difference in bacterial community diversity and abundance in the same depths/strata between healthy and footrot interdigital skin contribute to determining disease outcome.

While a number of studies have been conducted on the aetiopathogenesis of ovine footrot, most of these studies applied culture and/or molecular based approaches to target known bacterial species, thereby limiting information on the diversity of bacterial aetiology to a few known pathogens (Beveridge, 1941; Parsonson, Egerton & Roberts, 1967; Roberts & Egerton, 1969; Egerton, Roberts & Parsonson, 1969; Calvo-Bado *et al.*, 2011; Witcomb *et al.*, 2014; Maboni *et al.*, 2016).

As previously mentioned, a few studies have employed metagenomics sequencing approach to survey bacterial diversity in ovine interdigital skin 196

of healthy and footrot affected feet. However, none of these studies examined microbial abundance or diversity at different strata/depths of the skin, although investigation of the microbiota in progressive skin strata of papillomatous digital dermatitis (PDD) affected bovine feet have been published (Yano *et al.*, 2010; Santos, Pereira & Caixeta, 2012). These studies compared bacterial localisation in healthy and diseased (PDD) condition. Furthermore, the studies observed the localisation of treponemes in deep PDD affected skin lesions, thereby providing further evidence to implicate treponemes in the pathogenesis of the disease.

Generally, studies on healthy ovine and bovine interdigital skin reported Proteobacteria and Firmicutes as the predominant bacterial phyla with Proteobacteria being the more diverse (Zinicola *et al.*, 2015; Nielsen *et al.*, 2016; Maboni *et al.*, 2017). On the other hand, Firmicutes predominate in the superficial and intermediate depths of bovine interdigital skin while Spirochetes was the major phylum in the deep skin tissues (Koniarova, Orsag & Ledecky, 1993). This is in concordance with the studies of Maboni *et al.*, (2016; 2017) which reported a higher presence and abundance of *Treponema* in ovine footrot biopsy samples in comparison to healthy samples. Together, it may be inferred that treponemal species probably inhabit deep tissues in the ovine interdigital skin.

While metagenomic whole genome sequencing is the most appropriate to determine bacterial diversity and profile in progressive sections of the skin, with in-tissue thin biopsy sections (40  $\mu$ m) in this study, bacterial DNA is considerably small. In ovine interdigital skin whole biopsies, bacterial DNA constitute less than 1% of the total DNA and is greatly overwhelmed by

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host DNA (Maboni *et al.*, 2017), and thereby requires enrichment (Meisel *et al.*, 2016). Bacterial DNA enrichment may be achieved by host DNA depletion, 16S rRNA amplification or increased depths of sequencing. In this study, thin sections (40  $\mu$ m) were cut across each biopsy with 200  $\mu$ m sections pooled into a bin and each biopsy normalised into 11 bins. Considering the prohibitive cost of sequencing (11 bins x 9 samples), the bacterial survey was limited to the 16S rRNA amplicon approach of total bacteria, *D. nodosus* and *F. necrophorum*.

Although the number of samples in the bacterial localisation study were not enough to allow for a definitive biological conclusion, this study (Chapter five) clearly observed variable localisation of *D. nodosus* in sequential depths of healthy interdigital skin while *F. necrophorum* was detected in deep healthy tissues ( $\geq 600 \ \mu$ m). In footrot samples, *D. nodosus* and *F. necrophorum* were abundantly localised across skin depths including the dermis. Similarly, previous studies have shown localisation of *D. nodosus* and *F. necrophorum* within the dermal layers of ovine feet (Egerton, Roberts & Parsonson, 1969; Witcomb *et al.*, 2015). These observations suggest that bacterial components are able to localise in deep skin tissues where they may induce low, chronic, but persistent inflammatory response.

Finally in this study, eubacterial load was observed to correspond with depths of hair follicles in healthy skin samples but not in footrot samples. This may suggest that hair follicles play a role in bacterial localisation in the healthy skin. Hair follicles have been implicated as bacteria reservoirs

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in the skin (Lange-Asschenfeldt *et al.*, 2011), hence, may be an additional route of bacteria colonisation of the skin.

#### 7.6 Summary and conclusion

In conclusion, a contribution of external factors including increased bacterial localisation on the skin surface probably lead to disruption of ovine interdigital skin and development of histological lesions. The interactions between skin epidermal cells, histological lesions and the bacterial components of the skin including the virulent *D. nodosus* initiate local inflammatory response which probably drive the progression of footrot disease.

### 7.7 Application

- Histological quantification systems developed in this study will serve as a template which can be applied in other disease conditions to determine severity of lesions.
- ii. Characterisation of histological lesions as well as bacterial pathogens in healthy and disease samples in this study suggest the need for prophylactic treatment of animals once there is suspected infection in the flock. Apparently healthy feet may be incubating infection.
- iii. The presence of micro-abscesses in ovine feet as well as bacteria in deep skin tissues suggest a need for topical and systemic therapy to ensure adequate penetration and efficacy in the skin.
- iv. Methods and data analysis techniques used in this study provide additional tools for researches to investigate tissues for bacteria and histological lesions.

 Further analysis of histological lesions including inflammatory cell infiltration and micro-abscesses against tissue microbiome will provide insight on the effect of these lesions on bacterial composition in tissues.

### 7.8 Recommendation

Methods developed and applied in this study would be beneficial in other similar studies for the characterisation of histological lesions as well as bacterial abundance in successive strata of the skin. In addition, DNA from pooled sections could be sequenced to determine spatial composition of bacteria in different strata of the skin.

#### 7.9 Future work

A number of possible future studies using the same experimental set up are apparent. First, a study employing a larger sample set will be useful to establish statistical significance in D. nodosus and F. necrophorum load within and across clinical conditions (minimum 18 samples; see section 5.6.2). In this study, nine samples (4 healthy and 5 footrot) were employed. Secondly, the spatial determination of D. nodosus phenotype (benign or virulent) across skin depths will aid to determine if skin invasion is dependent on strain type and further increase our knowledge of the pathogenesis of footrot. Thirdly, the use of immuno-histochemistry/FISH or technique will allow in-situ visual assessment of bacterial presence in the epidermis, dermis and the associated hair follicles in healthy and footrot condition, thereby determining the existing relationship between them. Finally, next generation sequencing of bacterial DNA at known depths to determine composition and abundance within disease state and across disease state is imperative. This will allow elucidation of greater diversity of invasive pathogenic bacteria which may be contributing to the footrot pathogenesis.

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# Appendix 1

## a. Calibration of image

1. Open image pro. If it ceases to work or displays an error message such as 'netHASP not working', re-booth the system.

- 2. If there is a dropdown menu such as Macro-player, click done
- 3. Open document and select image to be analysed from the tool bar

4. On the Image pro tool bar, click on **Measure** in the main menu and select

- Calibration. Choose Spatial in the submenu that appears (spatial calibration)
- $\checkmark$  click 'New' and set name =e.g Mike 10x objective)
- ✓ Ensure (Unit= microns)
- ✓ Select (Unit/pixel)
- ✓ By clicking the first 'image button' immediately below units/pixel, a dialogue box and reference marker line appears on the image.

| ×    | · · /          |                   |       |
|------|----------------|-------------------|-------|
| Scal | ing            |                   | ×     |
| Posi | tion line over | length reference. |       |
| Refe | rence represe  | ents how many ur  | nits? |
|      | h              | ÷                 |       |
|      | OK             | Cancel            |       |

✓ Carefully click on the reference line and place it perfectly on the image scale bar (e.g 1µm above). Then fill in the scale bar (1µm) into the dialogue box and click the green coloured (good √ icon). The reference line disappears.

5. Select 'Apply' on the spatial calibration submenu (repeat the calibration process each time you change your image).

## b. Measurement of area of interest (AOI)

- 1. Up-load image of interest
- 2. Calibrate the image as previously described

3. Select **Measure** from image pro main menu and then click on **Measurements** from the dropdown menu and select a line icon.

- Place the line icon on the scale bar to double check the correctness of the calibration. If the line measurement is the same as length of as the scale bar, it confirms the calibration was rightly done, if the measurement differs, re-calibrate.
- ✓ Select the polygon icon
- Left click and hold down the mouse button on the starting point of AOI
- ✓ Trace along the area of interest, which will be highlighted in red line, until the red line, returns to the starting point
- ✓ When finished, right click the mouse and the area will be completely captured. The length of the AOI appears in a measurement window in the calibrated unit.

# Appendix 2

| Observ   | ver scores for inflammatory cell infiltrations |           |     |    |   |    |         |      |     |      |        |     |    |   |     |    |          |   |    |      |   |    |    |   |    |    |   |   |   |      |            |            |      |
|----------|--|-----------|-----|----|---|----|---------|------|-----|------|--------|-----|----|---|-----|----|----------|---|----|------|---|----|----|---|----|----|---|---|---|------|------------|------------|------|
| Tierree  |  | Epidermis |     |    |   |    |         |      |     | _    | Dermis |     |    |   |     |    |          |   |    | Clim |   |    |    |   |    |    |   |   |   |      |            |            |      |
| code     | 510  |           | 1   |    |   | 2  |         |      | 3   |      |        | 4 5 |    |   | 1 2 |    |          |   |    |      | 3 |    |    | 4 |    |    | F |   |   | Cin. |            |            |      |
| couc     |  | М         | G   | С  | м | G  | С       | М    | G   | С    | М      | G   | С  | М | G   | С  | ┢        | М | G  | С    | м | G  | С  | М | G  | С  | м | G | С | М    | G          | С          | conu |
| mm008    | 6  | 0         | 0   | 0  | 0 | 0  | 1       | 0    | 0   | 1    | 0      | 0   | 0  | 0 | 2   | 0  |          | 2 | 2  | 1    | 2 | 2  | 2  | 2 | 0  | 2  | 2 | 0 | 1 | 2    | 2          | 1          | н    |
|          | 16   | 3         | 4   | no | 2 | 2  | 3       | 1    | 0   | 2    | 2      | 1   | 1  | 2 | 1   | 2  |          | 2 | no | no   | 2 | no | 2  | 3 | 0  | 2  | 2 | 0 | 2 | 2    | 0          | 2          |      |
|          | 26   | 3         | 3   | no | 2 | 1  | 3       | 0    | 0   | 0    | 3      | 3   | 2  | 0 | 0   | 1  |          | 3 | 1  | 3    | 3 | 3  | no | 3 | 3  | no | 3 | 3 | 4 | 2    | 3          | no         |      |
| mm030    | 6  | 2         | 3   | 1  | 2 | 3  | 3       | 0    | 0   | 0    | 1      | 1   | 2  | 0 | 0   | 0  | -        | 2 | 1  | 0    | 3 | 1  | 1  | 2 | 1  | 2  | 2 | 0 | 1 | 3    | 0          | 2          | н    |
|          | 16   | 0         | 0   | 1  | 0 | 0  | 0       | 0    | 0   | 0    | 0      | 0   | 0  | 3 | 3   | 2  |          | 2 | 1  | 1    | 2 | 0  | 0  | 2 | 1  | 1  | 2 | 1 | 1 | 3    | 1          | 2          |      |
|          | 26   | 2         | 3   | 3  | 1 | 0  | 0       | 0    | 0   | 1    | 3      | 3   | no | 3 | 3   | 1  |          | 2 | 1  | 2    | 2 | 2  | 1  | 2 | 3  | 2  | 2 | 3 | 2 | 2    | 2          | 2          |      |
| mm031    | 6  | 3         | 0   | 2  | 1 | 4  | no      | 0    | 0   | 0    | 0      | 2   | no | 2 | 0   | 2  |          | 2 | 2  | 2    | 2 | 2  | 2  | 2 | 1  | 2  | 2 | 1 | 1 | 2    | 1          | 2          | н    |
|          | 16   | 0         | 0   | 1  | 1 | 1  | 1       | 2    | 1   | no   | 1      | 1   | 1  | 3 | 3   | no |          | 2 | 1  | 2    | 2 | 1  | 2  | 2 | 2  | 2  | 2 | 2 | 2 | 2    | 2          | 2          |      |
|          | 26   | 0         | 1   | 2  | 1 | 1  | 1       | 2    | 1   | 1    | 3      | 4   | no | 2 | 1   | 1  |          | 2 | 2  | 3    | 2 | 2  | 2  | 2 | 2  | 2  | 2 | 2 | 2 | 2    | 3          | 3          |      |
| mm022    | 6  | 2         | 1   | 2  | 0 | 0  | 1       | 0    | 0   | 1    | 2      | 1   | 2  | 2 | 2   | 2  | -        | 2 | 2  | 2    | 2 | 0  | 2  | 1 | 1  | 2  | 2 | 1 | 2 | 2    | 2          | 2          | TD   |
| 11111022 | 16   | 2         | 0   | 2  | 2 | 0  | 1       | 2    | 1   | 1    | 2      | 0   | 2  | 2 | 2   | no |          | 2 | 2  | 2    | 2 | 2  | 2  | 3 | 2  | 2  | 2 | 1 | 2 | 3    | 2          | 2          | 10   |
|          | 26   | 2         | 2   | no | 2 | 2  | 3       | 2    | 2   | 2    | 0      | 0   | 0  | 0 | 0   | 0  |          | 3 | 2  | 2    | 2 | 0  | 1  | 2 | 0  | 1  | 3 | 2 | 3 | 2    | 2          | 2          |      |
|          |  |           |     |    |   |    |         |      |     |      |        |     |    |   |     |    |          |   |    |      |   |    |    |   |    |    |   |   |   |      |            |            |      |
| mm024    | 6  | 0         | 0   | 1  | 2 | 0  | 2       | 0    | 0   | 1    | 3      | 3   | 3  | 0 | 0   | 0  |          | 2 | 0  | 1    | 2 | 1  | 2  | 3 | 2  | 2  | 3 | 1 | 2 | 2    | 0          | 1          | ID   |
|          | 26   | 1         | 0   | 0  | 0 | 0  | 1       | 0    | 0   | 1    | 0      | 0   | 2  | 0 | 0   | 0  | -        | 2 | 0  | 1    | 2 | 0  | 2  | 2 | 0  | 2  | 2 | 2 | 2 | 2    | 1          | 2          |      |
|          | 20   | -         |     | Ŭ  | Ū |    | -       | Ŭ    | Ū   | -    | Ŭ      | •   | Ŭ  | • |     |    |          | - | Ŭ  | -    | _ | Ŭ  |    | _ | •  |    | _ | - | Ű | _    | -          | _          |      |
| mm074    | 6  | 1         | 0   | 0  | 0 | 0  | 1       | 0    | 0   | 0    | 1      | 0   | 2  | 1 | 0   | 1  |          | 2 | 0  | no   | 2 | 1  | 2  | 2 | 1  | no | 2 | 1 | 2 | 2    | 1          | no         | ID   |
|          | 16   | 2         | no  | no | 0 | 0  | 1       | 0    | 0   | 1    | 0      | 0   | 0  | 1 | 0   | no |          | 2 | 0  | 2    | 2 | 0  | 2  | 2 | 1  | 1  | 3 | 1 | 2 | 2    | 2          | 1          |      |
|          | 26   | 1         | no  | no | 1 | 0  | 1       | 0    | 2   | 1    | 0      | 0   | no | 1 | no  | no |          | 2 | 1  | 1    | 2 | 1  | 2  | 2 | 1  | 1  | 3 | 0 | 1 | 2    | 1          | 2          |      |
| mm077    | 6  | 1         | 0   | 3  | 1 | 1  | 2       | 0    | 0   | 3    | 1      | 1   | 2  | 2 | 1   | 1  |          | 2 | 0  | 2    | 3 | 2  | 2  | 3 | 2  | 2  | 3 | 2 | 2 | 2    | 1          | 2          | ID   |
|          | 16   | 0         | 0   | 0  | 0 | 0  | 0       | 0    | 0   | 2    | 0      | 0   | 2  | 0 | 0   | 2  |          | 2 | 0  | 2    | 2 | 1  | 2  | 3 | 1  | 2  | 2 | 1 | 2 | 3    | 1          | 2          |      |
|          | 26   | 3         | 3   | 2  | 2 | 1  | 1       | 2    | 2   | 1    | 2      | 1   | 1  | 0 | 0   | 1  |          | 2 | 2  | 2    | 3 | 2  | 2  | 2 | 2  | 2  | 2 | 2 | 2 | 2    | 2          | 1          |      |
| mm047    | 6  | 0         | 0   | 0  | 0 | 0  | 1       | 4    | 4   | 4    | 4      | 4   | 4  | 2 | 4   | Δ  | _        | 2 | 1  | 2    | 2 | 2  | 1  | 2 | 2  | 2  | 2 | 1 | 2 | 2    | 2          | 2          | FR   |
|          | 16   | 0         | 0   | 2  | 3 | 4  | 4       | 4    | 4   | 4    | 3      | 3   | 3  | 0 | 0   | 1  |          | 3 | 2  | 3    | 3 | 2  | 2  | 3 | 2  | 3  | 3 | 2 | 2 | 3    | 2          | 2          |      |
|          | 26   | 0         | no  | no | 3 | 4  | no      | 2    | 2   | 2    | 3      | 3   | 4  | 0 | 0   | 2  |          | 2 | 1  | 2    | 2 | 1  | 2  | 3 | 2  | 2  | 2 | 2 | 2 | 3    | 3          | 3          |      |
| mm055    | 6  | 0         | 0   | no | 0 | 0  | no      | 1    | 1   | 1    | 0      | 0   | 0  | 0 | 0   | 0  | -        | 2 | 0  | 2    | 2 | 1  | 2  | 2 | 1  | 1  | 2 | 1 | 1 | 2    | 0          | 2          | FR   |
|          | 16   | 2         | 0   | 0  | 2 | 0  | 2       | 0    | 0   | 0    | 1      | 1   | 2  | 0 | 0   | 0  |          | 3 | 1  | 2    | 2 | 0  | 1  | 2 | 1  | 2  | 2 | 1 | 1 | 2    | 1          | 2          |      |
|          | 26   | 0         | 0   | no | 0 | 1  | 1       | 2    | 1   | 1    | 0      | 0   | 0  | 0 | 0   | 0  |          | 2 | 0  | 2    | 2 | 0  | 1  | 2 | 0  | 1  | 2 | 1 | 2 | 2    | 0          | 2          |      |
|          |  |           |     |    |   |    |         |      |     |      |        |     |    |   | _   |    |          |   |    |      |   |    |    |   |    |    |   |   |   |      |            |            |      |
| mm056    | 6  | 0         | 0   | 0  | 0 | no | no      | 2    | 1   | 1    | 0      | no  | no | 1 | 1   | 1  | _        | 2 | 0  | 1    | 2 | 1  | 2  | 2 | 0  | 1  | 2 | 1 | 1 | 2    | 2          | 2          | FR   |
|          | 26   | 0         | 0   | 0  | 1 | 0  | 0       | 1    | no  | no   | 2      | 1   | 1  | 0 | 0   | 0  | -        | 2 | 2  | 2    | 2 | 2  | 2  | 2 | 2  | 2  | 3 | 1 | 2 | 3    | - J<br>- 1 | - J<br>- 1 |      |
|          |  |           |     |    |   |    |         |      |     |      |        | _   |    |   |     |    |          |   | _  |      | - |    |    | - |    |    | _ | _ |   | -    |            |            |      |
| mm060    | 6  | 1         | 0   | 0  | 2 | 2  | 2       | 1    | 0   | 2    | 1      | 0   | 0  | 0 | 0   | 0  |          | 2 | 0  | 2    | 2 | 0  | 2  | 2 | 0  | 2  | 2 | 1 | 2 | 2    | 0          | 0          | FR   |
|          | 16   | 2         | 1   | 2  | 2 |    | no<br>1 | 3    | 4   | 4    | 2      | 1   | 10 | 0 | 1   | 1  | _        | 2 | 0  | 2    | 2 | 0  | 0  | 2 | 1  | 2  | 2 |   | 2 | 2    |            | 2          |      |
|          | 20   | 2         | 3   | 4  | 1 | 0  |         |      | -0  | 0    | 0      | -1  | 1  | 2 |     |    | ┝        | 2 | 1  | 2    | 2 | 0  |    | 2 | -1 | 1  | 2 | 0 | 1 | 2    | 0          | 0          |      |
| mm081    | 6  | 1         | 0   | 0  | 0 | 0  | 0       | 0    | 0   | 1    | 0      | 0   | 0  | 0 | no  | no | L        | 2 | 0  | 0    | 2 | 1  | 2  | 2 | 0  | 2  | 2 | 0 | 1 | 2    | 0          | 1          | FR   |
|          | 16   | 0         | 0   | 1  | 0 | 0  | 0       | 1    | 0   | 1    | 0      | 0   | 0  | 0 | 0   | no |          | 2 | 2  | 3    | 2 | 2  | 4  | 2 | 0  | 2  | 2 | 0 | 2 | 2    | 0          | 1          |      |
|          | 26   | 0         | 0   | 0  | 0 | 0  | 1       | 1    | 0   | 1    | 0      | no  | no | 0 | 0   | 0  | _        | 2 | 1  | 2    | 2 | 0  |    | 2 | 0  | 2  | 3 | 3 | 3 | 2    | 2          | 3          |      |
| mm092    | 6  | 2         | 0   | 2  | 1 | 0  | 0       | 1    | 0   | 0    | 1      | 0   | no | 0 | 0   | 1  | $\vdash$ | 1 | no | no   | 2 | 0  | 2  | 1 | 0  | 1  | 2 | 1 | 2 | 2    | 1          | 2          | FR   |
|          | 16   | 1         | 0   | 0  | 1 | 0  | no      | 0    | 0   | 1    | 0      | 0   | 0  | 0 | 0   | 1  | t        | 1 | 2  | 1    | 1 | 2  | 1  | 2 | 0  | 0  | 1 | 0 | 1 | 0    | 0          | 2          |      |
|          | 26   | 0         | 0   | 0  | 0 | 0  | 0       | 0    | 0   | 0    | 0      | 0   | 0  | 0 | 0   | no |          | 0 | 0  | 1    | 0 | 0  | 0  | 0 | 0  | 1  | 1 | 0 | 1 | 2    | 0          | 2          |      |
| mm094    | 6  | 0         | 0   | 0  | 0 | no | no      | 0    | 0   | 0    | 0      | 0   | 0  | 0 | 0   | 0  | $\vdash$ | 2 | 0  | 2    | 2 | 1  | 2  | 2 | 1  | 3  | 2 | 1 | 2 | 2    | 1          | 2          | FR   |
|          | 16   | 0         | 0   | 0  | 1 | 0  | 0       | 0    | 0   | 0    | 0      | 0   | 0  | 0 | 0   | 0  | F        | 2 | 1  | 2    | 2 | 1  | 2  | 2 | 1  | 1  | 2 | 0 | 1 | 2    | 0          | 1          |      |
|          | 26   | 0         | 0   | 2  | 0 | 0  | 0       | 0    | 0   | 0    | 0      | 0   | 2  | 0 | 0   | 0  |          | 1 | 1  | 2    | 2 | 0  | 1  | 2 | 0  | 1  | 2 | 1 | 2 | 2    | 0          | 1          |      |
| 3-obser  | vers   | agre      | eed |    |   |    | 0n      | e-pc | int | disa | gree   | eme | nt |   | _   |    | -        |   |    |      |   |    |    |   |    |    |   |   |   |      |            |            |      |

# Appendix 3

# Comparing variability of inflammatory cell infiltration scores between observers 1 and 2

|     |                        | Inflammatory cell infiltration scores |                             |                           |  |  |  |  |  |
|-----|------------------------|---------------------------------------|-----------------------------|---------------------------|--|--|--|--|--|
| S/N | Tissue code            | Observer 1<br>(score)                 | Observer 2<br>(first score) | Observer 2 (second score) |  |  |  |  |  |
|     | Epidermis              |                                       |                             |                           |  |  |  |  |  |
| 1   | Mm092 slide 6 image 3  | 1                                     | 0                           | 0                         |  |  |  |  |  |
| 2   | Mm060 slide 6 image 1  | 1                                     | 0                           | 1                         |  |  |  |  |  |
| 3   | Mm081 slide 6 image 1  | 1                                     | 0                           | 1                         |  |  |  |  |  |
| 4   | Mm074 slide 6 image 5  | 1                                     | 0                           | 1                         |  |  |  |  |  |
| 5   | Mm031 slide 26 image 2 | 1                                     | 1                           | 1                         |  |  |  |  |  |
| 6   | Mm077 slide 6 image 2  | 1                                     | 1                           | 1                         |  |  |  |  |  |
| 7   | Mm055 slide 6 image 3  | 1                                     | 1                           | 1                         |  |  |  |  |  |
| 8   | Mm030 slide 6 image 4  | 1                                     | 1                           | 1                         |  |  |  |  |  |
| 9   | Mm022 slide 6 image 1  | 2                                     | 1                           | 1                         |  |  |  |  |  |
| 10  | Mm060 slide 16 image 1 | 2                                     | 0                           | 2                         |  |  |  |  |  |
| 11  | Mm008 slide 26 image 2 | 2                                     | 1                           | 2                         |  |  |  |  |  |
| 12  | Mm077 slide 26 image 2 | 2                                     | 1                           | 1                         |  |  |  |  |  |
| 13  | Mm022 slide 26 image 1 | 2                                     | 2                           | 3                         |  |  |  |  |  |
| 14  | Mm008 slide 16 image 2 | 2                                     | 2                           | 2                         |  |  |  |  |  |
| 15  | Mm022 slide 26 image 2 | 2                                     | 2                           | 2                         |  |  |  |  |  |
| 16  | Mm060 slide 6 image 2  | 2                                     | 2                           | 2                         |  |  |  |  |  |
|     |                        |                                       |                             |                           |  |  |  |  |  |
|     | Dermis                 |                                       |                             |                           |  |  |  |  |  |
| 17  | MM030 slide 6 image 1  | 2                                     | 1                           | 2                         |  |  |  |  |  |
| 18  | Mm031 slide 16 image 1 | 2                                     | 1                           | 2                         |  |  |  |  |  |
| 19  | Mm074 slide 26 image1  | 2                                     | 1                           | 2                         |  |  |  |  |  |
| 20  | Mm047 slide 6 image 1  | 2                                     | 1                           | 2                         |  |  |  |  |  |
| 21  | Mm008 slide 6 image 1  | 2                                     | 2                           | 2                         |  |  |  |  |  |
| 22  | Mm022 slide 16 image 1 | 2                                     | 2                           | 2                         |  |  |  |  |  |
| 23  | Mm077 slide 26image 1  | 2                                     | 2                           | 2                         |  |  |  |  |  |
| 24  | Mm056 slide 16 image 3 | 2                                     | 2                           | 2                         |  |  |  |  |  |
| 25  | Mm022 slide 6 image 1  | 3                                     | 2                           | 3                         |  |  |  |  |  |
| 26  | Mm047 slide 16 image 1 | 3                                     | 2                           | 3                         |  |  |  |  |  |
| 27  | Mm077 slide 26 image 2 | 3                                     | 2                           | 3                         |  |  |  |  |  |
| 28  | Mm024 slide 16 image 4 | 3                                     | 2                           | 3                         |  |  |  |  |  |
| 29  | Mm008 slide 26 image 3 | 3                                     | 3                           | 3                         |  |  |  |  |  |
| 30  | Mm056 slide 16 image 4 | 3                                     | 3                           | 3                         |  |  |  |  |  |
| 31  | Mm081 slide 26 image 4 | 3                                     | 3                           | 3                         |  |  |  |  |  |
| 32  | Mm047 slide 26 image 5 | 3                                     | 3                           | 3                         |  |  |  |  |  |