UNIVERSITY OF LIVERPOOL

A study on the role of the foot skin microbiome and the host's genetics in the development of lameness causing foot lesions in dairy cattle

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by Veysel Bay.

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Authors Declaration

Apart from the help and advice as acknowledged, all research described within this thesis has been completed solely by the author.

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Veysel Bay

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This research was carried out in the Department of Epidemiology and Population Health within the Institute of Infection and Global Health, University of Liverpool

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ABSTRACT

Lameness is one of the major health and welfare problems for the global dairy cattle industry causing detrimental economic losses. Lameness has been associated with foot lesions of both infectious and non-infectious aetiology. Digital dermatitis (DD) is one of the infectious disorders and has been associated with several bacteria, particularly of the genus *Treponema*. The main non-infectious lameness causing claw horn disruption lesions (CHDLs); sole ulcers (SU), white line disease (WLD), and toe necrosis (TN) lesions, can be secondarily infected with DD associated bacteria. The aim of this study was to examine the role of the foot skin microbiota in the development of DD lesions, to explore the microbiota profile of complicated lameness causing lesions, and to investigate the genomic regions that are linked to lameness associated traits and foot skin microbiota profiles.

A total number of 554 cows from three different farms were genotyped in the study. Foot skin swabs were collected from 259 of these cows. Furthermore, 51 cows from ten different farms were used to examine the microbiota profiles of complicated lameness causing lesions as a pilot study. Foot microbiome profiles of lameness associated lesions were determined using 16S rRNA gene amplicon sequencing and the data were analysed using multivariate analysis approaches. Cattle genomic DNA samples were genotyped using a 50K single nucleotide polymorphism (SNP) chip, and genome-wide association (GWA) and regional heritability mapping approaches (RHM) were performed to find out genomic regions associated with lameness associated traits.

The cows which did not acquire DD lesions during the study had significantly different foot skin microbiota profiles from those which acquired DD lesions. Besides different microbiota populations were also identified in different farms. DD, complicated CHDLs and interdigital phlegmon (IP) lesions were shown to have polymicrobial profiles consisting of similar anaerobes, such as *Treponema* spp., and *Porphyromonas* spp., but interdigital hyperplasia (IH) lesions were not associated with any specific bacteria. Furthermore, *Treponema* spp. were not observed as early colonizers in DD. *Fastidiosipila* spp. were shown to be associated with lameness causing lesions for the first time. In addition, lameness associated traits, including digital cushion thickness (DCT), were observed to have significant genomic variation, moderate heritability and partially oligogenic architecture. Lastly, some significant genomic regions were found to be potentially associated with the relative abundance of DD associated bacteria, namely, *Treponema* spp. and *Peptoclostridium* spp.

In conclusion, lameness associated microbiota profiles revealed in this study could contribute to a better understanding of the aetiopathogenesis of lameness causing foot lesions leading eventually to improved treatment and prevention strategies. Moreover, genomic regions determined in this study could be included in cattle breeding programs.

CHAPTER I

1 Literature Review

1.1 Cattle Lameness

Dairy cattle lameness refers to the locomotion and postural abnormalities adopted by cows in order to alleviate pain in certain affected areas (Almeida et al. 2008). Lameness is a major problem for the dairy industry causing economic losses, and reduced animal welfare (Barkema et al. 1994; Kossaibati and Esslemont 1997; Whay et al. 1998; Rajala-Schultz and Gröhn 1999).

Lower milk yield is one of the reasons for the economic losses. In a study on two New York dairy farms, a significant decrease in milk production was shown to be a result of lameness. The decrease was greater for cows in second or later lactations, and for cows with more severe lameness (Warnick et al. 2001). In another study in the US, 465 cows were scored for lameness using a 0 to 5 locomotion scoring system and their milk yields were evaluated during the first 100 days after parturition. Lame cows (locomotion score ≥4) in their second or later lactations produced less milk than moderately lame or non-lame cows (Hernandez et al. 2005).

Furthermore, reduction in their reproductive performance can be seen for lame cows. In a study performed on 13 Dutch dairy farms, it was shown that lameness was associated with a longer interval between calving and first service, and between first service and conception without any effect on pregnancy rate at first service (Barkema et al. 1994). The effect of lameness on ovarian activity was investigated in a cohort of 238 cows. Lame cows with claw lesions had a longer period of return to cyclicity compared to healthy cows (Garbarino et al. 2004). In another study using 70 cows, 29% of lame cows showed no ovarian activity from 30 to 80 days post calving (Morris et al. 2011).

In addition, culling rate due to lameness was found to be 5-6% in a survey of 50 Holstein/Friesian dairy herds in England (Esslemont and Kossaibati 1997). Lame cows were also shown to incur increased veterinary expenses, and more labour (Harris et al. 1988; Cha et al. 2010).

Lameness is also a serious welfare problem since it contradicts the principal of five freedoms: freedom from hunger and thirst, freedom from discomfort, freedom from pain, injury and disease, freedom from fear and distress, and freedom to express

normal behaviour (Farm Animal Welfare Council 1992; Whay and Shearer 2017). Lame cows were shown to have impaired eating behaviour (González et al. 2008; Norring et al. 2014) and reduced water intake (Kramer et al. 2009). In terms of discomfort that is caused by lameness, lame cows have more difficulty while standing up and lying down (Yunta et al. 2012; K. Ito et al. 2010) and longer lying times than non-lame cows (Calderon and Cook 2011). As an indicator of pain, mechanical nociceptive thresholds of lame cows were shown to be lower than the thresholds of healthy cows (Ley et al. 1996; Whay et al. 1998). Lame cows also have desynchronization in daily activities that cause isolation from the rest of the herd. Increased isolation makes cows feel more vulnerable with having fear and distress (Whay and Shearer 2017).

The prevalence of lameness in the United Kingdom (UK) in 1996 was found to be more than 20% (Faull et al. 1996), and more than 30% (Clarkson et al. 1996; Murray et al. 1996) in other studies; in 2010 it was found to be 35% (Barker et al. 2010) and it has recently been found to be 28% in England and Wales (Griffiths et al. 2018). In other countries, the prevalence of lameness has been found to be 21% in Canada (Solano et al. 2015), 31% in China (Chapinal et al. 2014), and 14% for dry cows in the USA (Foditsch et al. 2016).

Lameness is a clinical sign rather than a disease, and causes of lameness show variation between farms, regions and countries (Potterton et al. 2012). More than 90% of lameness causing lesions are observed in the foot (Clarkson et al. 1996), and predominantly in the hind limbs (Archer et al. 2010). Most of the lameness cases can be categorized as infectious, such as digital dermatitis (DD) and interdigital phlegmon (IP), and non-infectious claw horn disruption lesions (CHDLs), such as sole ulcer (SU) and white line disease (WLD) (van der Waaij et al. 2005; Bicalho & Oikonomou 2013; Green et al. 2002). Interdigital hyperplasia (IH) is another prevalent lameness causing lesion (Barker et al. 2010); its aetiopathogenesis has not been completely explained.

1.2 Foot lesions commonly associated with cattle lameness

1.2.1 Digital Dermatitis

Digital dermatitis is a bacterial disease seen on the skin of the heels of cattle, predominantly in the plantar parts of the hind limbs (Figure 1.1) (Murray et al. 1996; Rodriguez-Lainz et al. 1996; Read and Walker 1998; Schultz and Capion 2013). DD

was reported for the first time in Italy in the 1970s by Cheli and Mortellaro (Cheli and Mortellaro 1974), thus it is also named as 'Mortellaro's disease'(Holzhauer et al. 2006). DD is also known as papillomatous DD, strawberry or raspberry heel warts, strawberry foot rot, hairy foot warts, hairy heel warts, and foot warts (Wells et al. 1999; Brown et al. 2000). Prevalence of DD was reported to be more than 30% in England and Wales (Murray et al. 1996), 10.5% in Chile (Rodriguez-Lainz et al. 1998), 30% in Brazil (Cruz et al. 2001) and 21.2% in The Netherlands (Holzhauer et al. 2006).

Figure 1.1. Digital dermatitis lesion

DD is an important problem for the dairy industry since it results in reduced animal welfare and economic losses in many countries (Kossaibati and Esslemont 1997; Whay et al. 1998). Bovine digital dermatitis (BDD) is a prevalent cattle disease and its active form (ADD) is characterized by lesions with an ulcerative area, whilst the inactive form (IDD) is characterized by the presence of firm scab, hyperkeratosis, proliferative overgrowth, and absence of an ulcerative area (Zinicola et al. 2015a). It results in reduced milk production and reproductive performance, increased risk of culling (Argaez-Rodriguez et al. 1997; Losinger 2006) and high treatment costs (Cha et al. 2010).

DD is a polymicrobial disease (Plummer and Krull 2017; Moreira et al. 2018), and its pathology was first described by detection of filamentous, spirochete-like organisms in the skin of the heels of cattle (Read et al. 1992; Blowey et al. 1992). These organisms were identified to be members of the genus *Treponema*. (Walker et al. 1995). *Treponema* spp. have been detected in other DD studies by using culture (Walker et al. 1995; Trott et al. 2003), fluorescence in situ hybridization (FISH) (Moter et al. 1998; Klitgaard et al. 2008), amplification of 16S rRNA gene by polymerase chain reaction (PCR) (Demirkan et al. 1998; Evans et al. 2008, 2009), and metagenomic techniques (Zinicola et al. 2015a; Krull et al. 2014; Moreira et al. 2018). Other genera including *Porphyromonas* spp. (Moe et al. 2010),

Mycoplasma spp., *Bacteroides* spp. (Collighan and Woodward 1997), *Campylobacter* spp. (Döpfer et al. 1997), *Dichelobacter nodosus* (Rasmussen et al. 2012), *Guggenheimella* spp. (Schlafer et al. 2008), *Borrelia* spp. (Blowey et al. 1994), *Fusobacterium necrophorum, and Candidatus Aemobophilus asiaticus* (Zinicola et al. 2015a; Zinicola et al. 2015b; Krull et al. 2014) were also detected in DD lesions using various methods. However, the aetiopathogenesis of DD has not been completely elucidated (Moreira et al. 2018).

Treatment of DD lesions is usually performed by direct application of topical antimicrobials, such as tetracycline and oxytetracycline to the lesions; systemic injection of antimicrobials can also be used (Evans et al. 2016; Plummer and Krull 2017). For management or prevention of the lesions foot bathing with $CuSO_4$, $ZnSO_4$, formalin and antibiotic solutions is used (Zinicola et al. 2015a; Plummer & Krull 2017).

1.2.2 Interdigital phlegmon

Interdigital phlegmon, also known as interdigital necrobacillosis, foot rot or foul in the foot, is characterised by fissures, subcutaneous necrosis located in the interdigital space, and digital swelling (Figure1.2) with an unpleasant smell and is caused by *Fusobacterium necrophorum* (Berry 2001). Besides *Fusobacterium necrophorum*, *Porphyromonas levii* and *Prevotella intermedia* bacteria have also been isolated from IP lesions (Morck et al. 1998; Nagaraja et al. 2005). Prevalence of IP was shown to be 21.1% in cows with DD lesions in The Netherlands (Holzhauer et al. 2006) and 9% in the United States (Hernandez et al. 2002).

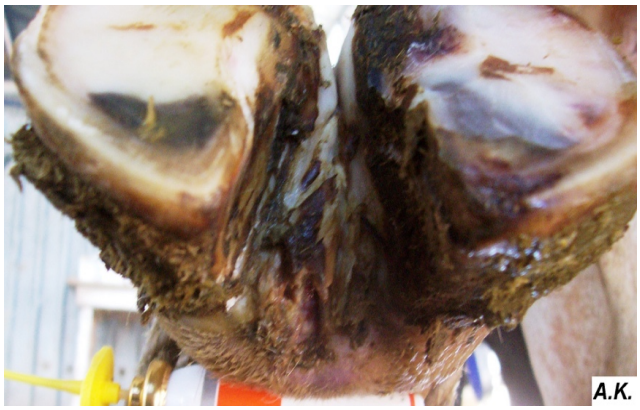

Figure 1.2. Interdigital phlegmon lesion (Courtesy of Dr. Alexandros Kougioumtzis)

1.2.3 Interdigital Hyperplasia

Interdigital hyperplasia refers to the proliferation of the interdigital skin in the interdigital space (Figure 1.3) (Kofler et al. 2011). It may occur as a result of outward spreading of the claws, poor structure of ligaments, and finally stretching of the interdigital skin (Desrochers et al. 2008). Poor hygiene, moisture, overgrown or poorly shaped claws, and slippery surfaces may also predispose cows to acquire IH lesions (Berry 2001). Prevalence of IH was shown to be 5% in England and Wales (Murray et al. 1996), 1.8% in Sweden (Manske et al. 2002), 10.4% in Brazil (Cruz et al. 2001), 2.56% as an average in Denmark (Capion et al. 2009), and 8% in France (Croué et al. 2017). IH was shown to be associated with the prevalence of DD (Holzhauer et al. 2006; Sullivan et al. 2013; van Metre 2017), however, there is no study proving an infectious aetiology for IH.

Figure 1.3. Interdigital hyperplasia lesion

1.2.4 Sole Ulcers and Sole Haemorrhages

Sole haemorrhages (SH), also known as bruising of the sole (Cramer et al. 2008), is the haemorrhagic discoloration of the sole of the foot (Figure 1.4) (Manske et al. 2002). Bruises are usually pink or yellow at early stages, then get darker, and might be confused with black pigmentation (Baggott and Russell 1988). Prevalence of SH was found to be 8% in England and Wales (Murray et al. 1996), 30% in Sweden (Manske et al. 2002).

Figure 1.4. Sole haemorrhage lesion

Sole ulcers are defined as localized loss of horny sole and exposure of the corium (Figure 1.5); their development has been associated with housing, diet, foot trimming, and genetic conformational

problems (Enevoldsen et al. 1991; Shearer and van Amstel 2017). Both SH and SU have been thought to be associated with impairment of the vascular system in the corium causing a disintegrated dermal-epidermal junction, sinking of the distal phalanx, and lesion formation in the horn capsule including the suspensory apparatus and digital cushion (Ossent and Lischer 1998; Lischer and Ossent 2002; Shearer and van Amstel 2017). The digital cushion is a tissue complex which mainly consists of fat situated under the distal phalanx functioning as a shock absorber (Räber et al. 2004; Bicalho et al. 2009; Shearer and van Amstel 2017). In addition, these lesions are associated with predisposing causes such as housing, nutrition, foot trimming and genetic conformational problems (Enevoldsen et al. 1991; Vermunt and Greenough 1994). These predisposing causes can lead to mechanical loading and metabolic/enzymatic alterations which impair the suspensory apparatus or the digital cushion resulting in vascular injury. It has been hypothesised that the digital cushion becomes thinner due to fat mobilization after calving (Bicalho et al. 2009). Moreover, calving has been associated with increased laxity in the connective tissue around the distal phalanx (Tarlton et al. 2002), possibly due to the hormonal effect of relaxin or oestrogen (Shearer and van Amstel 2017). Besides, the suspensory apparatus might be weakened by the degradation of the connective fibres via activated matrix metalloproteinases due to inflammation around calving (Shearer and van Amstel 2017). Standing and walking on concrete surfaces might also be associated with mechanical weakening of the digital cushion (Bicalho and Oikonomou 2013). Prevalence of SU was found to be 40% in England and Wales (Murray et al. 1996), 8.6% in Sweden (Manske et al. 2002), 5.6% in The Netherlands (Holzhauer et al. 2008b), 4.2% for parity 1, and 27.8% for parity >1 cows in the United States (Bicalho et al. 2009).

Figure 1.5. Sole ulcer lesion

1.2.5 White Line Disease

White line disease is seen when the sole is separated from the side wall of the hoof (Figure 1.6). Similarly to the development of SU lesions, the impaired vascular system may cause poor keratinization in the horn, which becomes less resistant to physical forces and is more open to intrusion of foreign materials, such as stones (Shearer and van Amstel 2017). Separation of the sole leads to a weak point which is more open to infections (Baggott and Russell 1988). The prevalence of WLD was found to be 29% in England and Wales (Murray et al. 1996), 1% for parity 1, and 6.5% for parity >1 cows in the United States (Bicalho et al. 2009).

Figure 1.6. White line lesion (Courtesy of Bethany Griffiths)

1.2.6 Toe Necrosis/ Toe Ulcers

Toe ulcer (TU) is defined as exposure of fresh or necrotic toe corium and formation of a ulcer, and the necrosis of the tip of the pedal bone after a bacterial infection (Figure 1.7) is defined as toe necrosis (TN) (Kofler 2017). Prevalence of TU/TN was found to be 12.6% (Nuss et al. 1990) and 4.8% (Kofler 1999) in Central Europe (Kofler 2017). They are thought to be a result of excessive trimming or excessive wearing of the sole horn.

Figure 1.7. Toe necrosis lesion (Courtesy of Bethany Griffiths)

1.3 The Role of the Microbiome in Health and Disease

The term "Microbiome" refers to the genome of a community (microbiota) of microorganisms (bacteria, archaea, viruses and lower and higher eukaryotes), which inhabit a defined environment (Marchesi and Ravel 2015). All higher organisms including humans are populated by microorganisms. There is a growing agreement that symbiotic microorganisms are very important factors in shaping phenotypes of their hosts including fitness; ability to survive and reproduce in the environment (Bosch and McFall-Ngai 2011; Ross et al. 2013).

The human microbiome concept was first proposed by Joshua Lederberg (Lederberg and McCray 2001) as the commensal, pathogenic and symbiotic communities that share our body space. It is estimated that the human body consists of at least 10 times more bacteria than nucleated human cells (excluding non-nucleated red blood cells) (Sender et al. 2016). In addition, the number of microbial genes in the body could be 100 times more than the number of human genes (Ezenwa et al. 2012).

The colonization of the gastrointestinal tract of new-born infants with bacteria was shown to be seeded with the maternal gut microbiota (Korpela et al. 2018). After initial colonization, pioneer bacteria can alter the gene expression of the host and create a favourable environment for themselves and inhibit the growth of other bacteria (Hooper et al. 2001). The colonization pattern is affected by the type of birth

(natural or caesarean) and diet, (breast or formula feeding). Additionally, since there is a significant difference between birth in developed and developing countries, and as a result of the differences between hospital circumstances, country of birth and the hospital environment might alter the colonization pattern (Knight et al. 2003).

The human gut microbiota makes functional contributions to human metabolism; it helps to harvest the foods' energetic value by its digestive capacity. If microbiota did not exist in the human gut, the nutrient/energetic value of food would become lower. Harvesting the energetic value of foods is accomplished by the fermentation and absorption of the main products of fermentation, short-chain fatty acids (SCFA), that have approximately 40-50% energy of the carbohydrate. Among these fatty acids, butyrate is metabolized in the colonic epithelium, propionate is metabolized in the liver, and acetate is metabolized in muscle. Moreover, the microbiota has a role in the synthesis of vitamins B and K. Additionally; it enables the metabolism of xenobiotics and other metabolic phenotypes by its own catabolic activities (Cummings and Macfarlane 1997). In mice, it has been demonstrated that obesity-related microbiome provides more energy harvest from ingested nutrients. It is also confirmed by the transfer of gut microbiota from obese mice to wild-type germ-free mice, which causes an increase in fat mass compared to the transfer of those from lean mice (Turnbaugh et al. 2006). There are two dominant groups of bacteria in mice gut, Bacteroidetes, and Firmicutes, and the relative abundance of these groups in obese mice is different compared to lean mice. Obese mice have 50% fewer Bacteroidetes and respectively, more Firmicutes than their lean siblings (Ley et al. 2005). In the case of the human gut, there are similarities with mice. Obese people have fewer Bacteroidetes and more Firmicutes than lean people. It has been shown that weight loss in obese people regardless of diet type, is associated with an increase in the relative abundance of Bacteroidetes and a decrease in the abundance of Firmicutes (Ley et al. 2006). Moreover, the colonization of germ-free mice with microbiota taken from the distal intestine results in 60 % increase in body fat content and insulin resistance within two weeks, although there is a reduction in food intake. The microbiota also promotes triglyceride production in the liver and storage of triglycerides in adipocytes by inhibition of the expression of circulating lipoprotein lipase inhibitor in the intestine, that causes fatty acid release from triglyceride-rich lipoproteins in muscle, heart and fat. In addition, suppression of fasting-induced adipocyte factor (*Fiaf*) by microbiota in the intestine leads to adiposity (Bäckhed et

al. 2004). In addition, studies with germ-free mice, that have slower cell renewal rate than normal, show that communication between gut microbiota and hosts' immune cells could enhance renewal of gut epithelial cells. Besides that, the microbiome contributes to maturation and activity of innate and adaptive immune systems. The studies in germ-free animals also showed that it can affect the sizes of different organs, such as the heart. It is yet to be answered if the microbiota has any effect on the behaviour of the host (Cummings and Macfarlane 1997; Turnbaugh et al. 2007). Moreover, in mice lacking Toll-like receptor 5 (TLR5), which is expressed in the gut mucosa and has an important role in the innate immune system, symptoms of metabolic syndrome are seen. It is shown that these symptoms are correlated with the composition of the gut microbiota, and the transfer of gut microbiota from TLR5 deficient mice to wild-type germ-free mice results in observation of metabolic syndrome symptoms in the recipients (Vijay-kumar et al. 2010).

The microbiota in the other parts of the body is quite different from the gut microbiota depending on the temperature, pH and oxygen availability. The human skin hosts approximately one billion bacteria in a square centimetre despite having low temperature and pH, and shedding (Cooper et al. 2015). Using 16S rRNA gene sequencing, Actinobacteria, Firmicutes, Bacteroidetes, and Proteobacteria were identified as the major phyla, and *Propionibacterium* spp., *Staphylococcus* spp., *Corynebacterium* spp. were identified as major genera residing on healthy skin (Grice and Segre 2011). Alterations in the skin microbiota are associated with several disorders including psoriasis, acne vulgaris and atopic dermatitis (Grice and Segre 2011). Atopic dermatitis has been shown to be associated with increased abundance of *Staphylococcus aureus* (Kong et al. 2012), *Clostridium* spp., and *Serratia* spp (Oh et al. 2013). Psoriasis lesions were shown to have increased abundance of *Streptococcus* spp. and *Propionibacterium* spp. compared to healthy skin (Fahlen et al. 2012). Acne vulgaris were shown to have six different strains of *Propionibacterium acnes* compared to healthy skin dominated by one unique strain (Eady and Layton 2013). In addition, other commensal bacteria such as *Streptococcus epidermidis* were shown to coexist in acne, but not in healthy skin (Bek-Thomsen et al. 2008).

The studies mentioned above have prompted new questions about the effects of the microbiota of different body niches on ruminants, particularly regarding their health and productivity.

1.4 Microbiome studies in Ruminants; Associations with Health and Productivity

Ruminants, especially cattle, sheep, and goats, are among the most important animals for humans due to their ability to convert low nutrient density organic materials into food for the human population. This is mainly achieved by anaerobic microbial fermentation, in the rumen, of fibrous plant materials (Chaucheyras-Durand and Ossa 2014). This ability of ruminants makes them an important intermediate between light energy harvested via photosynthesis and the production of edible compounds, such as milk and meat (Jami et al. 2014).

Jami and Mizrahi (2012) demonstrated that the microbiota composition in the rumen of different individuals shows variation. They used real-time PCR to detect similarities between ruminal bacteria with different metabolic functions in 16 individual cows by amplification of 16S rRNA to explore the relative abundance of 13 bacterial species. Furthermore, to examine the degree of similarity for dominant bacterial species, they used automated ribosomal intergenic spacer analysis (ARISA). They identified a bacterial community with 32% operational taxonomic units (OTUs) in at least 90% of individuals and 19% OTUs in all individuals. Regarding only the absence or presence of each OTU, they found out 75% similarity between each cow pair and when they add the abundance of each OTU the similarity was less than 60%. Hence, they concluded that there is a core rumen microbiome, which can greatly vary in taxa level (Jami and Mizrahi 2012) The same research group characterized the bovine ruminal bacterial populations in five different age groups, from 1-day-old calves to 2-year-old cows using 454 amplicon pyrosequencing. As a result, they showed that there is a decrease in the ecosystem of aerobic and facultative taxa and an increase in the anaerobic taxa by age. They also showed each age group has its own distinct bacterial species and this bacterial composition changes over time. When the 6-month and 2-year groups were fed the same diet, their microbiota composition was still different (Jami et al. 2013). In addition, the same research group described the potential role of the bovine rumen microbiome in modulating milk composition. They characterized the rumen bacterial composition in 15 lactating dairy cows by pyrosequencing and some physiological parameters such as milk yield, milk content (carbohydrate, fat, and protein) and pH. One of the main findings was the strong correlation between the relative abundance

ratio of the phyla Firmicutes to Bacteroidetes and the milk-fat yield (Jami et al. 2014).

Pitta et al. (2016) characterized the rumen microbiome of five primiparous (first lactation; L1) cows and five multiparous [second lactation; L2 (n=2), third lactation; L3 (n=2) and fifth lactation; L5 (n=1) (it was excluded from the study)] dairy cows for phylogeny and functional pathways by using a metagenomic approach and related it to productivity. They demonstrated milk fat and protein yields of the three lactation groups were similar. On the other hand, they identified that in terms of the phylogeny of the gene sequences, Bacteroidetes had more metabolic activity in L1 cows, whereas Firmicutes and Proteobacteria had more metabolic activities in L2 and L3 cows. As a result, they showed that the rumen microbiome composition is evolving during the dairy cows' lactation progress and age, and this could have a potential role in milk production. In addition, they found that diet can have important effects on the composition of rumen microbiome (Pitta et al. 2016).

In terms of the associations between microbiota and health, ruminants might have microbial disorders, such as lameness that also affect their productivity.

To investigate the associations between microbial profiles and lameness, Zinicola et al. (2015a) performed Shotgun metagenomic sequencing to compare samples from ADD, IDD, and healthy cows. They used 8 healthy, 4 ADD and 4 IDD cows, characterized their microbiome, and examined for the first time, the composition of functional genes. They found that Firmicutes and Actinobacteria were highly abundant in healthy skin, Spirochaetes were dominant in active stages of the disease, while Firmicutes, Bacteroidetes, and Proteobacteria dominated in chronic or inactive stages. In addition to the dominant phyla explained above, they discovered a specific higher abundance of *Treponema* species such as *T. denticola-like, T. vincentii-like* and *T. phagedenis-like* in ADD and IDD samples compared to the healthy samples. Furthermore, genes related to flagella structure and synthesis were represented excessively in ADD and IDD samples compared to the healthy samples. In both ADD and IDD samples, they identified higher proportion of genes for iron acquisition, which is important for bacterial growth, and for resistance to antibiotics and toxic compounds, such as copper and zinc compared to the healthy samples (Zinicola et al. 2015a).

Zinicola et al. (2015b) also characterized the microbiome composition of healthy skin and different stage DD lesions for 89 DD and 51 healthy dairy cows. They identified the foot skin microbiome using 16S rRNA metagenomic techniques and additionally they examined the gut microbiome for its potential role as a reservoir of DD pathogenesis using Shotgun and 16S rRNA metagenomic techniques. They identified that the relative abundance of *Treponema denticola*, *Treponema maltophilum*, *Treponema medium*, *Treponema putidum*, *Treponema phagedenis* and *Treponema paraluiscuniculi* in active lesion samples was higher than in healthy and inactive lesion samples and these species were also abundant in rumen and faecal samples. A novel bacterium, *Candidatus Amoebophilus asiaticus* also had greater abundance in both active and inactive lesions samples compared to the healthy samples (Zinicola et al. 2015b).

In addition, Krull et al. (2014) used both Shotgun metagenomic and 16S amplicon sequencing to investigate the changes in the bacterial populations during the progression of BDD. They examined 48 staged BDD specimens, which were collected during a 3-year study. They also developed a morphological lesion scoring system. According to the results of Shotgun sequencing, there was no correlation between fungal or viral components and disease progression. The scoring system showed that each stage had a significantly different microbial diversity than other stages. Moreover, *Treponema* species were dominant in advanced lesions, however, they were in low abundance in early lesions. In conclusion, they found that DD is a polybacterial disease and temporal changes in microbiota profiles occur throughout the progression of DD (Krull et al. 2014).

Moore et al. (2005) investigated dominant bacteria related to the severe ovine foot disease known as Contagious Ovine Digital Dermatitis (CODD). They used microscopy, 16S rRNA PCR and anaerobic culture for 13 healthy and 50 diseased animal samples. In the feet of animals with CODD, they identified *D. nodosus*, *F. necrophorum*, and Spirochaetes of the genus *Treponema* that is similarly found in BDD samples (Moore et al. 2005). Similarly, Sullivan et al. (2015) analysed 58 CODD and 56 healthy sheep foot tissues using PCR with the primers specific to BDD associated treponemes. They showed that BDD associated treponemes were detected in CODD samples, but were not detected in healthy foot tissues (Sullivan et al. 2015).

Studies mentioned above show that shifts in microbiota profiles are associated with health and disease for ruminants. However, more research is warranted that will describe what kind of alterations lead to certain diseases and/or what kind of microbial profiles may provide protection against diseases.

1.5 Genetics and Genomics

Genetics is the study of genes and their roles in heredity, and genomics refers to the study of genes with their functions and interactions (Guttmacher and Collins 2002).

Traditional selection for economically important traits in animals and plants used to be based on observed quantitative records of the individual and its relatives owing much to Mendel's discovery of internal factors that can be inherited by the next generations (Henderson 1984; Korte and Farlow 2013). Use of DNA information using recent advances in molecular genetic techniques led to marker maps and detection of quantitative trait loci (QTL) (Georges et al. 1995; Meuwissen et al. 2001). Principally, phenotypic differences are detected and linked back to causative loci in the genome (Korte and Farlow 2013).

The earliest use of DNA markers started with restriction fragment length polymorphism (RFLP), which is based on the usage of restriction enzymes to cut DNA. Then restricted patterns are visualized by gel electrophoresis or radioactively labelled gene specific probes via hybridization (Saiki et al. 1985; Williams 2005). PCR amplification of microsatellite markers which contain 5-20 copies of a 2-4 base pair short tandem repeats was also used in genetic studies (Williams 2005). However, marker based selection provided tracing of only a small number of major genes, not the genes with small genetic effects (VanRaden et al. 2009). Recently, the availability of single nucleotide polymorphism (SNP) data has provided high-throughput analysis of genome wide associations. SNPs are a highly convenient way to investigate genome wide associations since they are densely found in the genome (in every 1000-2000 bases); they are binary, hence suitable to automated genotyping; and they are more resistant to recurrent mutations compared to microsatellites (The International SNP Map Working Group 2001).

For dairy cattle, genomic evaluations were included in breeding programs in US, Canada, France, The Netherlands, New Zealand and the Scandinavian countries (Ibanez-Escriche and Gonzalez-Recio 2011). Genomic evaluations for dairy cows

mainly rely on the availability of genotype information of the sires and their daughters' phenotypic measurements, there is usually a lack of genomic information from the dams (Ibanez-Escriche and Gonzalez-Recio 2011; Lourenco et al. 2015; Vukasinovic et al. 2017). To genotype cattle, several types of SNP-chips, for example low-density chips such as 15K, 25K, and 50K and high-density chips such as 777K, have been developed and used (Guo et al. 2017; Lopes et al. 2018). In order to increase the success of breeding strategies, a reference population of both males and females with regular phenotyping might be created, and genomic evaluation of this population might be performed using high-density genotyping. Thus, the animals out of the reference population could be genotyped with low-density chips for a cheaper cost, and the missing genomic data could be imputed from the data of the reference population (Ibanez-Escriche and Gonzalez-Recio 2011; Guo et al. 2017). However, imputation may lead to contradictory results since the reliability of the imputation process depends on the number of animals, selected variants, and number and analysis method of the data sources (Frischknecht et al. 2017; VanRaden et al. 2017; Lopes et al. 2018).

1.6 Lameness Genetics and Genomics

Lameness can be associated with the environment, nutrition and genetic factors (Olmos et al. 2009), and it is the third most important health condition after fertility problems and mastitis (Green et al. 2002; Buitenhuis et al. 2007; Cha et al. 2010). Improvement in the productive performance of animals by genomic selection used to be based upon productivity traits and did not take health traits into account (Williams 2005). Recently, the focus of breeding programs has shifted to non-productions traits (Buitenhuis et al. 2007) such as health (Holmberg and Andersson-Eklund 2006), fertility (Royal et al. 2002) and behavioural traits (Schmutz et al. 2001). Claw health status data have started to be recorded at foot trimming in many countries, and can be used for genetic evaluation (Heringstad et al. 2018). For genetic evaluation of claw health, direct traits and indirect traits have been used (Heringstad et al. 2018). Direct traits include foot trimmer records, and veterinary records. Routinely recorded foot trimmer data are the most advantageous data for the genetic improvement of claw health (Koenig et al. 2005; Häggman et al. 2013), and foot trimmer data also provide early detection of disorders, thus they provide economical and welfare benefits. However, bias caused by the recorder, level of education and of the trimmer, and trimming time affect the data quality. In case of

veterinary records, data reflect especially more severe cases, and it may lead to bias by ignoring early cases (Heringstad et al. 2018). Indirect traits include lameness and mobility scores, conformational traits, and the trait data collected by automated sensors. Lameness scoring could be binary or based on clinical gait score that quantifies lameness from absent to severe. Moreover, mobility is scored based on the length and direction of the steps (Heringstad et al. 2018). Since mobility scoring might not be that sensitive for early cases, it should be performed regularly (Bicalho and Oikonomou 2013; Heringstad et al. 2018). Some studies show that conformational traits such as dorsal wall length and heel depth (Gitau et al. 1997), are helpful (van der Linde et al. 2010), or not (Häggman et al. 2013) for claw health evaluation. However, they were measured once in animals' life and there is little information about the association between conformation changes and claw health. For the traits recorded by automated systems, it has been shown that in automatic milking systems, lameness can be predicted by monitoring daily activity of animals such as movement in the milking parlour, rumination and feeding (Miguel-Pacheco et al. 2014). Infrared thermography has also been used to detect inflammation caused by foot lesions (Alsaaod & Büscher 2012; Oikonomou et al. 2014a).

Heritability is the proportion of the phenotypic variance that is due to genetic factors (Willerman and Plomin 1973). The genetic variation for lameness scored by farmers was shown in different studies with the heritability estimates being 0.1 (Boettcher et al. 1998), and 0.06 (Zwald et al. 2004). Heritability for locomotion was shown to be 0.09; and for conformational traits it was shown to be 0.14 for rear leg rear view, 0.19 for rear leg side view, 0.13 for foot angle (Laursen et al. 2009). From the routine trimming data, the heritability was shown to be 0.073 for DD lesions, 0.086 for SU lesions, 0.104 for WLD lesions, and 0.115 for IH lesions. In a recent study, the heritability of DD was shown to be between 0.19 and 0.52 (Schöpke et al. 2015).

Due to relatively low heritability estimates and the inability to measure lameness related traits in early life, identification of lameness associated QTLs could provide selection of lameness resistant animals more accurately (Buitenhuis et al. 2007). Buitenhuis et al. (2007) used the data from granddaughters of 19 Danish Holstein grandsires using a regression and variance component method. They identified 2 QTLs for lameness in the first lactation on *Bos taurus* autosomes (BTA) BTA5 and BTA26, and 2 QTLs for lameness in the second lactation on BTA19 and

BTA22 (Buitenhuis et al. 2007). In another study, a strong association between IQ motif-containing GTPase-activating protein 1 (*IQGAP1*) on BTA21 and SH was detected using mixed threshold analysis of the data from 1183 German Holstein cows and a custom-made SNP array of 384 SNPs which were preselected based on the literature. After inclusion of this SNP into a commercial SNP chip (Illumina BovineSNP50), it was found to be associated with feet and leg traits (Swalve et al. 2014). In a relatively small-scale study with 169 cows, 2 SNPs on BTA6 and BTA26 were identified associated with DD, and these SNPs were considered to be associated with over-proliferation of the skin cells, and the immune response mechanism, respectively (Scholey et al. 2012). In another study using deregressed estimated breeding values of 5,334 Danish Holstein, 4,237 Nordic Red Dairy Cattle, and 1,180 Danish Jersey bulls with a modified linear mixed model, 5, 3, and no QTLs were found to be associated with feet and leg disorders, respectively (Wu et al. 2016). The main limitations in genomic studies are inconsistency between different studies, large standard errors due to small population sizes, lack of historical data for new traits, and bias caused by the quality of data recording (Heringstad et al. 2018). Various results from different studies showed that routine recording of foot and claw health data and using more improved analysis methods could facilitate identifying problems more accurately and this could be used in breeding programs more beneficially.

1.7 Host Genotype-Microbiome Interactions

The microbiota in specific body parts varies between individuals. However, family members tend to have more microbial similarity than non-familial individuals. This could be explained by environmental factors but may also be the result of related host genome. The relationship between host genome and microbiota is still unclear (Spor et al. 2011). Benson et al. (2010) investigated whether there was an interaction between the host genotype and the gut microbiota composition in mice using a large very well controlled inter-cross murine line (n=645). They identified 19 genera and 64 conserved taxonomic groups as core measurable microbiota, which can vary between individuals. They investigated 530 SNP markers and found 18 host QTL that have a significant relationship with relative abundances of specific microbial taxa. As a result, they explained the QTL effect on microbiota composition in three ways. 1) some loci control only individual microbial species, 2) some loci control a group of related taxa 3) some loci can have pleiotropic effects on distantly

related groups. Finally, they concluded that host genome and microbiota composition co-segregate as quantitative traits (Benson et al. 2010).

McKnite et al. (2012) used the BXD mouse line, which was extensively characterized at molecular and phenotypic level, to investigate how the gut microbiota composition may be influenced by host genetic factors. They combined next generation sequencing of the gut microbiota and genetic mapping by using faecal samples of 30 BXD strains. They demonstrated that a QTL region on chromosome 4, which is related to the interferon genes, had associations with *Bacteroides* population, and potentially on phyla Bacteroidetes and Firmicutes. They found a QTL on chromosome 15 that was associated with *Irak4*, a signalling molecule in the Toll-like receptor pathways, as a candidate modulator for Rikenellaceae population. They also found a candidate QTL on chromosome 12 related with *Tgfb3*, a cytokine, for modulating Prevotellaceae population (McKnite et al. 2012).

In humans, Khachatryan et al. (2008) examined the effect of one gene *MEFV*, that encodes a protein called pyrin /marenostrin and mutations in this gene, which cause an autoinflammatory disorder (Familial Mediterranean fever, FMF), on the gut microbial community structure. They genotyped 19 FMF patients and 8 healthy individuals and performed bacterial diversity analysis by 16S rRNA and FISH techniques. They found a smaller number of total bacteria, loss of diversity and important shifts in populations of Bacteroidetes, Firmicutes, and Proteobacteria phyla in the presence of the disorder compared to the healthy state. Additionally, in the acute stage of the disease the percentage of Porphyromonadaceae, Phascolarctobacterium, Faecalibacterium, and Parabacteroides phyla increased and the percentage of Prevotellaceae, Dialister and Prevotella phyla decreased compared to the control (Khachatryan et al. 2008).

Goodrich et al. (2014) investigated the associations between the host genome and the gut microbiome. They compared more than 1000 faecal samples of 977 individuals from the TwinsUK population: 171 of them were monozygotic and 245 of them were dizygotic twins, two of them were with unknown zygosity and 143 of them from just one twin of twin pairs that was used as an unrelated sample. They found that the relative abundance of microbiota in monozygotic twins was more similar than those in dizygotic twins. They also showed that the family

Christensenellaceae was the most heritable taxon, which was more prevalent in healthy individuals and those with a low body mass index (BMI) (Goodrich et al. 2014).

In a genome-wide association study in humans, Blekhman et al. (2015) used Human Microbiome Project (HMP) data to identify genes and pathways correlated with microbiome composition. In the HMP, there are some sampled and catalogued microbial taxa in more than ten body sites. Subsequently, they extracted data from human contaminant reads and gathered genome-wide genetic variation data of 93 individuals. They found 615 microbiome abundance traits in 15 body sites and to reduce the number of statistical tests they selected SNPs which are only present in protein coding regions and finally found 83 associations. Moreover, they found SNPs on the immune-related genes, such as the *HLA-DR* gene which correlated with the abundance of *Selenomonas* in the throat and *TLR1* gene correlated with the abundance of *Lautropia* in the tongue dorsum. In addition, they found SNPs in the *LCT* gene, that encodes lactase, correlated with the abundance of *Bifidobacterium*, that can metabolize lactose, in the GI tract. They also found correlations between the microbiome composition and complex disease-linked genes (Blekhman et al. 2015).

Davenport et al. (2015) investigated the faecal microbiome of an isolated community, the Hutterites, because of their conserved background and stable and non-diverse environment. They had used these data for only temporary seasonal effects between summer and winter and in this study, they examined samples from 93 individuals collected in winter, 91 in summer and 57 in both seasons for sex, age, and genetic effects. They found that only the genus *Bifidobacterium* was correlated with age. They also found that there was at least 4 bacterial taxa related to sex and this could be caused by different daily activities. In terms of heritability, they found at least 8 taxa correlated with at least one SNP. Furthermore, the bacterial genus *Akkermansia* was shown to be enriched in DNase hypersensitivity peaks of endothelial cells and was linked to BMI; this genus is thought to be an important factor in obesity. According to gene enrichment analysis in this study, SNPs function in various mechanisms including immunity, metabolism, and energy regulation was identified. However, the number of samples should be larger and there should be other independent studies in order to confirm these results (Davenport et al. 2015)

The studies summarized above could lead to the conclusion that microorganisms play important roles in host organisms and they can have significant associations with the host genome.

The roles of the foot skin microbiome, host genetic factors, and their interactions in the development of lameness causing foot lesions have not been studied adequately until now. In this study, genomic DNA samples from 554 cows were genotyped, foot microbiome profiles of 259 of those cows were determined, DCT for 360 of those cows was measured, and lameness causing lesions were recorded for each individual. Foot skin microbiome profiling was performed with pre-calving foot swabs sampled about 1 month before calving and those cows were followed as freshly calved, 30 days and 60 days after calving to observe and compare the difference in microbiome profiles between healthy cows and diseased cows while recording the foot lesions and measuring DCT. Moreover, cow genotype data were analysed in order to find the genomic regions associated with lameness causing lesions, DCT, and some of the most significantly prevalent bacteria in DD lesions. In addition to these, the microbiome profile of IH, IP, complicated SU, WLD, and TU/TN lesions were investigated.

Hypotheses of the study

1- The bovine foot skin microbiota is associated with the development of infectious lameness causing lesions and complicated claw horn disruption lesions, and is also associated with the host's genetic background.
2- There are bovine genomic regions associated with the DCT at the animal's foot and predisposition to lameness causing lesions in dairy cattle.

Objectives of the study;

- To determine the role of the foot skin microbiota in the aetiology of DD, one of the major lameness causing lesions in cattle, and identify bacteria that are associated with a predisposition to DD lesions or have potential protective properties.
- To characterize the microbiota profile in the skin of cow feet with complicated CHDLs, IH, and IP.

- To investigate the bovine genomic regions that are linked to various different lameness associated traits.

CHAPTER II

2 Investigating the Role of the Foot Skin Microbiota in the Development of Digital Dermatitis Lesions in Dairy Cattle

2.1 Summary

Dairy cattle lameness is a serious health and welfare problem causing economic losses in the dairy industry globally. One of the most common diseases associated with dairy cattle lameness is the infectious disease, digital dermatitis (DD).

In the present study, 16S rRNA gene amplicon sequencing was used to investigate the foot skin microbiota profiles of 259 dairy cows from 3 different farms. Foot skin swab samples were collected one month before calving, and the cows were followed for 3 months, if they already had DD at the beginning of the study or they developed lesions during this period. Animals were classified into four different groups according to their DD lesion status. The microbiota profiles of these groups were compared to each other in order to find out potential bacteria that may be associated with a predisposition to DD, and to discover potential bacteria that have protective roles against DD.

Different DD lesion types had similar relative abundances for the 20 most prevalent genera and phyla. The microbiota of those cows which did not acquire DD lesions in the study and those which did acquire lesions had significantly different opportunistic anaerobes present. Farm level differences in microbiota populations were also identified.

The results have shown that there are other pathogens potentially acting together with the Treponemes in the development of DD lesions, and these pathogens might provide more favourable environment for the proliferation of the main causative pathogens. Furthermore, it is indicated that changes in the microbiome profile are associated with the development of DD foot lesions and exist before any clinically detectable manifestation of the disease.

2.2 Introduction

Dairy cattle lameness is an important issue for the dairy industry as it results in reduced animal welfare, and economic loss (Kossaibati and Esslemont 1997; Whay et al. 1998). It is a serious welfare issue due to discomfort and pain (Whay et al. 1998; Warnick et al. 2001; Green et al. 2002). Economic loss occurs due to reduced milk yield (Alawneh et al. 2011), poor reproductive performance (Barkema et al. 1994; Garbarino et al. 2004), culling of lame animals, and increased veterinary expenses (Harris et al. 1988; Rajala-Schultz and Gröhn 1999). Lameness is a clinical sign rather than a single disease, and it has various causes between farms, regions and countries (Potterton et al. 2012). Lameness can be caused by foot lesions of infectious aetiology, such as digital dermatitis (DD), interdigital phlegmon (IP), as well as non-infectious, claw horn disruption lesions (CHDLs), such as sole ulcer (SU), toe necrosis (TN), and white line disease (WLD) (Green et al. 2002; van der Waaij et al. 2005; Bicalho and Oikonomou 2013).

DD is one of the major lameness associated diseases having higher impact on welfare compared to other lameness associated lesions due to its' high incidence, and long duration (Zinicola et al. 2015b; Bruijnis et al. 2012; Holzhauer et al. 2006). The first case of DD was reported in Italy in the 1970s by Cheli and Mortellaro (Cheli and Mortellaro 1974), therefore, it is also known as 'Mortellaro's disease'(Holzhauer et al. 2006). Internationally, it has several other names such as papillomatous DD, strawberry or raspberry heel warts, strawberry foot rot, hairy foot warts, hairy heel warts, and foot warts (Wells et al. 1999; Brown et al. 2000). In the UK, the incidence of DD was reported to be more than 30% in 1996 (Clarkson et al. 1996; Murray et al. 1996). DD lesions are mostly observed in the plantar/palmar parts of hind limbs (Murray et al. 1996; Rodriguez-Lainz et al. 1996; Read and Walker 1998; Schultz and Capion 2013), and these lesions are observed more frequently in lactating heifers (Rodriguez-Lainz et al. 1996; Read and Walker 1998). Interdigital dermatitis has the same histopathology with DD, but it is observed in interdigital skin (Walker et al. 1995).

Development of DD lesions occurs as series of morphological stages which were first described by Döpfer et al. (1997) with stages; M1 ("M" referring Mortellaro) for a small ulcerative area, M2 for a mature ulcerative area, M3 for healing lesions, and M4 for chronic lesions. Krull et al. (2014) developed a new scoring system (The IOWA DD scoring system) to be able to describe two extra

early lesion stages. The IOWA DD scoring system consists of stage 0 for normal skin, stage 1 for initial onset, stage 2 for developing lesions, stage 3 for acute lesions, and stage 4 for chronic lesions. Additionally, stage 1 and 2 consist of two subtypes; A and B. The "A" type lesions form in the interdigital space and appear to be more ulcerative, whereas the "B" type lesions are more diffuse across the heel with more thickening and a scab. These subtypes proceed to stage 3 and 4 with the same morphology (Krull et al. 2014). The scoring system used in the present study was modified by Zinicola et al. (2015b) (Figure 2.1).

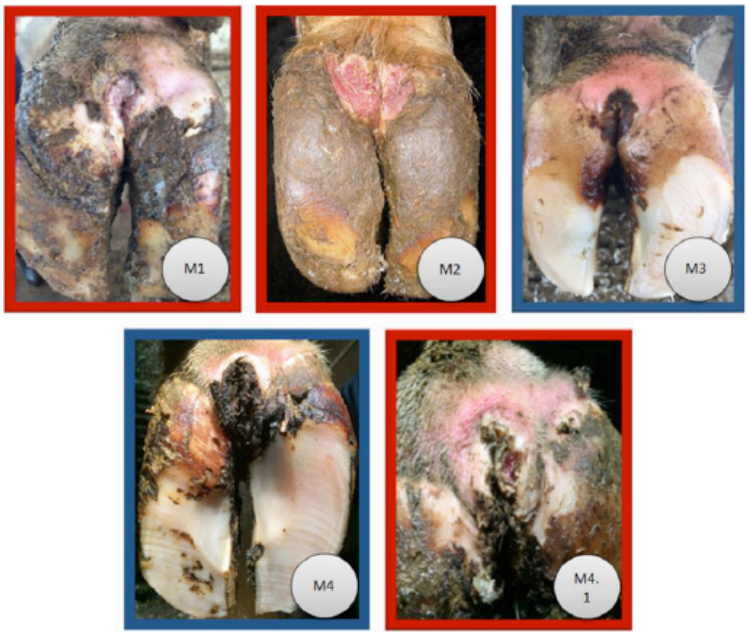

Figure 2.1. M-stage scoring system used in the present study modified by Zinicola et al. (2015b) from Döpfer et al. (1997) and Berry et al. (2012) (Döpfer et al. 1997; Berry et al. 2012; Zinicola et al. 2015b). M1: early active lesions with <2 cm diameter, M2: active lesions with >2 cm diameter, M3: healing lesions with a scab, and M4: chronic lesions, M4.1: small active lesions with <2 cm diameter on a chronic M4 lesion (M0: without any lesion).

DD infections have polymicrobial characteristics and have been associated with a variety of bacteria. However, the genus *Treponema* is strongly associated with DD. *Treponema* spp. have been consistently found in high numbers and isolated from DD lesions (Zinicola et al. 2015a; Choi et al. 1997; Cruz et al. 2005; Evans et al. 2009; Nordhoff et al. 2008). Besides *Treponema* spp., *Porphyromonas* spp. (Moe et al. 2010), *Mycoplasma* spp., *Bacteroides* spp. (Collighan and Woodward 1997),

Campylobacter spp. (Döpfer et al. 1997), *Dichelobacter nodosus* (Rasmussen et al. 2012), *Guggenheimella* spp. (Schlafer et al. 2008), *Borrelia* spp. (Blowey et al. 1994), *Fusobacterium necrophorum, and Candidatus Aemobophilus asiaticus* (Zinicola et al. 2015a; Zinicola et al. 2015b; Krull et al. 2014) were also shown to be associated with the disease.

DD presents in an active form (ADD) characterized by the presence of an ulcerative area, and an inactive form (IDD) characterized by the presence of firm scab, hyperkeratosis, proliferative overgrowth, and absence of an ulcerative area (Zinicola et al. 2015a). Zinicola et al. (2015a) performed whole genome-Shotgun sequencing to characterize and compare the microbiome profile of ADD (n=4), IDD (n=4), and healthy cows (n=8). They found out that *Treponema denticola-like, Treponema vincentii-like* and *Treponema phagedenis-like* spp. were more prevalent in ADD and IDD samples compared to the healthy samples. In addition, they showed that the phyla Firmicutes and Actinobacteria were highly abundant in healthy skin, whereas Spirochaetes were dominant in active stages of the disease, and Firmicutes, Bacteroidetes, and Proteobacteria were dominant in chronic or inactive stages (Zinicola et al. 2015a). In another study, the same group characterized the microbiome composition of healthy skin (n=51), and different stages of DD lesions (n=89) for dairy cows. They described the foot skin microbiome by sequencing the 16S rRNA gene. They showed that relative abundances of *Treponema denticola*, *Treponema maltophilum*, *Treponema medium*, *Treponema putidum*, *Treponema phagedenis* and *Treponema paraluiscuniculi* in ADD samples were higher than healthy and IDD lesion samples, and these species were also detected in rumen and faecal samples. As a novel finding, they showed that the relative abundance of *Candidatus Amoebophilus asiaticus* was higher in both active and inactive lesions samples compared to the healthy samples (Zinicola et al. 2015b).

Krull et al. (2014) used both Shotgun and 16S rRNA metagenomic sequencing to investigate the changes in the foot skin bacterial population through the progression of DD by using a morphological lesion scoring system. They collected 48 staged DD biopsies during a 3-year period. They showed that each stage of DD had a significantly different microbial diversity compared to other stages. Moreover, *Treponema* species were dominant in latter stage lesions, however, they were in low abundance in early lesions. They concluded that DD is a polybacterial disease and temporal changes in the foot skin microbiota occur in each stage during

the progression of the disease (Krull et al. 2014). However, it is not clear what bacteria are present on healthy skin, and the kind of alterations on the skin microbiota that may provide a suitable environment for the proliferation of *Treponema* spp. and other pathogens. In addition to that, the bacteria that predispose healthy skin to acquire DD lesions, or the bacteria having potential protective roles are not well described.

Skin microbiota studies in humans showed that there is a stable core skin microbiota, although the skin is exposed to various environmental conditions over time (Oh et al. 2016). The interaction between the skin microbiota and the host varies, even different strains of the same species can affect the health of host in opposite ways (Byrd et al. 2017; Erin Chen et al. 2018). There is also microbe-microbe interactions which can impact human health (Bomar et al. 2016; Ramsey et al. 2016). Hence, unravelling the host-microbiome and microbiome-microbiome interactions, and partial alterations in local microbiome contents might help treating diseases and creating more disease-resistant individuals (Erin Chen et al. 2018).

The aim of this study was to investigate the associations between foot microbiota profiles and DD status, and identify bacteria that may cause a predisposition to DD and bacteria with potential protective roles against DD.

2.3 Materials and Methods

2.3.1 Ethics and sampling

Ethical approval for the study was granted by University of Liverpool Research Ethics Committee. The sample population consisted of 259 cows in total from three different dairy farms in in Cheshire and North Wales (UK) examined between October 2016 and June 2017. 91 cows were from farm 1, 56 cows were from farm 2 and 112 cows were from farm 3.

The milking cows were housed in cubicle sheds with grooved concrete flooring on all 3 farms. Farms 1 and 3 had rubber flooring in the milking parlours. On farm 1 the cubicle bedding was sawdust and rubber, on farm 2 the bedding was sawdust and sand, and on farm 3 the cubicle bedding was sand. Cows were managed on straw yards during the dry period in farms 1 and 2. Dry cows on farm 3 were kept on sand bedded cubicles. Foot trimming was performed about 2 months after calving and 1 week before drying off in the first and third farm, at dry off in the second farm. Foot bathing was applied twice a week with 4% formalin and once a week with 4%

copper sulphate in the first farm. On farm 2, foot bathing was with 5% formalin every week day. Farm 3 had regular foot bathing with 2% formalin every day. Farm 1 and 2 were not foot bathing their dry cows. Dry cows in farm 3 were walking through a 4% formalin foot bath three times every week. The second farm has access to grazing between May and October, and pregnant cows could stay outside until 3 weeks before calving. Farm 1 and farm 3 did not have access to grazing. Diet was mainly forage based in all farms.

Sampling was performed in a cattle foot trimming crush by lifting animals' left hind foot (Figure 2.2). The foot was cleaned thoroughly using clean paper towels in order to remove gross faecal contamination. Sterile swab samples were then taken from the skin above the heel bulbs. After that, swabs were placed in sterile/DNA-free tubes and labelled with animal ID, and date. Samples were kept and transported on ice and stored at -80°C within a few hours from collection.

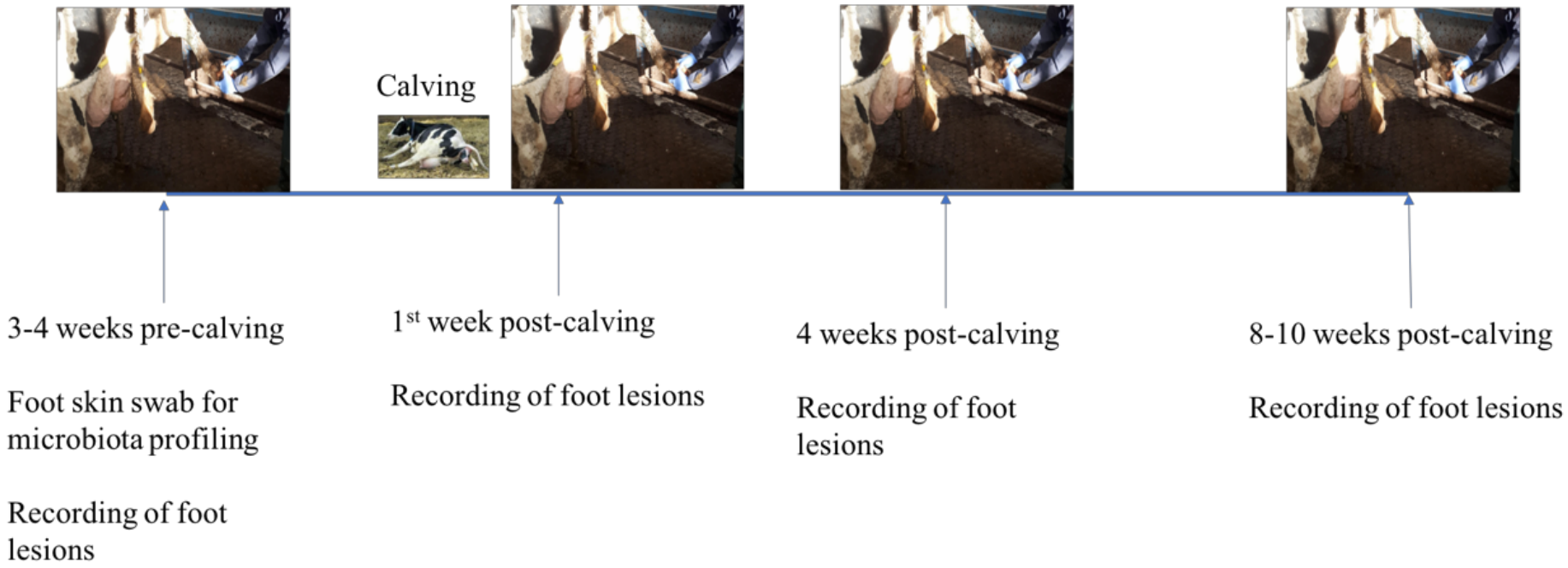

Figure 2.2. Study design

Lesions were categorized using a 5-point scale, as M0 for the animals without any lesion, M1 for the animals with early active lesions with less than 2 cm diameter, M2 for the animals with active lesions with more than 2 cm diameter, M3 for the animals with healing lesions, and M4 for the animals with chronic lesions; M4.1 animals had small active lesions with less than 2 cm diameter on a chronic M4 lesion (Zinicola et al. 2015b). These cows were then re-examined when they were within one week after calving, and approximately one month and two months after calving.

Based on DD foot lesion status (on any foot) during the study cows were classified into four groups to give a cow level DD status: Group HtHt animals which remained healthy (i.e. no DD lesions on any foot) during the whole study; group HtIn animals which were healthy at initial check point but developed DD lesions (in any foot); group InIn animals which had DD lesions in any foot at all check points during the study; and group InHt animals, which had DD in initial pre-calving check point but had no DD lesions for the remainder of the study in any foot. Cows were also classified similarly using only information pertaining to the back-left foot (BL_HtHt, BL_HtIn etc.).

2.3.2 DNA extraction

Microbial DNA was extracted from collected swabs using the PureLink™ Microbiome DNA Kit (Invitrogen, Carlsbad, CA, USA) and following the manufacturer's instructions. The incubation of the samples in bead tubes at 65°C was extended to 15 minutes, and bead beating was performed for 15 minutes. Extracted DNA samples were stored at -20°C.

2.3.3 16S rRNA gene amplification, and library construction

The Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, Fair Lawn, NJ, USA) was used to measure DNA concentrations before PCR. The sample with the lowest concentration (1.28 ng/µl) was used at the maximum volume (10 µl) for PCR, so to equalize the amount of DNA in each tube, 12.8 ng from each sample was used as template DNA for amplification of the V3-V4 hypervariable region of 16S rDNA with negative (Nuclease-Free Water (not DEPC-Treated) Thermo) and positive controls (ZymoBIOMICS™ Microbial Community DNA Standard). The 341F (Illumina_16S_341F 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCC TACGGGNGGCWGCAG), and 805R (Illumina_16S_805R 5′-GTCTCGTGGGCT CGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) universal

primers with adapter sequences were used (Zheng et al. 2015). For the first step PCR, 1.25 µl of amplicon PCR forward primer (2.5 µM), 1.25 µl of amplicon PCR reverse primer (2.5 µM), and 12.5 µl of NEBNext High-Fidelity 2X PCR Master Mix (New England Biolabs) at 95 °C initial denaturation for 3 min, followed by 12 cycles of 95 °C for 30 s, 62.3 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min as described by Zheng et al. (Zheng et al. 2015). PCR products were cleaned up with Agencourt AMPure XP beads (Beckman Coulter Genomics, Fullerton, CA, USA) following the manufacturer's protocol.

In a second PCR step, dual indices and Illumina sequencing adapters were attached using 7.5 µl of amplicon PCR product DNA, 2.5 µl of Illumina Nextera XT Index Primer 1 (N7xx), 2.5 µl of Nextera XT Index Primer 2 (S5xx), and 12.5 µl of NEBNext High-Fidelity 2X PCR Master Mix, with thermocycling at 95 °C for 3 min, followed by 13 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. The final PCR products were cleaned with Agencourt AMPure XP beads, and final concentrations of samples were measured with Qubit™ dsDNA HS Assay Kit.

2.3.4 Pooling of PCR amplicons

Amplified libraries were pooled with 10 ng/µl, and 40 ng/µl concentrations with low-concentration and high-concentration samples, respectively. After measuring the concentrations of these two pools, they were mixed as 24.8 ng/µl from each amplicon with maximum volume from negative controls. The final pool was purified with Agencourt AMPure XP beads, and eluted in 30 µl to increase the final concentration.

2.3.5 Sequencing

Concentration and quality of the pooled PCR amplicons was evaluated with the Qubit™ dsDNA HS Assay Kit, and a fragment analyser (High Sensitivity NGS Fragment Analysis Kit, Advanced Analytical Technologies, Inc., Ankeny, IA, USA). The fragment analyser traces indicated that no primer-dimers or other non-target PCR products were present; thus, size selection was not required. A quantitative real-time PCR (qPCR) assay, designed to specifically detect adapter sequences flanking the Illumina libraries, was performed using an Illumina® KAPA Library Quantification Kit (Kapa Biosystems, Wilmington, USA). This assay was used to specifically quantify the number of DNA templates that had both adaptor sequences

on either end and therefore those that would successfully form clusters on a flowcell for sequencing. Briefly, a 20 µl PCR reaction (performed in triplicate for each pooled library) was prepared on ice with 12 µl SYBR Green I Master Mix and 4 µl diluted pooled DNA (1:1000 to 1:100000 depending on the initial concentration determined by the Qubit® dsDNA HS Assay Kit). PCR thermal cycling conditions consisted of initial denaturation at 95°C for 5 minutes, 35 cycles of 95°C for 30 seconds (denaturation) and 60°C for 45 seconds (annealing and extension), melt curve analysis to 95°C (continuous) and cooling at 37°C (LightCycler® LC48011, Roche Diagnostics Ltd, Burgess Hill, UK).

The template DNA was denatured with freshly diluted 0.1 N sodium hydroxide (NaOH) for 5 minutes at room temperature and the reaction was terminated by the addition of the hybridization buffer (HT1) supplied with the Illumina HiSeq Kit. Following calculation of the molarity using qPCR data, template DNA was diluted to a loading concentration of 7.5 pM using the same HT1 buffer. Each pool of amplicon libraries was sequenced on a lane of the Illumina® HiSeq 2500 platform, in rapid mode with version 2 chemistry using sequencing by synthesis (SBS) technology to generate 2 x 300 bp paired-end reads. 15% PhiX fragment library was added to increase sample diversity.

In addition to the foot swab samples, ZymoBIOMICS™ Microbial Community DNA Standard was used as a mock microbial community. This community comprises eight microbial and two fungal strains; seven out of eight bacterial strains were successfully observed at species level; one of them was only described at the genus level. The relative abundances obtained were the expected ones. Only 0.3% in the positive control sequences were found to belong to other unexpected bacteria. Despite being in extremely low concentrations, and not visible on the agarose gel, three PCR negative controls were also sequenced. All these negative controls yielded less than 1000 sequences.

2.3.6 Sequence analyses

Initial processing and quality assessment of the sequence data were done with the use of an in-house pipeline. CASAVA version 1.8.2 (Illumina) was used for base-calling and de-multiplexing of indexed reads to produce 242 samples across the two runs, in FASTQ format. PCR primer sequences and Illumina adapter sequences were trimmed by using Cutadapt version 1.2.1 (Marcel Martin 2011). Sequencing

errors were corrected to improve base quality in both forward and reverse reads using the error-corrected module within SPAdes sequence assembler (version 3.1.0) (Bankevich et al. 2012). Read pairs were converted to single sequences that span entire amplicons using PEAR (version 0.9.10) (Zhang et al. 2014). Sequences with uncalled bases were removed. Size selection was applied to select sequences between 200bp and 750bp, thus removing sequences from potential PCR primer dimers or spurious amplification events. Sequences matching PhiX (E-value<10^{-5}) were filtered out of the dataset.

For each sample, sequences that passed the filters were merged into a single file. This final sequence file with its own metadata file containing description for each sample, was analysed using a custom pipeline based on QIIME 1.9.0 (Caporaso et al. 2010). The Silva database (version 123) (Quast et al. 2013) was used along all the analysis, except for the phylogenetic tree alignment step, which was performed using the GreenGenes precomputed 16S rRNA gene tree. The obtained amplicon sequences were sorted in groups to identify the sequence variability in each sample. This step was performed using SWARM (Mahé et al. 2014) with the strictest parameters (default parameters). Potential chimeric sequences were discarded. Sequences were then aligned on the identified clusters to calculate the abundance of each cluster using a minimum similarity threshold of 97% for the entire length of the sequence in VSEARCH1.1.3. In total, 48,991,273 analysed sequences were clustered in 75.643 different operational taxonomic units (OTU). Taxonomic assignment of each cluster was carried out using QIIME and the RDP classifier (Wang et al. 2007) to match a representative sequence from each OTU to a sequence from the database. The most abundant sequence within each OTU's cluster was used as the representative sequence.

The sequencing depth of each sample was evaluated to exclude any possible outliers as well as to choose the most appropriate data normalization step to perform before performing any further analyses (i.e. alpha- and beta-diversity studies). In this study, to explore the sequencing depth of all samples, the ‘Chao 1’ (Chao 1984) richness index was plotted as a rarefaction curve. The sequencing depth was sufficient to cover most of the species diversity within samples, although few samples seemed under sequenced in rarefaction curve. To compensate for this variation, a normalisation step was performed by subsampling the reads for each sample at the same value. Thus, samples with a lower number of sequences than the

selected threshold (135,000 sequences per sample) were excluded from the following part of the analysis. This normalization step was performed using the sampling without replacement method within QIIME.

2.3.7 Statistical analyses

To visualize the community composition of each sample, the OTUs abundance table obtained after normalization (at 135,000 sequences per sample) was used to summarise taxon abundance for a given taxonomic rank (from kingdom to species), using QIIME. The OTUs abundance table was also used to investigate the richness and evenness of the samples using the following estimators: total observed sequence variants (*i.e.* number of OTUs in the sample), Chao1, Shannon, and Simpson indexes. Comparisons of these indexes between the different groups of samples were performed using a series of t-tests. Since the data weren't normally distributed, nonparametric Wilcoxon/Kruskal Wallis tests were performed, and *P*-values from t-tests and nonparametric tests were compared.

To consider how the taxa composition changed in relation to groups for each metadata category, the rarefied abundance table was used to build pairwise sample distance matrices, using the Bray-Curtis (Bray and Curtis 1957) and the Weighted and Unweighted UniFrac (Lozupone et al. 2011) dissimilarity measures. Nonmetric Multidimensional Scaling (NMDS) analysis was performed on the obtained dissimilarity matrices and statistical significance for the obtained dissimilarity matrices calculated using analysis of similarities (ANOSIM). Subsequent *P*-values were calculated using Student's t-test.

Mean relative abundances of the twenty most prevalent phyla and genera were charted for each lesion. Log fold changes (Log10) of the genera with at least 0.5 % prevalence were calculated for each sample. In JMP Pro 12 (SAS Institute Inc., Cary, NC), robust response screening was performed to evaluate the differences in OTU (genus level assignments) relative abundance between the samples with different DD status, and also between the samples from different farms. Response screening automates performing tests across large number of responses. Mean relative abundances were logit transformed before response screening. False discovery rate (FDR) (2 that is equivalent of p-value=0.01) was used for significance and it is indicated as Robust FDR LogWorth. Then, log fold change of genera was plotted versus Robust FDR LogWorth using Y mean relative abundance as size, and

effect size as colouring (Ganda et al. 2016). Since there was a clustering between farm 3 and farms 1 and 2, response screening with the same parameters was performed to evaluate the differences between farm 3 and farms 1 and 2.

2.4 Results

Swab samples were collected from 259 cows in three dairy farms. However, 17 samples could not pass the selected rarefaction threshold (135000) in quality control steps. The classification of the animals in terms of their DD status in both back-left feet and cow level are shown in Table 2.1. Four groups were defined: "HtHt" represents the cows which remained healthy during the study, "HtIn" represents the cows which were healthy at the initial check point then developed DD, "InIn" represents the cows which had DD in all checkpoints, "InHt" represents the cows which had DD at initial check point then recovered. The unknown category represents the cows which could not be recorded adequately, died or were sold during the study. During the study, 118 cows remained healthy (45.5%). 54 cows were healthy at enrolment and subsequently developed DD lesions (20.8%). 60 cows had DD lesions throughout the study (23.2%). 19 cows recovered from DD (7.3%). DD status of 8 cows could not be recorded properly because the cows could not be followed adequately, died or sold during the study (3.1%).

Table 2.1. Number of DD cases per each different farm; **a)** back-left (BL) foot level analysis, **b)** cow level analysis. (HtHt: The cows which remained healthy during the study, HtIn: The cows which were healthy at sampling, then developed DD, InIn: The cows which had DD in all checkpoints, InHt: The cows which had DD at initial check point then recovered, Unknown: The cows which could not be followed adequately, died or sold during the study.)

a)

Farm	BL_HtHt	BL_HtIn	BL_InIn	BL_InHt	BL_Unknown
1	54	11	10	7	1
2	24	14	7	1	5
3	70	16	20	1	1
Total	148	41	37	9	7

b)

Farm	HtHt	HtIn	InIn	InHt	Unknown
1	40	16	14	12	1
2	13	18	14	1	5
3	59	14	30	4	1
Total	112	48	58	17	7

In total 48,991,273 sequences passed the quality filtering stage and could be used for further analyses (Mean=183,298, SD=43,426). Alpha diversity indexes; Chao1, Shannon, and Simpson, are shown in Table 2.2 for back-left feet level and Table 2.3 for cow level. Significant differences between different groups of animals (HtHt, HtIn, InIn, InHt including active/inactive DD status) stated with *P*-values (<0.05) from both t-tests and nonparametric Wilcoxon/Kruskal Wallis tests are shown in bold, including the differences with tendency to be significant (*P*-value between 0.05 and 0.1).

Table 2.2. Alpha diversity analyses (back-left (BL) foot level). Significant P-values were shown in bold, including the differences with tendency to be significant (BL_HtHt: The cows which had healthy BL feet during the study, BL_HtIn: The cows which were healthy at sampling, then developed DD on their BL feet, BL_InIn: The cows which had BL feet DD in all checkpoints, BL_InHt: The cows which had BL DD at initial then check point then recovered, active: M1, M2 and M4.1 lesions, inactive: M3, M4 lesions, SE: standard error, N_P_value: P-value of nonparametric Wilcoxon/Kruskal Wallis tests). Table continues in the next page.

	n	Chao1	SE	*P*-value	N_*P*-value	Shannon	SE	P-value	N_*P*-value	Simpson	SE	*P*-value	N_*P*-value
BL_HtHt	148	13532.40	173.38	**0.0167**	0.1053	10.60	0.06	**0.0116**	0.2616	0.05	0.002	0.5797	0.6584
BL_HtIn	41	12633.60	329.41			10.27	0.11			0.04	0.003		
BL_HtHt	148	13532.40	179.65	**0.0052**	**0.0369**	10.60	0.06	**0.0001**	**0.0002**	0.05	0.002	**0.0107**	**0.0152**
BL_InIn	37	12396.40	359.29			10.07	0.12			0.04	0.003		
BL_HtHt	148	13532.40	166.97	**0.0125**	0.1450	10.60	0.06	**0.0001**	**0.0056**	0.05	0.002	**0.0014**	**0.0014**
BL_InHt	9	11769.90	677.08			9.52	0.22			0.02	0.007		
BL_InIn	37	12396.40	506.10	0.5868	0.8139	10.07	0.19	0.2159	0.2505	0.04	0.003	**0.0513**	**0.0509**
BL_InHt	9	11769.90	1026.20			9.52	0.39			0.02	0.006		

	n	Chao1	SE	*P*-value	N_*P*-value	Shannon	SE	P-value	N_*P*-value	Simpson	SE	*P*-value	N_*P*-value
BL_HtHt	148	13532.40	168.88			10.60	0.06			0.05	0.002		
BL_InIn (active)	20	13185.10	459.41	0.5034	0.7502	10.15	0.15	**0.0055**	**0.0070**	0.03	0.004	**0.0046**	**0.0054**
BL_HtHt	148	13532.40	169.68			10.60	0.06			0.05	0.002		
BL_InIn (inactive)	17	11468.40	500.65	**0.0001**	**0.0036**	9.97	0.17	**0.0006**	**0.0038**	0.04	0.005	0.3841	0.4562
BL_HtHt	148	13532.40	165.48			10.60	0.05			0.05	0.002		
BL_InHt (active)	9	11163.2	760.90	**0.0028**	**0.0745**	9.37	0.25	**0.0001**	**0.0043**	0.02	0.008	**0.0032**	**0.0032**
BL_HtHt	148	13532.40	159.30			10.60	0.05			0.05	0.002		
BL_InHt (inactive)	2	13893.40	1370.70	0.7940	0.8186	10.06	0.44	0.2190	0.5553	0.03	0.014	0.1953	0.1899
BL_InIn (active)	20	13185.10	669.70			10.15	0.25			0.03	0.004		
BL_InHt (active)	9	11163.20	1132.00	0.1368	0.2235	9.37	0.43	0.1299	0.1503	0.02	0.006	0.1704	0.2032
BL_InIn (inactive)	17	11468.40	730.30			9.97	0.31			0.04	0.005		
BL_InHt (inactive)	2	13893.40	2129.10	0.2964	0.2879	10.06	0.90	0.9303	0.7905	0.03	0.014	0.3324	0.2319

Table 2.3. Alpha diversity analyses comparisons in cow level. Significant P-values were shown in bold, including the differences with tendency to be significant (HtHt: The cows which remained healthy during the study, HtIn: The cows which were healthy at sampling, then developed DD, InIn: The cows which had DD in all checkpoints, InHt: The cows which had DD at initial then check point then recovered, active: M1, M2 and M4.1 lesions, inactive: M3, M4 lesions, SE: standard error, N_P_value: P-value of nonparametric Wilcoxon/Kruskal Wallis tests). Table continues in the next page.

	n	**Chao1**	**SE**	***P*-value**	**N_*P*-value**	**Shannon**	**SE**	**P-value**	**N_*P*-value**	**Simpson**	**SE**	***P*-value**	**N_*P*-value**
HtHt	112	13412.22	181.74	0.5575	0.8406	10.61	0.06	**0.0903**	0.8319	0.05	0.002	0.1899	0.2888
HtIn	48	13217.19	277.61			10.41	0.10			0.04	0.003		
HtHt	112	13412.22	218.41	**0.0165**	**0.0839**	10.61	0.08	**0.0002**	**0.0048**	0.05	0.002	**0.0494**	**0.0595**
InIn	59	12511.40	300.93			10.12	0.11			0.04	0.003		
HtHt	112	13412.20	190.76	0.6445	0.7079	10.61	0.06	**0.0166**	0.2100	0.05	0.002	**0.0647**	**0.0452**
InHt	16	13162.60	504.69			10.20	0.16			0.04	0.005		
InIn	59	12511.40	404.08	0.4590	0.3257	10.12	0.15	0.8114	0.7661	0.04	0.003	0.5691	0.4532
InHt	16	13162.60	775.94			10.20	0.29			0.04	0.005		
HtHt	112	13412.20	204.29	0.8801	0.9464	10.61	0.07	**0.0001**	**0.0144**	0.05	0.002	**0.0024**	**0.0027**
InIn (active)	32	13005.70	382.18			10.02	0.13			0.03	0.004		

	n	Chao1	SE	*P*-value	N_*P*-value	Shannon	SE	P-value	N_*P*-value	Simpson	SE	*P*-value	N_*P*-value
HtHt	112	13412.20	194.21			10.61	0.06			0.05	0.002		
InIn (inactive)	27	11925.60	395.55	**0.0010**	**0.0058**	10.23	0.13	**0.0105**	**0.0567**	0.05	0.004	0.8822	0.8148
HtHt	112	13412.20	190.61			10.61	0.06			0.05	0.002		
InHt (active)	12	12649.50	582.32	0.2156	0.6481	10.10	0.18	**0.0101**	0.2504	0.03	0.006	0.1507	0.1261
HtHt	112	13412.20	168.15			10.61	0.05			0.05	0.002		
InHt (inactive	4	14702.00	889.78	0.1571	0.1087	10.48	0.26	0.6380	0.5653	0.03	0.010	0.1984	0.1422
InIn (active)	32	13005.70	576.68			10.02	0.22			0.03	0.004		
InHt (active)	12	12649.50	941.71	0.7487	0.8125	10.10	0.36	0.8501	0.6733	0.04	0.006	0.6002	0.5800
InIn (inactive)	27	11925.60	537.90			10.23	0.20			0.05	0.004		
InHt (inactive	4	14702.00	1397.50	**0.0739**	**0.0339**	10.48	0.53	0.6622	1.0000	0.03	0.010	0.1894	0.0990

Weighted UniFrac distances were used to calculate Beta-diversity of the samples, and non-metric multidimensional scaling (NMDS) values were charted in 3D scatterplots. There was a clear clustering between the third farm and other two farms (ANOSIM R= 0.36, *P*= 0.001). Farm 1 and farm 2 were grouped together with a slight clustering between them (Figure 2.3). There was no clear clustering between HtHt and HtIn samples (ANOSIM R= 0.13, *P*= 0.003) (Figure 2.4), and between HtHt and InIn samples (ANOSIM R= 0.18, *P*= 0.001 (Figure 2.5) in both back-left feet and cow level.

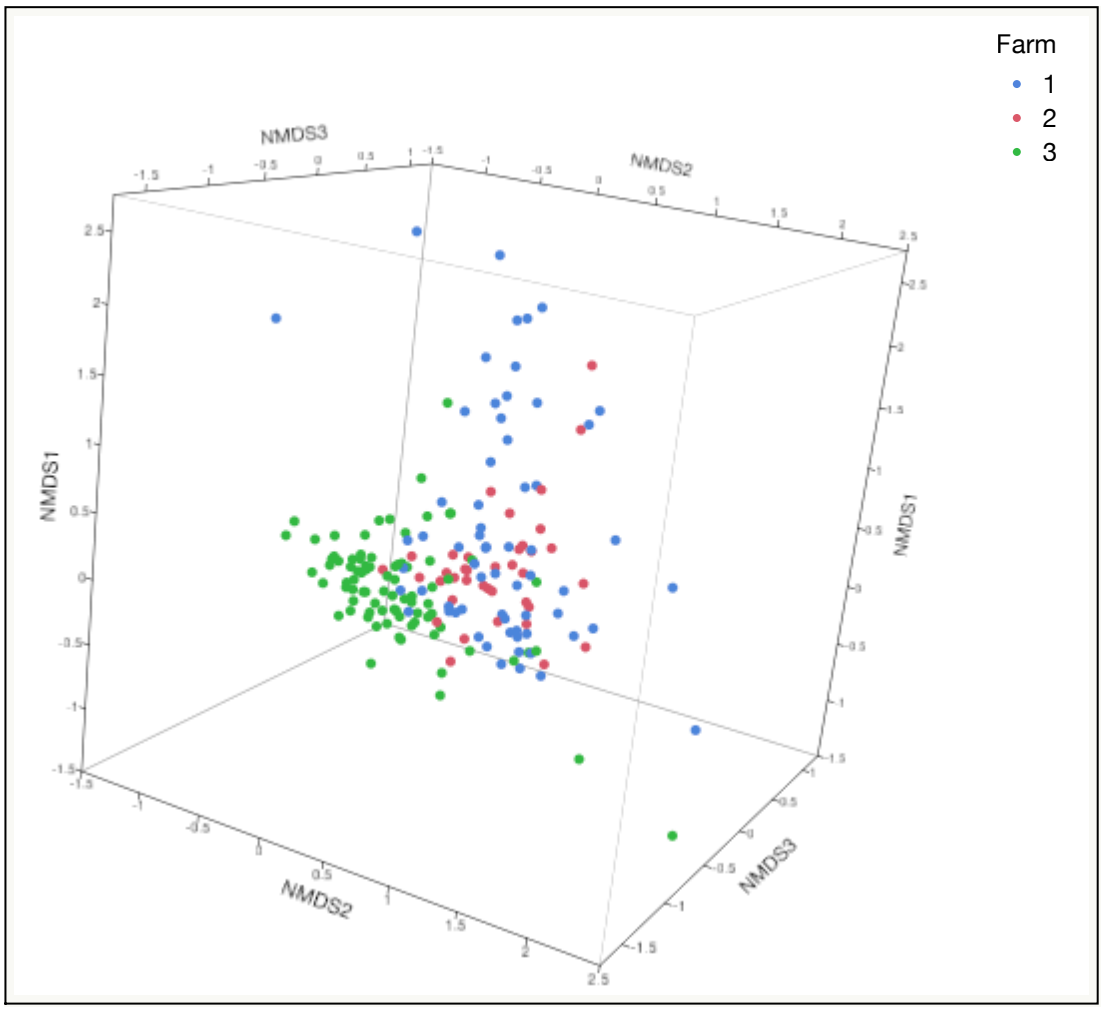

Figure 2.3. NMDS plot of weighted UniFrac distances (comparisons between farms) (Analysis of Similarity (ANOSIM), R= 0.36, P= 0.001)

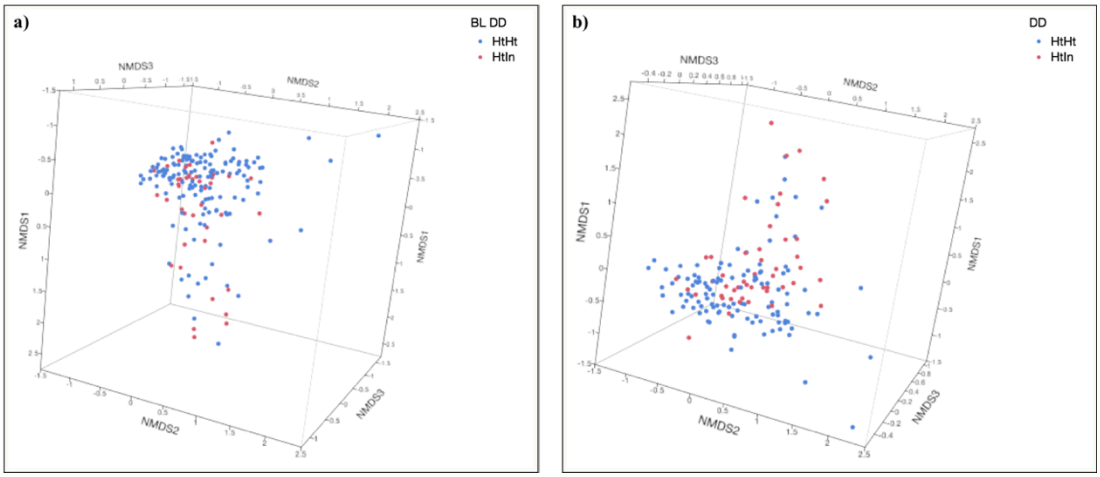

Figure 2.4. NMDS plot of weighted UniFrac distances of HtHt compared to HtIn samples for **a)** back-left (BL) feet level, **b)** cow level. (HtHt: The cows which remained healthy during the study. HtIn: The cows which were healthy at initial check point, then developed DD.

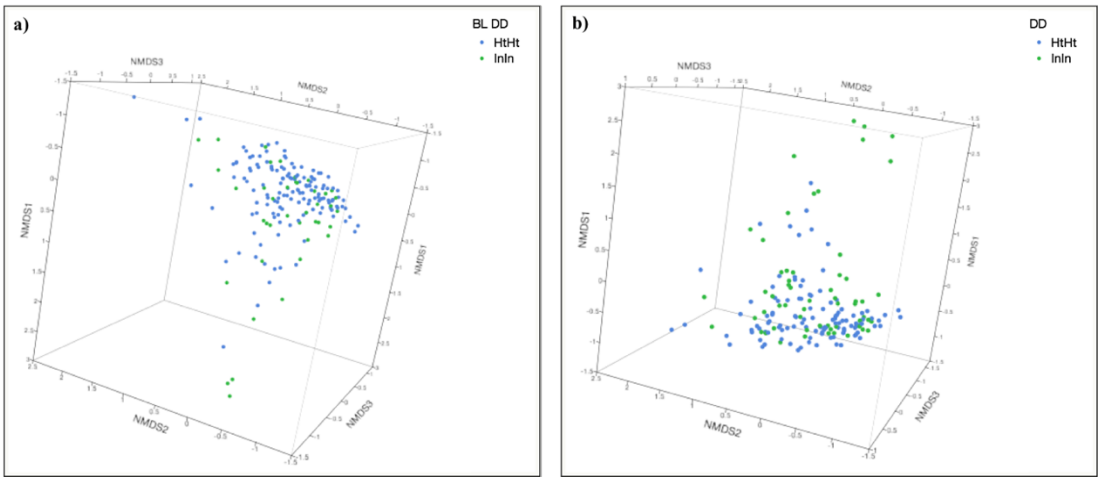

Figure 2.5. NMDS plot of weighted UniFrac distances of HtHt compared to InIn samples for **a)** back-left (BL) feet level, **b)** cow level. (HtHt: The cows which remained healthy during the study. InIn: The cows which had DD in all checkpoints.)

Relative abundances of the 20 most prevalent phyla in all samples and at the cow level are shown in Figure 2.6. There were no striking differences in relative abundances of phyla amongst the samples from cows with different DD status in the

study by looking at the figures. The main phyla for all the samples were Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria.

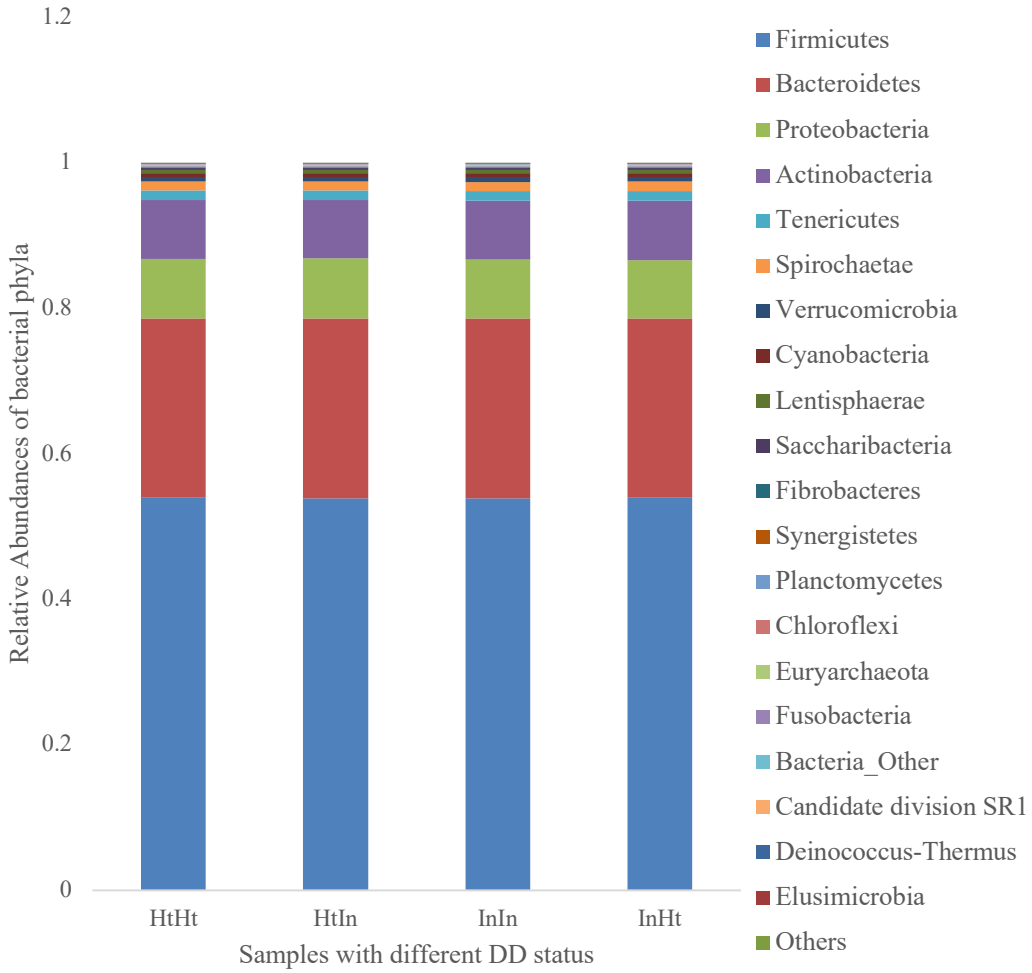

Figure 2.6. Relative abundances of 20 most prevalent bacterial phyla in the samples with different DD status (HtHt: The cows which remained healthy during the study. HtIn: The cows which were healthy at initial checkpoint, then developed DD. InIn: The cows which had DD in all checkpoints. InHt: The cows which had DD at initial checkpoint, then recovered.)

The relative abundances of the 20 most prevalent genera in all samples are shown at the back-left feet level (Figure 2.7), and at the cow level (Figure 2.8). Relative abundances of genera in the samples with different DD status in both cow and back-left feet level were similar. However, some genera were observed to have different relative abundances when each cow level DD status was investigated separately. For instance, the relative abundance of *Porphyromonas* spp., were higher in HtIn and InIn samples compared to HtHt samples. Moreover; *Treponema* spp. were not present in the 20 most prevalent genera in HtHt samples, but they were present in HtIn and InIn samples. In addition to that, *Fastidiosipila* spp. were present only in InIn samples, and not present in HtHt and HtIn samples (Appendix Figure A.1, Figure A.2, and Figure A.3).

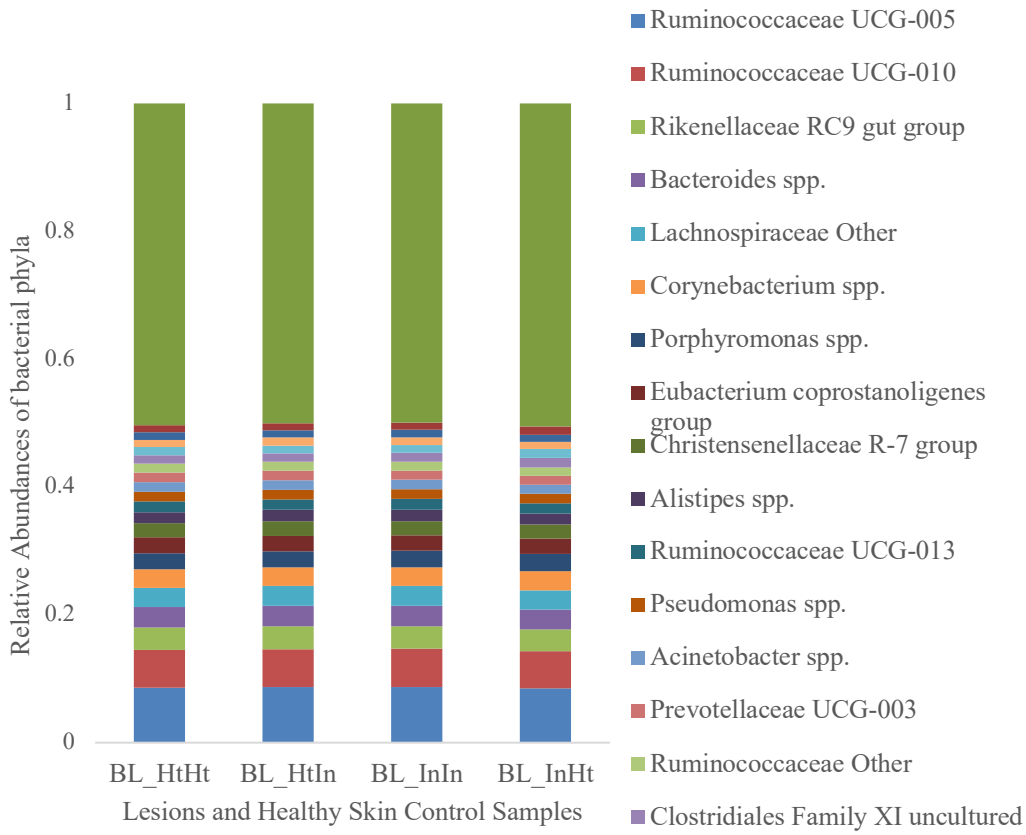

Figure 2.7. Relative abundances of 20 most prevalent bacterial genera in the samples with different DD status in back-left foot level (BL_HtHt: The cows which remained healthy during the study. BL_HtIn: The cows which were healthy at initial checkpoint, then developed DD. BL_InIn: The cows which had DD in all checkpoints. BL_InHt: The cows which had DD at initial checkpoint, then recovered.)

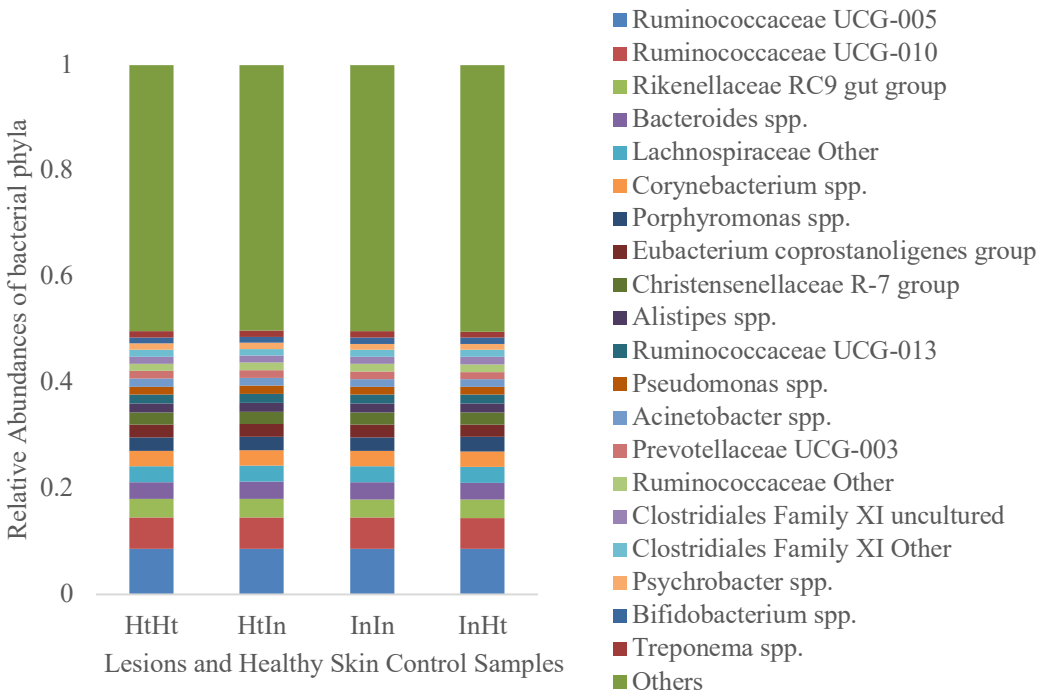

Figure 2.8. Relative abundances of 20 most prevalent bacterial genera in the samples with different DD status in cow level (HtHt: The cows which remained healthy during the study. HtIn: The cows which were healthy at initial checkpoint, then developed DD. InIn: The cows which had DD in all checkpoints. InHt: The cows which had DD at initial checkpoint, then recovered.)

Differences at the genus level were investigated in more details using response screening analysis. To display the differences between HtHt and HtIn samples, and HtHt and InIn samples bubble plots were plotted with response screening results.

For back-left foot level HtIn samples (BL_HtIn), *Succiniclasticum* spp., *Porphyromonas* spp., *Mycoplasma* spp., *Anaerococcus* spp. and *Peptoniphilus* spp were significantly more prevalent compared to HtHt samples, whereas *Macrococcus* spp. were significantly more prevalent in HtHt samples compared to HtIn samples (Figure 2.9).

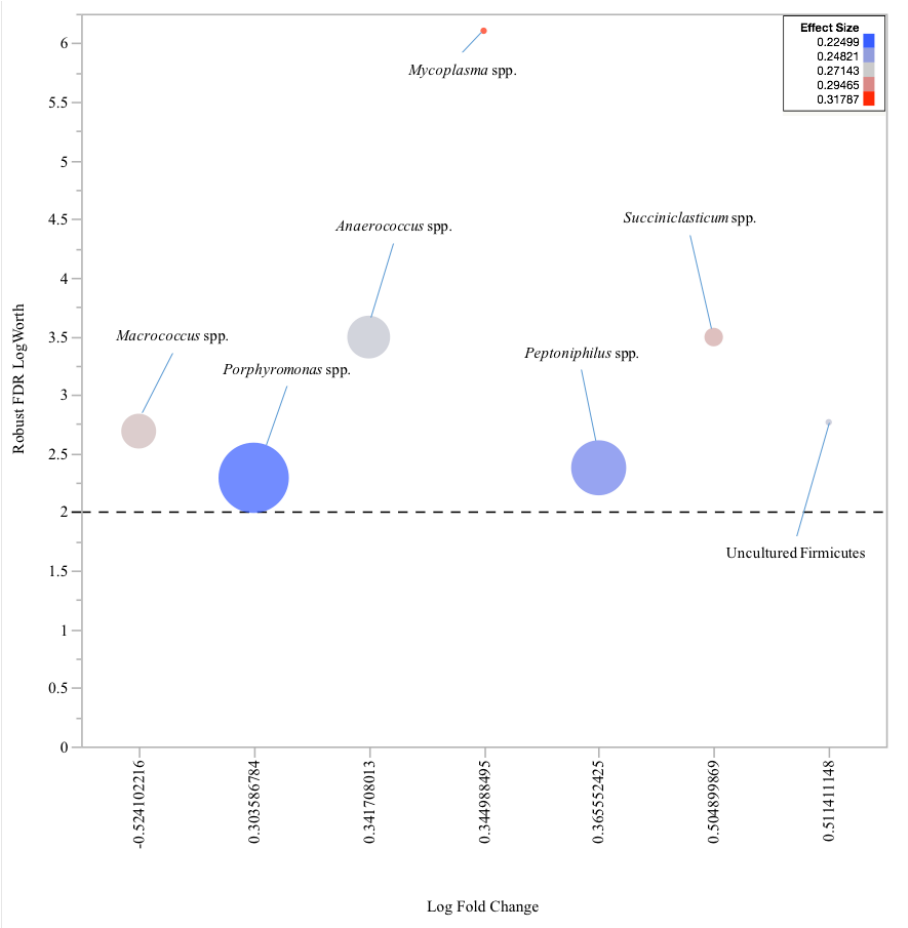

Figure 2.9. Comparison of microbiota profiles of HtHt and HtIn samples at the back-left foot level. Size of the circle represents the prevalence of each genus, and colour represents the effect size. The graph shows log fold change in 16S rRNA gene abundance in HtIn samples relative to HtHt control samples versus corrected robust false discovery rate (FDR) logWorth (i.e. log10P). The dashed line shows the P-values (0.01) adjusted for FDR. (HtHt: The cows which remained healthy during the study. HtIn: The cows which were healthy at initial checkpoint, then developed DD.)

In cow level HtIn samples, *Succiniclasticum* spp., *Porphyromonas* spp., *Acholeplasma* spp., *Anaerococcus* spp., *Fastidiosipila* spp., *Peptoclostridium* spp., *Prevotella* spp., other genera of the family Porphyromonadaceae, other genera of the family Bacteroidetes, other genera of the family Sphingobacteriales, and other genera of the family Clostridiales Family XI were significantly more prevalent compared to HtHt samples; *Macrococcus* spp., and *Brachybacterium* spp., were significantly more prevalent in HtHt samples compared to HtIn samples (Figure 2.10).

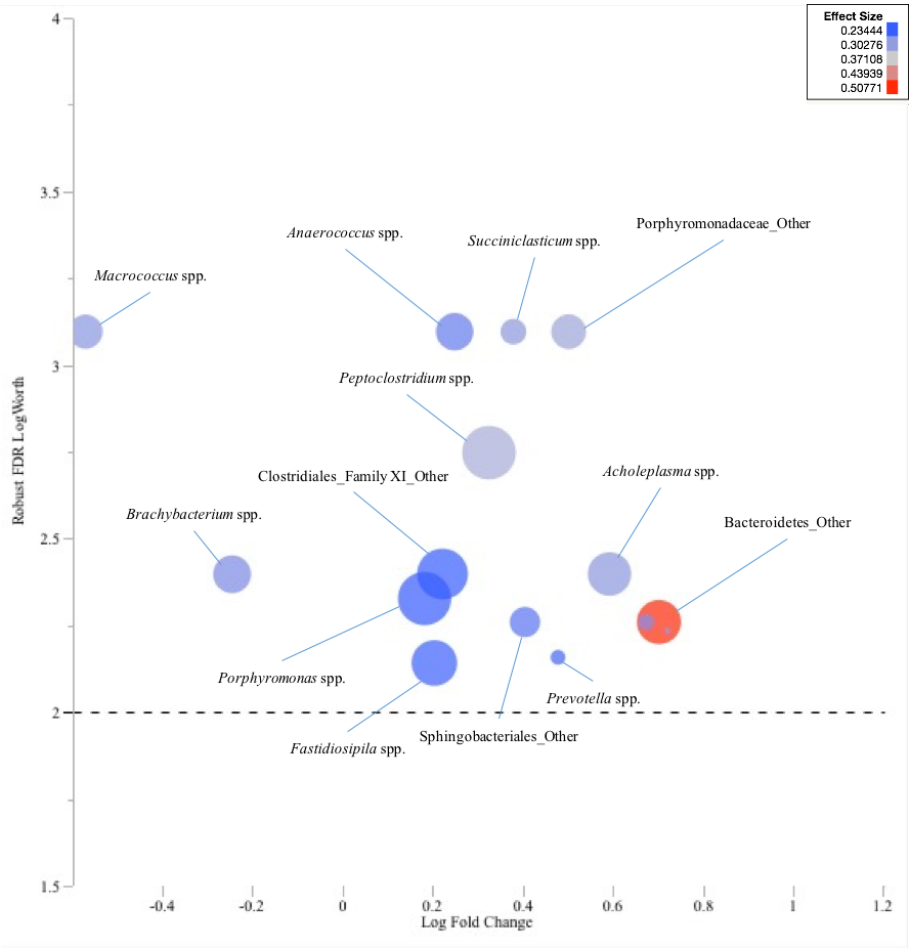

Figure 2.10. Comparison of microbiota profile of HtHt and HtIn samples at the cow level. Size of the circle represents the prevalence of each genera, and colour represents the effect size. The graph shows log fold change in 16S rRNA gene abundance in HtIn samples relative to HtHt control samples versus corrected robust false discovery rate (FDR) logWorth (i.e. log10P). The dashed line shows the P-values (0.01) adjusted for FDR. (HtHt: The cows which remained healthy during the study. HtIn: The cows which were healthy at initial checkpoint, then developed DD.)

In back-left foot level InIn samples (BL_InIn), *Succiniclasticum* spp., *Porphyromonas* spp., *Treponema* spp., *Acholeplasma* spp., *Anaerococcus* spp., *Fastidiosipila* spp., *Peptoclostridium* spp., *Murdochiella* spp., *Ezakiella* spp., *Peptoniphilus* spp. other genera of the family Porphyromonadaceae, and other genera of the family Bacteroidetes were significantly more prevalent compared to in HtHt samples, while *Moraxella* spp., *Corynebacterium* spp., and *Pseudomonas* spp., were

significantly more prevalent in HtHt samples compared to InIn samples (Figure 2.11).

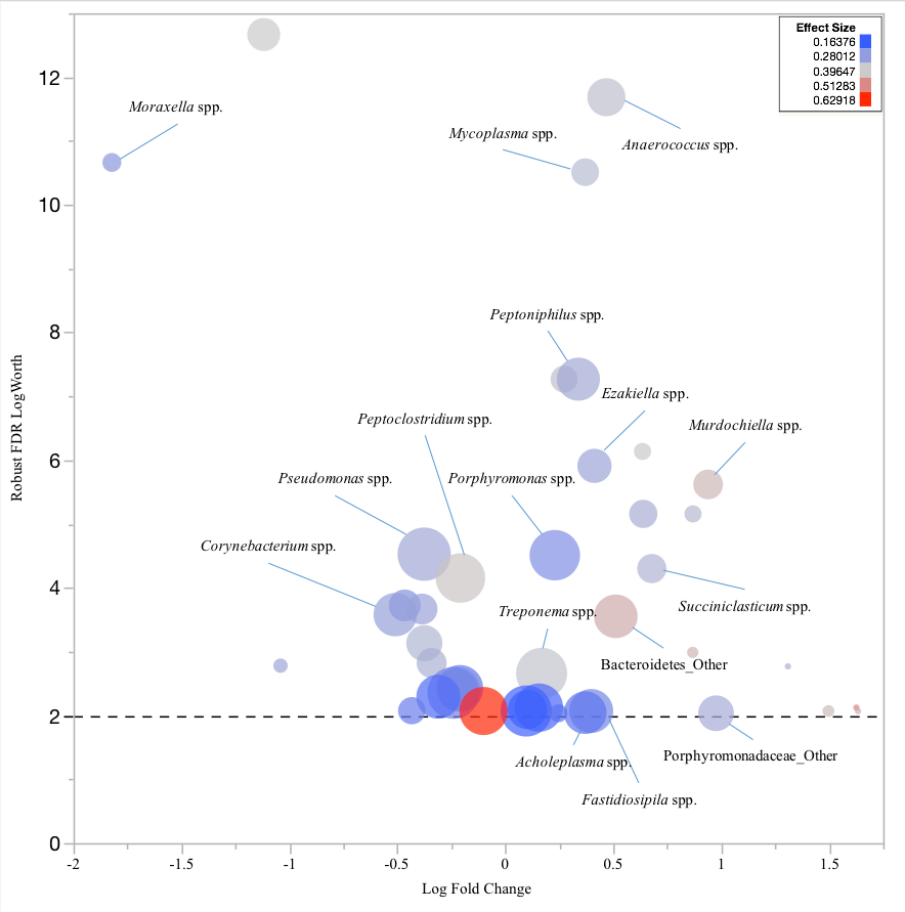

Figure 2.11. Comparison of microbiota profile of HtHt and InIn samples at the back-left foot level. Size of the circle represents the prevalence of each genus, and colour represents the effect size. The graph shows log fold change in 16S rRNA gene abundance in InIn samples relative to HtHt control samples versus corrected robust false discovery rate (FDR) logWorth (i.e. log10P). The dashed line shows the P-values (0.01) adjusted for FDR. (HtHt: The cows which remained healthy during the study. InIn: The cows which had DD in all checkpoints.)

In cow level InIn samples, *Succiniclasticum* spp., *Porphyromonas* spp., *Treponema* spp., *Acholeplasma* spp., *Anaerococcus* spp., *Fastidiosipila* spp., *Peptoclostridium* spp., *Murdochiella* spp., *Ezakiella* spp., *Peptoniphilus* spp. other genera of the family Porphyromonadaceae, other genera of the family Bacteroidetes, uncultured bacteria of the phyla Bacteroidetes, Firmicutes, and the family

Clostridiales Family XIII were significantly more prevalent compared to in HtHt samples, while *Macrococcus* spp., *Moraxella* spp., *Kocuria* spp., *Jeotgalicoccus* spp., *Acinetobacter* spp., Ruminococcaceae NK4A214 group, other genera of the family Alcaligenaceae and the family Clostridiales were significantly more prevalent in HtHt samples compared to InIn samples (Figure 2.12).

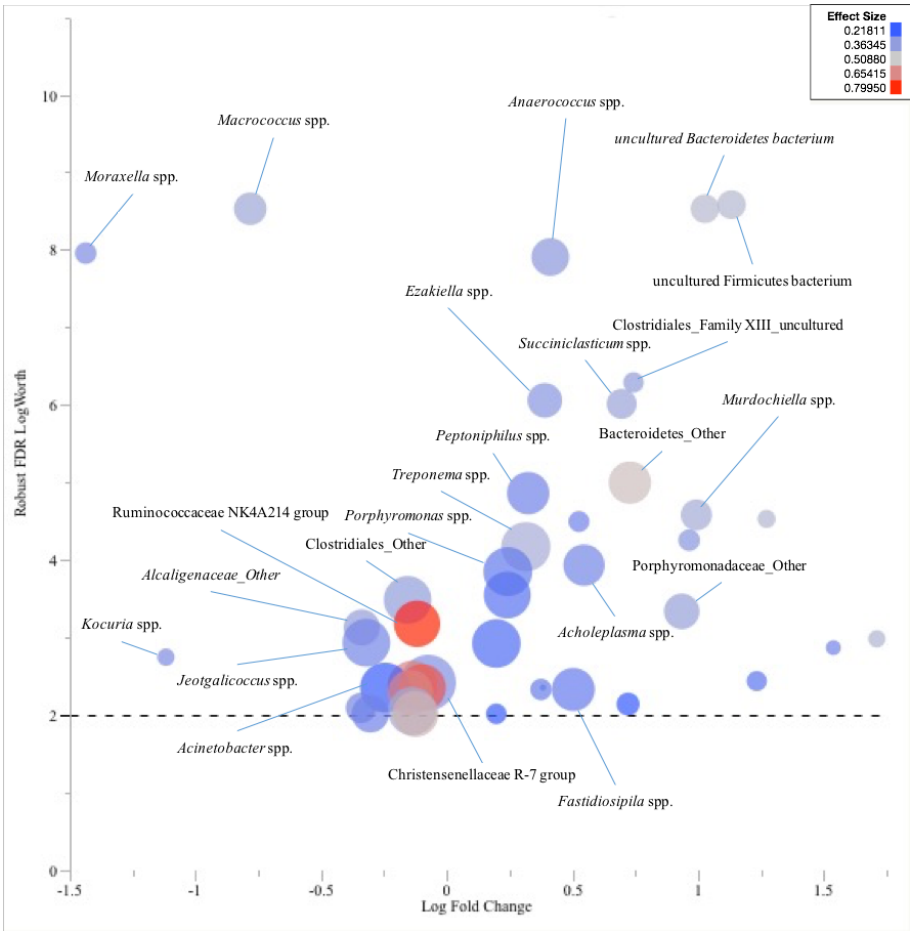

Figure 2.12. Comparison of microbiota profile of HtHt and InIn samples at the cow level. Size of the circle represents the prevalence of each genus, and colour represents the effect size. The graph shows log fold change in 16S rRNA gene abundance in InIn samples relative to HtHt control samples versus corrected robust false discovery rate (FDR) logWorth (i.e. log10P). The dashed line shows the P-values (0.01) adjusted for FDR. (HtHt: The cows which remained healthy during the study. InIn: The cows which had DD in all checkpoints.)

InHt samples did not show any significant difference at genus level from HtHt samples (back-left foot level and cow level analysis).

Response screening between farm 3 and farms 1 and 2 indicated several genera passing the significance threshold, but most of them were around zero log fold change (Appendix Figure A.4). Therefore, the ones with a difference below log fold 1 (at least 10 times difference) were discarded to decrease the complexity in the figure. In farm 3, relative abundances of *Salinimicrobium* spp., *Ornithobacterium* spp., and *Ornithinicoccus* spp. were significantly higher compared to farms 1 and 2, while relative abundances of *Aliidiomarina* spp., *Wautersiella* spp. MBG55, and uncultured Prevotellaceae were significantly higher in farms 1 and 2 compared to farm 3 (Figure 2.13).

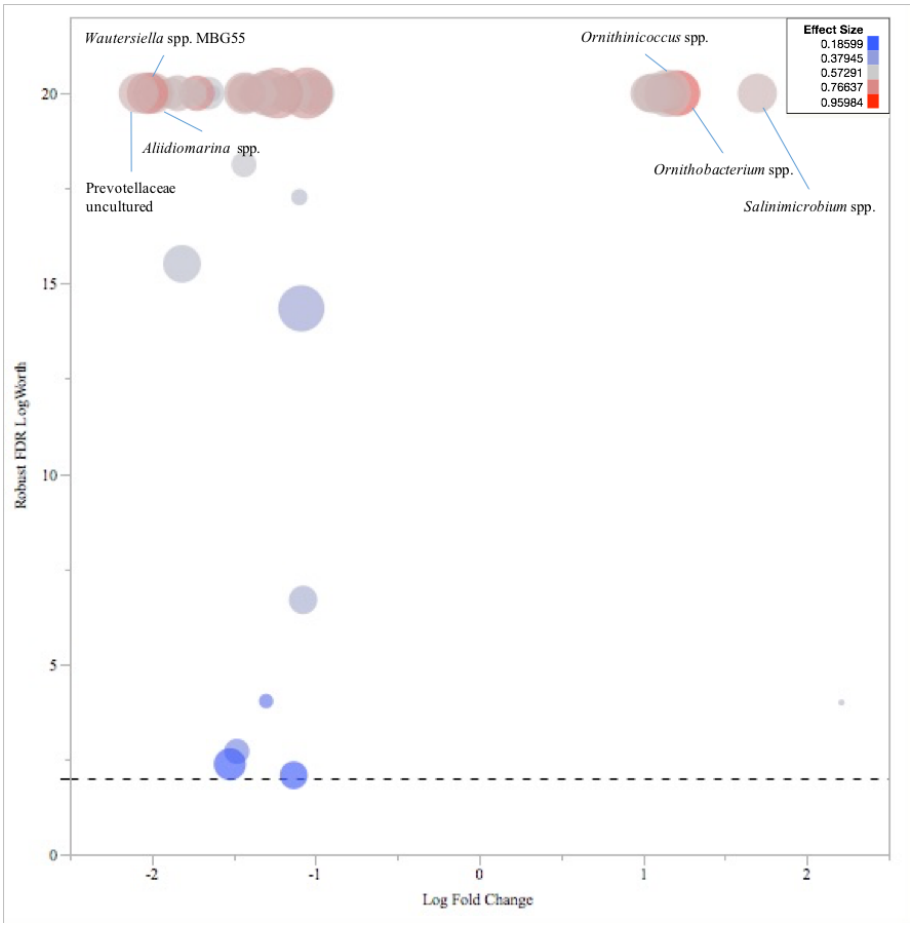

Figure 2.13. Comparison of microbiota profile of farms 1 and 2 and farm 3 samples. Size of the circle represents the prevalence of each genera, and colour represents the effect size. The graph shows log fold change (>1 and <-1 showing at least 10 times difference) in 16S rRNA gene abundance in farm 3 samples relative to farms 1 and 2 samples versus corrected robust false discovery rate (FDR) logWorth (i.e. log10P). The dashed line shows the P-values (0.01) adjusted for FDR.

2.5 Discussion

The potential role of the foot skin microbiota in the aetiology of DD lesions for dairy cattle was explored with the use of samples from three different farms with differences in management systems. Weighted UniFrac analysis showed that microbiota profiles of farms 1 and 2 were similar and clearly distinct from farm 3. The cows were categorized into four groups; HtHt, HtIn, InIn, InHt, according to lesion status at the back-left foot level and at the cow level. HtHt, HtIn, InIn, InHt samples did not show any clear clustering in the Weighted UniFrac analysis. On the other hand, response screening analysis at the genus level allowed the description of statistically significant differences between different lesion status groups.

DD has been associated with many bacterial agents. *Treponema* spp. were identified as a major bacteria associated with DD by cultivation (Walker et al. 1995), fluorescence in situ hybridization (Knappe-Poindecker et al. 2013), polymerase chain reaction (Rijpkema et al. 1997; Demirkan et al. 1998), and metagenomics (Klitgaard et al. 2013; Krull et al. 2014). Immunological studies confirmed that antibodies against *Treponema* spp. were increased in individuals with DD (Walker et al. 1997; Demirkan et al. 1999). Besides, DD lesion induction trials with pure *Treponema* spp. cultures were successful when favourable conditions for the growth of treponemes, such as a moist environment, were created (Gomez et al. 2012; Krull et al. 2016). On the other hand, vaccination against *Treponema* spp. did not affect DD incidence or severity (Angel. Ertze et al. 2006). In addition, being represented by a diversity of species instead of single species (Evans et al. 2008; Yano et al. 2009; Krull et al. 2014) causes problems for fulfilling the Koch's postulates for DD development (Orsel et al. 2018).

In the current study, *Treponema* spp. were significantly more prevalent in InIn samples compared to HtHt samples at the back-left foot level and at the cow level. In addition, *Porphyromonas* spp., *Fastidiosipila* spp., *Succiniclasticum* spp., *Acholeplasma* spp., *Anaerococcus* spp., *Peptoclostridium* spp., other genera of the family Porphyromonadaceae, and other genera of the family Bacteroidetes were significantly more prevalent in both HtIn and InIn samples compared to the HtHt samples. *Porphyromonas* spp. were previously shown in different human and animal infections (Finegold and Jousimies-somer 1997; Walter and Morck 2002), and especially in DD lesions (Moe et al. 2010). Furthermore, *Fastidiosipila* spp. were previously shown to be associated with human osteitis lesions (Beauruelle et al.

2014). *Acholeplasma* spp. were shown to be present in bovine mastitis infections (Ayling et al. 2004). *Anaerococcus* spp. were isolated from both human and animal infections (Jiménez et al. 2003; Fenollar et al. 2006). *Peptoclostridium* spp. are ruminal bacteria that have also been previously reported in both human and animal infections (Creevey et al. 2014; Nycz et al. 2018; Zheng et al. 2018). *Succiniclasticum* spp. are ruminal bacteria that have never reported in any infection (van Gylswyk 1995).

HtHt samples displayed higher relative abundances of *Macrococcus* spp. compared to HtIn and InIn samples. *Macrococcus* spp. were generally considered to be a part of healthy skin microflora (Calvo-Bado et al. 2011; Maboni et al. 2017). In addition, HtHt samples had higher relative abundances of *Moraxella* spp., and *Kocuria* spp. which were also previously shown to be associated with healthy skin (Yano et al. 2010; Braem et al. 2012).

Previous studies have demonstrated the prevalence of *Campylobacter* spp., *Dichelobacter nodosus*, and *Fusobacterium necrophorum* in DD lesions, however, the same bacteria were also present in healthy skin samples (Moe et al. 2010; Rasmussen et al. 2012; Knappe-Poindecker et al. 2013). In the present study, these species were also detected, but they did not show any significant prevalence in DD samples compared to healthy skin samples.

In the current study, although *Treponema* spp. were significantly more prevalent in InIn samples compared to HtHt samples, the comparison between the microbiota profile of HtIn and InIn samples versus HtHt samples reveals that *Treponema* spp. do not have a significantly higher relative abundance at early stages of the disease progression. The presence of other anaerobic pathogens might provide a better environment for the proliferation of *Treponema* spp., as previously shown for human periodontal disease (Edwards et al. 2003; Ito et al. 2010b; Plummer and Krull 2017). In progression of both DD and periodontal disease, it is considered that waves of bacterial colonization might happen. In periodontal disease, the early colonizers push the environmental conditions toward a more anaerobic niche. Moreover, the metabolic profiles of the bacterial community shift from mainly saccharolytic (using glucose and sugars as energy source) to mainly proteolytic metabolism of proteins. The early colonizers, which are mainly saccharolytic aerobes, drive the environment to a more favourable condition for the facultative

anaerobes which metabolise sugars to produce volatile fatty acids (VFAs). Thus the environment then becomes more favourable for the final anaerobic colonizers, which utilize VFAs for energy (Krull et al. 2014; Takahashi 2015; Plummer and Krull 2017). The findings of the current study concur with this hypothesis.

The microbiota profile of farm 3 diverged from microbiota profiles of farms 1 and 2. This might be caused by differences in management systems. For instance, farm 3 performs foot bathing and used deep sand cubicles for dry cows, whereas farms 1 and 2 do not perform foot bathing and keep dry cows in straw yards. On the other hand, samples from farm 1 were collected for a longer period of time, however, the ones from farms 2 and 3 were collected during a more a limited time interval. Therefore, the difference in microbiota profiles of these farms might also be a result of seasonal effects.

The aim of this study was to investigate the role of the foot skin microbiota in the aetiology of DD, and to identify bacteria that have potential associations with a predisposition to DD lesions or have potential protective properties. The results presented here suggest that there are some genera such as *Porphyromonas* spp. that probably contribute in the development of DD lesions by shifting the microenvironment of the foot skin to a more suitable environment for the proliferation of other pathogens like *Treponema* spp. On the other hand, some genera such as *Macrococcus* spp. might have potentially protective roles against disease development. However, further research is needed to confirm these results. For instance, sampling could be performed in each visit and more advanced sequencing techniques such as Shotgun sequencing could be performed to get more thorough results. After that, new therapeutic or preventive approaches could be developed. One example approach could be changing foot bathing solutions since the current ones in use are not without limitations; e.g. formalin is a carcinogen (Buesa 2008), copper sulfate causes environmental pollution (Flemming and Trevors 1989), and use of antibiotics could result in antibiotic resistance (Neu 1992). Alternatively, probiotic solutions including bacteria with potential protective roles against DD could be used in foot bathing. Similar probiotic approaches using *Lactobacillus* spp. to treat periodontal disease has yielded promising results (Shimauchi et al. 2008; Vivekananda et al. 2010; Vicario et al. 2013; Maekawa and Hajishengallis 2014).

2.6 Conclusion

In this study, the role of the foot skin microbiota in the aetiology of DD lesions was investigated using 16S rRNA gene amplicon sequencing and bacteria associated with a predisposition to DD or having potential protective roles were characterized. Multivariate analysis was used to identify significant differences between the foot microbiota profiles of cows with different DD status. It is shown here that shifts in the microbiota profiles of the foot skin could lead to a more favourable environment for opportunistic pathogens such as *Treponema* spp. Although *Treponema* spp. are well-known causative agents in the aetiology of DD, the present study suggests that other pathogens such as *Porphyromonas* spp., *Fastidiosipila* spp., *Succiniclasticum* spp., *Acholeplasma* spp., *Anaerococcus* spp., *Peptoclostridium* spp. may be acting synergistically with *Treponema* spp. in the development of DD lesions.

CHAPTER III *

3 16S rRNA amplicon sequencing reveals a polymicrobial nature of complicated claw horn disruption lesions and interdigital phlegmon in dairy cattle

3.1 Summary

Lameness represents an intractable problem for the dairy industry resulting in economic losses and seriously compromising animal welfare. Complicated claw horn disruption lesions (CHDLs) (complicated sole ulcer (SU), toe necrosis (TN), and white line disease (WLD), interdigital hyperplasia (IH), and interdigital phlegmon (IP) are important lameness causing foot lesions. Their aetiology is multifactorial, but infectious processes are likely implicated in disease pathogenesis. The aim of this study was to investigate the microbiota profile of these lesions using 16S rRNA gene amplicon sequencing of swab samples obtained from 51 cattle across ten different dairy farms in the UK.

In this study, IH (n=11), IH with signs of interdigital dermatitis (IIH) (n=4), IP (n=3), complicated SU (n=20), complicated TN lesions (n=3), and complicated WLD lesions (n=11) were investigated; corresponding healthy skin control samples from the same animals and for each one of these lesions were also analysed. All diseased tissues displayed reduced microbial richness and diversity compared to their healthy skin control samples. This study confirms the role of *Treponema* spp in some of these disorders. Furthermore, other anaerobic bacteria including *Fusobacterium* spp, *Fastidiosipila* spp and *Porphyromonas* spp were implicated (some for the first time) in the aetiology of all these lesions with the exception of IH.

Complicated CHDLs, and IP were found to have similar microbiota profiles. This would suggest that many of the various infectious agents detected in these foot lesions are acting opportunistically; this finding could contribute towards future treatment and/or control strategies for these disorders.

**This chapter has been accepted for publication in Scientific Reports with first authorship attributed to both Veysel Bay and Bethany Griffiths. Veysel Bay performed the laboratory work, analysed the data and wrote the manuscript. Bethany Griffiths contributed to the design of the study, collected the samples, assisted laboratory work and wrote part of the manuscript.*

3.2 Introduction

Lameness is a significant health issue within the dairy cattle industry due to reduced animal welfare and productivity, and associated economic losses (Bicalho and Oikonomou 2013; Huxley 2013). Lameness is associated with significant reductions in milk yield, increased discomfort and pain (Whay et al. 1998; Green et al. 2002; Bicalho et al. 2008), reduced fertility (Barkema et al., 2015; Bicalho et al., 2007), and higher culling rates and veterinary costs (Rajala-Schultz and Gröhn 1999; Oikonomou et al. 2013). Lameness, a clinical sign and not a disease per se, is multifactorial in nature with over 90% of lameness causing lesions found in the foot (Murray et al. 1996). These lesions can have either an infectious or a non-infectious aetiology. The most common lesions of infectious aetiology are interdigital phlegmon (IP) (also known as foul in the foot, interdigital necrobacillosis, or bovine foot rot) (van Metre 2017), and digital dermatitis (DD). The most common non-infectious lesions are described as claw horn disruption lesions (CHDLs) and include sole haemorrhage (SH), sole ulcer (SU), toe necrosis (TN), and white line disease (WLD) (Green et al. 2002; van der Waaij et al. 2005; Bicalho and Oikonomou 2013).

Bovine DD was reported for the first time in 1974 (Cheli and Mortellaro 1974) and usually affects the palmar or plantar aspect of the feet, caudal to the interdigital space. It has two forms; an active form consisting of lesions with a moist ulcerative area, and an inactive form which is characterized by the presence of a firm scab, hyperkeratosis, proliferative overgrowth, and an absence of an ulcerative area (Wilson-Welder et al. 2015; Zinicola et al. 2015a; Read & Walker 1998). *Treponema* spp. have been consistently detected in high numbers and routinely isolated from these lesions and are considered the primary aetiological agent (Choi et al. 1997; Evans et al. 2009; Nordhoff et al. 2008; Santos et al. 2012). However, a variety of other bacteria have also been associated with these lesions; *Porphyromonas* spp. (Moe et al. 2010), *Mycoplasma* spp., *Bacteroides* spp. (Collighan and Woodward 1997), *Campylobacter* spp. (Döpfer et al. 1997), *Guggenheimella* spp. (Schlafer et al. 2008), *Borrelia* spp. (Blowey et al. 1994), *Dichelobacter nodosus* (Rasmussen et al. 2012), *Fusobacterium necrophorum, and Candidatus Aemobophilus asiaticus* (Zinicola et al. 2015b; Krull et al. 2014; Zinicola et al. 2015a) have all been identified in DD lesions.

DD associated *Treponema* spp. have also been associated with complicated (non-healing) CHDLs (SU, TU and WLD), as they have been shown capable of

infecting exposed corium (Evans et al. 2011; Kofler et al. 2015). Specifically, Evans et al. (2011) described a strong association between the presence of all three identified, cultivable, DD treponemes; *Treponema medium-like*, *Treponema phagedenis-like* and *Treponema denticola-like* spirochaetes, within each of the three different non-healing bovine claw horn lesions: TN, non-healing WLD, and non-healing SU. In contrast to the typical CHDL lesions (which are of non-infectious aetiology), complicated lesions may display a topical granular appearance, with a typical pungent smell; presence of purulent discharge is also common (Evans et al. 2011; Kofler 2017).

IP is an acute or subacute necrotizing dermatitis located in the interdigital space. Swelling and redness of the skin are observed in the interdigital space and the coronary band, and as a result of swelling digits become separated. It is believed to be caused by *Fusobacterium necrophorum*; however, *Porphyromonas levii* and *Prevotella intermedia* bacteria have also been isolated from these lesions (Clark et al. 1985; Alban et al. 1995; Morck et al. 1998; Nagaraja et al. 2005; van Metre 2017). Interdigital hyperplasia (IH) refers to the formation of hyperplastic interdigital skin at the axial hoof wall in the interdigital space (Kofler et al. 2011). Despite being a prevalent lesion (Barker et al. 2010; Foditsch et al. 2016), its aetiopathogenesis is not fully elucidated. It has been speculated that outwards spreading of the claws and poor ligamentous structure leads to stretching of the interdigital skin resulting in hyperplasia (Desrochers et al. 2008). Furthermore, there is evidence for a genetic predisposition (Croué et al. 2017; Malchiodi et al. 2017). A potential association between IH and DD has been speculated; however, the evidence supporting this association remains scarce (Holzhauer et al. 2008a; Sullivan et al. 2013; van Metre 2017).

Culture-independent analysis of mixed microbial communities relying either on target-specific 16S rRNA gene sequencing or shotgun metagenomic sequencing (Shah et al. 2011; Addis et al. 2016), can aid the study of diseases of a potentially polymicrobial nature and lead to a better understanding of their aetiopathogenesis. 16S rRNA gene amplicon sequencing is cost effective and well established pipelines and substantial archived reference data exist for data analyses, but whole genome-Shotgun sequencing approaches can provide with substantially more information (Ranjan et al. 2016). *Treponema* spp. have recently been associated with bovine DD lesions using shotgun metagenomic and 16S rRNA gene sequencing (Zinicola et al.

2015a; Zinicola et al. 2015b; Krull et al. 2014). However, studies of the microbial communities of complicated CHDL, IP or IH lesions are still absent from the scientific literature. It could be valuable to characterise the microbiota composition of these lesions in order to identify microorganisms involved in disease pathogenesis.

The objective of this part of the present study was to characterize the microbial composition of each lesion type, and to identify the putative pathogens associated with those lesions. Amplicon-based 16S rRNA gene sequencing was performed on samples collected from dairy cows affected with IH, infected IH, IP, and complicated SU, TN, and WLD lesions. Control samples collected from healthy skin were also analysed for comparative purposes.

3.3 Materials and Methods

3.3.1 Ethics and sampling

Ethical approval for the study was granted by the University of Liverpool Research Ethics Committee (Reference Number: VREC547). As part of this cross-sectional study, a research student accompanied five different professional foot trimmers as they visited clients' farms throughout June and July 2017. Farm visits were organised to coincide with therapeutic foot trimming, as opposed to routine preventative foot trimming visits, to increase the likelihood of finding the targeted lesions. The lesions targeted within this study included; complicated SU, complicated WLD, complicated TN, IH, IH with signs of interdigital dermatitis, and IP. Cases were defined following the ICAR Claw Health Atlas definitions (Egger-Danner et al. 2015) with the only additional requirement for a CHDL to be enrolled being the presence of obvious signs of infection. Claw horn disruption lesions without signs of infection (not complicated) were not included in this study. The foot trimmer was observed during routine foot trimming and when a targeted lesion was identified, a picture was taken, and a sterile swab used to obtain a sample from within lesion. In the case of IP and IH the skin was cleaned from any gross contamination with the use of a paper towel before sample collection. As a control, a second swab sample was taken from the plantar/palmar aspect of the affected foot, targeting the normal skin proximal and adjacent to the interdigital cleft, just above the heel bulbs. The area was cleaned of any gross contamination with the use of a paper towel before sample collection. Once obtained, swabs were placed in sterile tubes and labelled with lesion type, animal id, date, and whether the swab was lesion

or control sample. Samples were transported on ice and stored at -80°C until DNA extraction.

3.3.2 DNA extraction, 16S rRNA gene amplification, and library construction, pooling of PCR amplicons

The methods described in the second chapter were used to extract DNA, amplify 16SrRNA gene, and pool the PCR amplicons. The sample with the lowest concentration (3.2 ng/µl) was used in maximum volume (10 µl) for PCR reaction, so to equalize the amount of DNA in each tube, 32 ng from each sample was used as template DNA for amplification of the V3-V4 hypervariable region of 16S rRNA gene. Amplified libraries were pooled at 8 ng/µl, and 30 ng/µl concentrations for low-concentration and high-concentration samples, respectively. After measuring the concentrations of these two pools, they were mixed with 8.1 ng/µl for each amplicon with maximum volume from negative controls.

3.3.3 Sequencing and sequence analyses

Sequencing and sequence analyses were performed following the methods in the second chapter for 102 samples. In total, 48,128,931 analysed sequences were clustered in 91,207 different OTUs.

Besides the lesion and healthy skin control samples, the ZymoBIOMICS™ Microbial Community DNA Standard was used as a mock microbial community. This community comprises eight microbial and two fungal strains; seven out of eight bacterial strains were successfully assigned at species level, and in their expected relative abundances. One bacterium was correctly identified at the genus level. Only 0.05% of the sequences from the positive control sample were assigned to other unexpected bacteria. Despite being in extremely low concentrations, and not visible on the agarose gel, three PCR negative controls were also sequenced. Two of these negative controls yielded less than 1,000 sequences, and one of them yielded 20,951 sequences; less than 5% of the average number of sequences obtained from the lesion and healthy skin samples. In addition to these, sterile swabs were taken out in the farm environment, and processed as negative controls for both farms and extractions. Since their concentrations were very low and they were invisible in the gel, these samples were not further analysed.

3.3.4 Statistical analyses

OTUs abundance table (at 260,000 sequences per sample) was used to investigate the richness and evenness of the samples using Chao1, Shannon, and Simpson indexes as described in the second chapter. In addition, distance matrices were built using Weighted UniFrac dissimilarity measures as explained in the second chapter. Nonmetric Multidimensional Scaling (NMDS) analysis was performed on the obtained dissimilarity matrices and statistical significance for the obtained dissimilarity matrices calculated using analysis of similarities (ANOSIM). Subsequent *P*-values were calculated using Student's t-test.

Mean relative abundances of the fifteen most prevalent phyla and genera were charted for each lesion. Log fold changes (Log10) in relative abundance of the genera with at least 1% abundance in one sample were calculated for each lesion compared to their respective healthy skin control samples. Robust response screening analysis was performed in JMP Pro 12 (SAS Institute Inc., Cary, NC) in order to evaluate the differences in OTU (genus level assignments) relative abundance between complicated CHDLs, IH, and IP and their corresponding healthy skin control samples. A false discovery rate (FDR) correction was applied and statistical significance was declared at FDR LogWorth of 1.3 (equivalent of a *P*-value of 0.05). To facilitate data presentation, the genera with a Robust FDR LogWorth of 20 or more were adjusted to 20 (corrected Robust FDR LogWorth). Subsequently, the log fold change was plotted versus the corrected Robust FDR LogWorth value using bubble plot graphs in JMP Pro 12. Genera mean relative abundance defined the bubbles' size, and effect size was indicated by the bubbles' colouring (Ganda et al. 2016).

3.4 Results

Samples were collected from 10 dairy farms in Cheshire and North Wales, UK. The numbers of each lameness causing lesion obtained from each different farm are described in Table 3.1.

Table 3.1. Distribution of lesions in each farm. (IH: Interdigital Hyperplasia, IHC: IH Control IIH: Infected Interdigital Hyperplasia, IIHC: IIH Control, IP: Interdigital Phlegmon, IPC: IP Control, SU: Sole Ulcer, SUC: SU Control, TN: Toe Necrosis, TNC: TN Control, WLD: White Line Disease, WLDC: WLD Control)

Farm	IH	IHC	IIH	IIHC	IP	IPC	SU	SUC	TN	TNC	WLD	WLDC
A	0	0	0	0	0	0	1	1	0	0	0	0
B	0	0	0	0	3	2	0	0	0	0	4	4
C	0	0	0	0	0	0	1	0	0	0	1	1
D	6	6	2	2	0	0	0	0	0	0	0	0
E	5	5	2	2	0	0	0	0	0	0	0	0
F	0	0	0	0	0	0	4	4	0	0	3	3
G	0	0	0	0	0	0	0	0	0	0	2	2
H	0	0	0	0	0	0	0	0	0	0	1	1
I	0	0	0	0	0	0	0	0	2	2	0	0
J	0	0	0	0	0	0	14	14	1	1	0	0
Total	11	11	4	4	3	2	20	19	3	3	11	11

After quality filtering processes, a total of 47,661,917 sequences were used for further analyses (Mean=433,290, SD = 58,425 sequences per sample). Chao1, Shannon, and Simpson alpha-diversity indices for different lesion groups and their control samples are shown in Table 3.2. WLD samples (n=11) had a lower Chao1 richness index than their control samples (*P*-value = 0.01). Chao1 index was also numerically lower for SU (n=20), and TN (n=3) samples comparing to their control samples (*P*-value = 0.09 for both comparisons). Shannon index was significantly lower for SU (*P*-value = 0.01) and WLD (*P*-value = 0.02) samples comparing to their control samples, and TN samples also showed a tendency to be different from their control samples (*P*-value=0.09). Analysis of the Simpson index suggested that IH (n=11) (*P*-value=0.04), SU (*P*-value=0.02) and WLD (*P*-value=0.03) samples were significantly different than their respective control samples.

Table 3.2. Alpha diversity analyses of all lesions compared to their healthy skin control samples. Comparisons were made with the use of a series of t-tests. (IH: Interdigital Hyperplasia, IHC: IH Control IIH: Infected Interdigital Hyperplasia, IIHC: IIH Control, IP: Interdigital Phlegmon, IPC: IP Control, SU: Sole Ulcer, SUC: SU Control, TN: Toe Necrosis, TNC: TN Control, WLD: White Line Disease, WLDC: WLD Control)

	Chao1	*P*-value	Shannon	*P*-value	Simpson	*P*-value
IH	16496.14 ± 2952.34	1.00	9.72 ± 0.47	0.10	0.02 ± 0.01	0.04
IHC	16593.19 ± 2982.26		10.26 ± 0.65		0.03 ± 0.02	
IIH	16791.32 ± 2013.63	0.99	9.56 ± 1.16	0.35	0.02 ± 0.01	0.75
IIHC	16593.19 ± 2982.26		10.26 ± 0.65		0.02 ± 0.01	
IP	9695.06 ± 2326.01	0.14	7.08 ± 1.72	0.28	0.01 ± 0.00	0.15
IPC	18163.51 ± 404.56		10.76 ± 0.05		0.03 ± 0.00	
SU	11036.77 ± 4006.81	0.09	7.54 ± 1.11	0.01	0.01 ± 0.00	0.02
SUC	18318.57 ± 2461.62		10.34 ± 0.60		0.02 ± 0.01	
TN	7812.94 ± 1988.10	0.09	6.62 ± 0.55	0.09	0.01 ± 0.00	0.06
TNC	18446.32 ± 720.82		10.51 ± 0.37		0.04 ± 0.01	
WLD	8930.12 ± 1956.27	0.01	6.97 ± 0.68	0.02	0.01 ± 0.00	0.03
WLDC	17842.96 ± 1489.88		10.51 ± 0.47		0.03 ± 0.01	

Beta-diversity was calculated through weighted UniFrac distances, and NMDS values were charted in 3D scatterplots. Bacterial composition of the samples did not indicate any clear clustering between farms (Figure 3.1). On the other hand, there was a clear distinction between healthy skin control samples and IP, SU, TN, and WLD lesions. IH and IIH samples were however grouped together with the control samples (Figure 3.2).

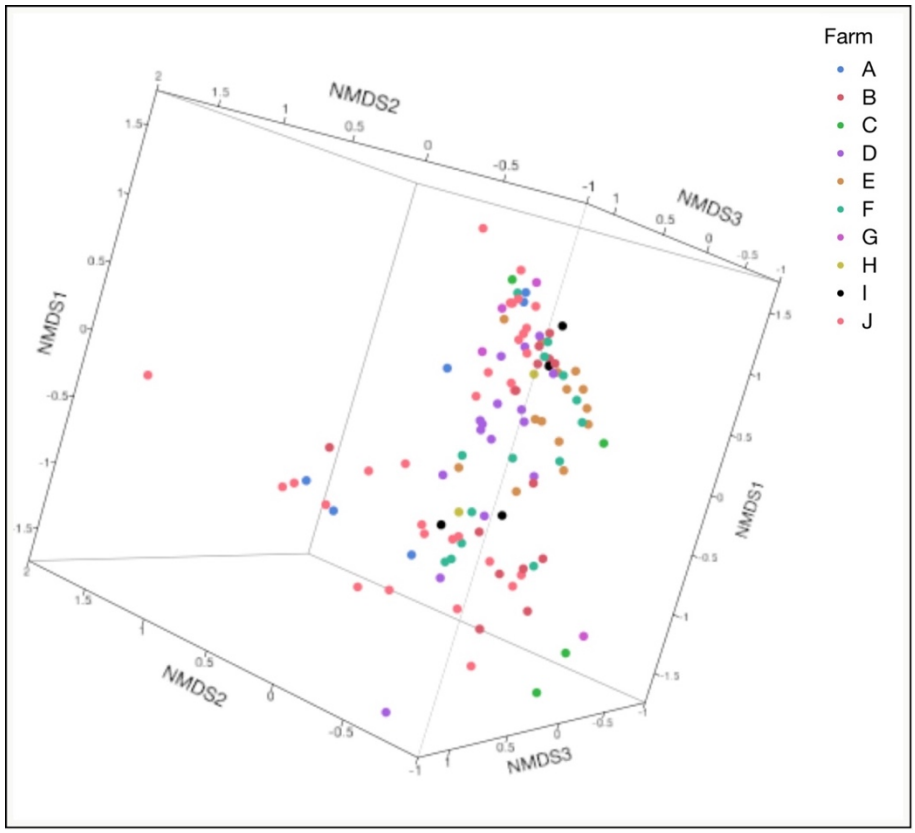

Figure 3.1. NMDS plot of weighted UniFrac distances for samples obtained from different farms (Analysis of Similarity (ANOSIM), R= 0.33, P= 0.001)

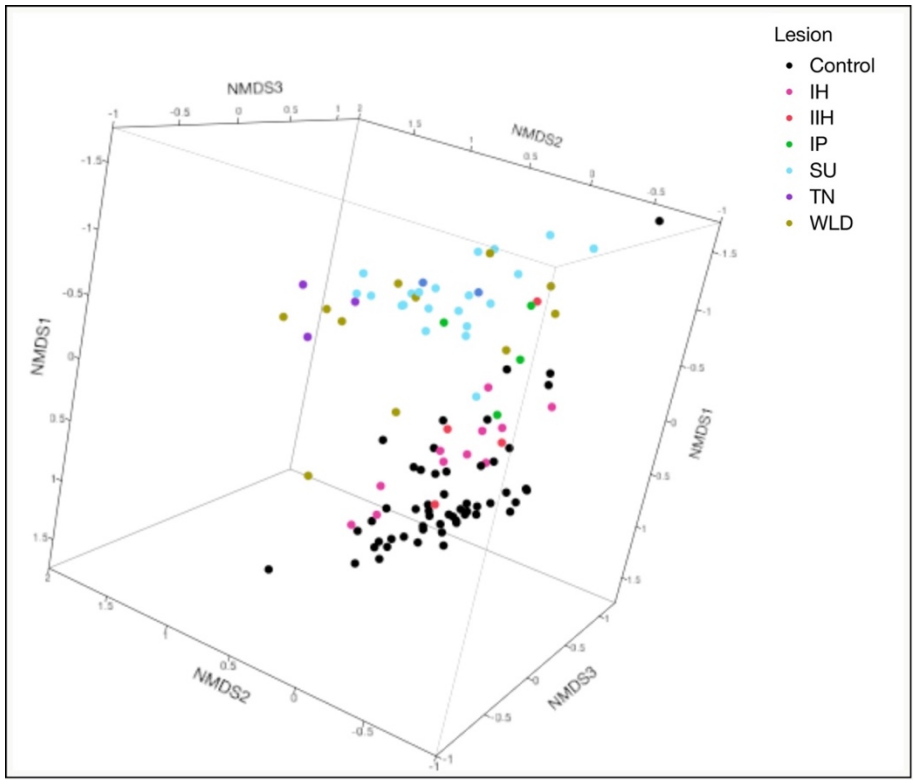

Figure 3.2. NMDS plot of weighted UniFrac distances of all lesions and their all healthy skin control samples (Analysis of Similarity (ANOSIM) R= 0.61, P= 0.001). (IH: Interdigital Hyperplasia, IIH: Infected Interdigital Hyperplasia, IP: Interdigital Phlegmon, SU: Sole Ulcer, TN: Toe Necrosis, WLD: White Line Disease)

Relative abundances of the 15 most prevalent phyla are shown in Figure 3.3 Main phyla for all the samples were Firmicutes followed by Bacteroidetes, Spirochaetes and Fusobacteria were found in increased relative abundances in SU, TN, and WLD lesions compared to their control samples.

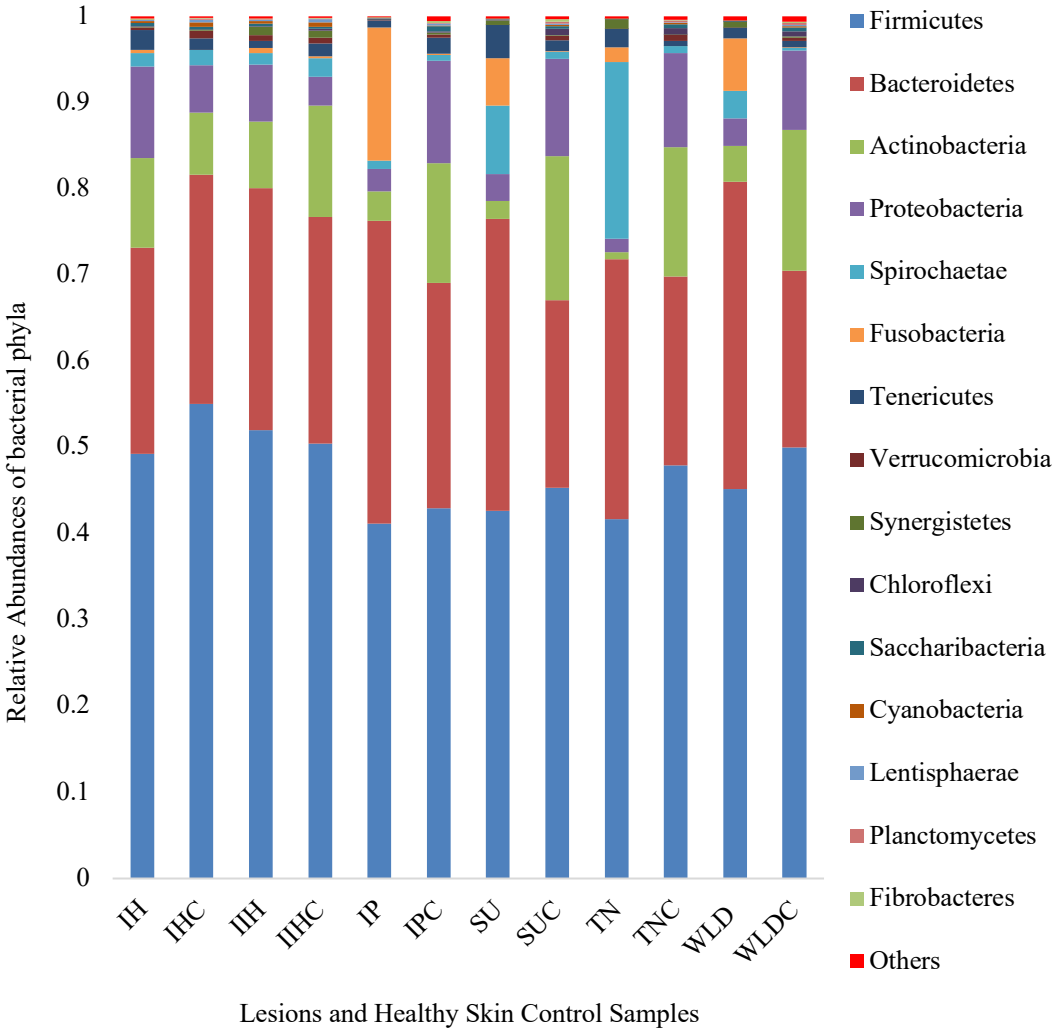

Figure 3.3. Relative abundances of fifteen most prevalent bacterial phyla in lesion and healthy skin control samples. (IH: Interdigital Hyperplasia, IHC: IH Control IIH: Infected Interdigital Hyperplasia, IIHC: IIH Control, IP: Interdigital Phlegmon, IPC: IP Control, SU: Sole Ulcer, SUC: SU Control, TN: Toe Necrosis, TNC: TN Control, WLD: White Line Disease, WLDC: WLD Control)

In Figure 3.4, relative abundances of the 15 most prevalent genera are shown. *Porphyromonas* spp., *Treponema* spp., *Fusobacterium* spp., Clostridiales Family XI, *Fastidiosipila* spp., and other genera of the family Porphyromonadaceae were more prevalent in IP, SU, TN, and WLD lesion samples compared to their healthy skin control samples. On the other hand, the prevalence of *Corynebacterium* spp., *Eubacterium coprostanoligenes* group, Ruminococcaceae, Rikenellaceae RC9 gut

group, family Lachnospiraceae, and Christensenellaceae R-7 group were higher in healthy skin control samples of IP, SU, TN and WLD compared to their lesion samples.

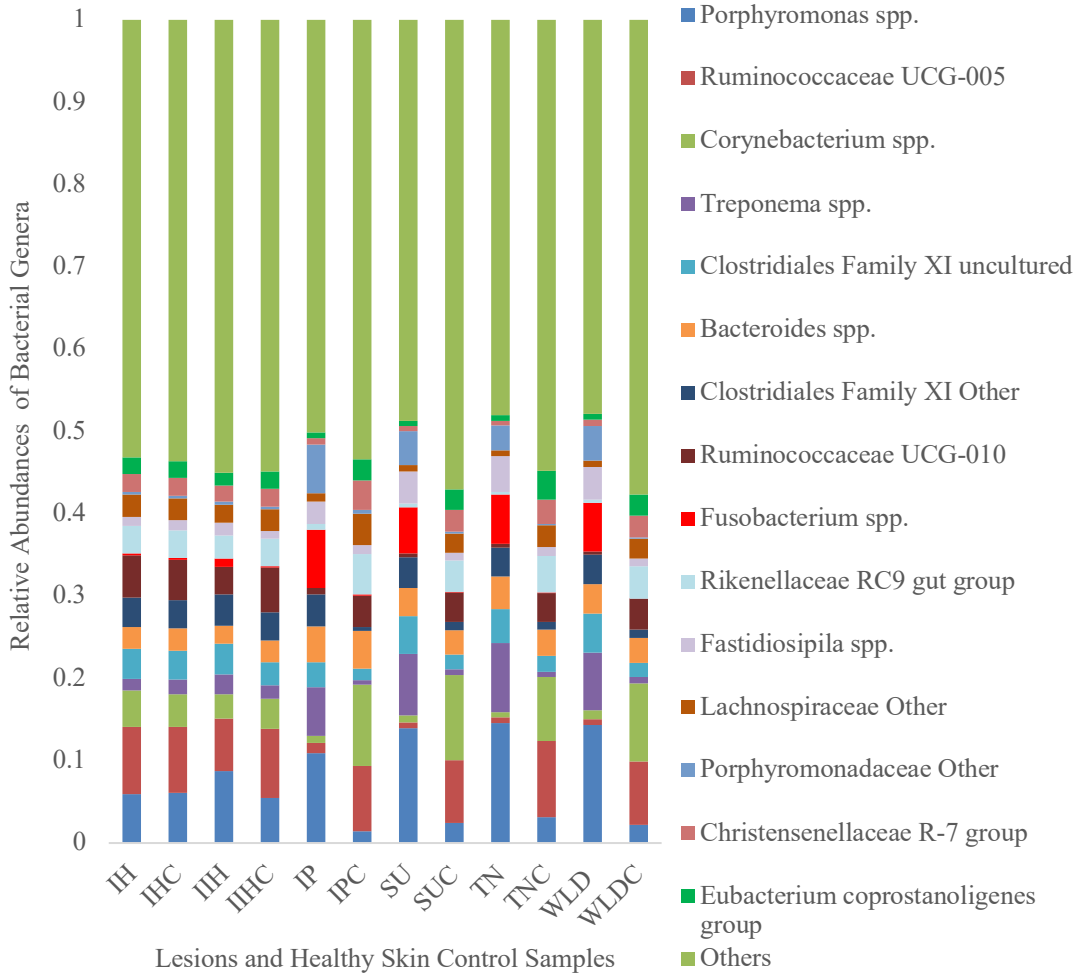

Figure 3.4. Relative abundances of fifteen most prevalent bacterial genera in lesion and healthy skin control samples. (IH: Interdigital Hyperplasia, IHC: IH Control IIH: Infected Interdigital Hyperplasia, IIHC: IIH Control, IP: Interdigital Phlegmon, IPC: IP Control, SU: Sole Ulcer, SUC: SU Control, TN: Toe Necrosis, TNC: TN Control, WLD: White Line Disease, WLDC: WLD Control)

The response screening analysis results provided with a more comprehensive analysis of the differences at genus level between lesion and control samples; these results are charted in bubble plots. *Fusobacterium* spp., *Porphyromonas* spp., *Helcococcus* spp., *Parvimonas* spp., and *Peptostreptococcus* spp. were found to be

significantly more prevalent in IP lesion samples compared to their healthy skin control samples. On the other hand, the prevalence of *Corynebacterium* spp., *Acinetobacter* spp., Christensenellaceae R-7 group, Ruminococcaceae UCG-005, Rikenellaceae RC9 gut group, and other genera of the family Corynebacteriaceae were significantly higher in healthy skin control samples compared to IP lesion samples (Figure 3.5).

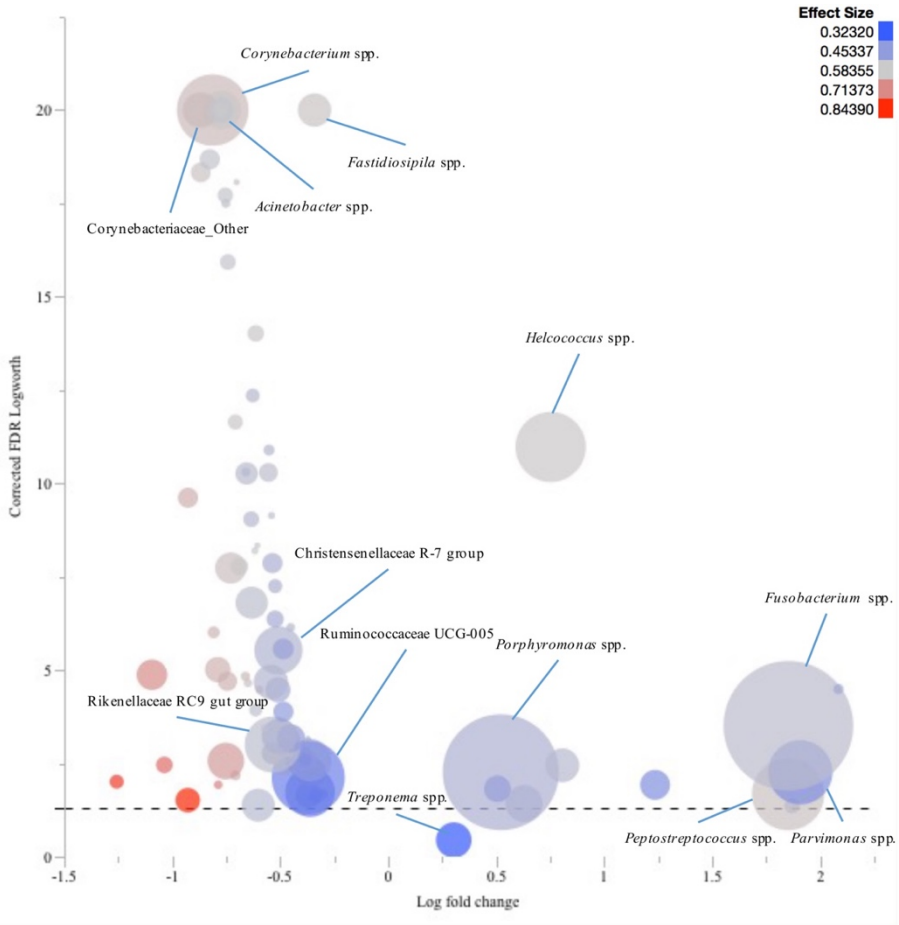

Figure 3.5. Comparison of microbiota profiles from IP samples and their healthy skin control samples. Size of the circle represents the prevalence of each genus, and colour represents the effect size. The graph shows log fold change in 16S rRNA gene abundance in IP lesion relative to their healthy skin control samples versus corrected robust false discovery rate (FDR) logWorth (i.e. log10P). The dashed line shows the P-values (0.05) adjusted for FDR.

In SU lesion samples, *Fusobacterium* spp., *Porphyromonas* spp., *Treponema* spp., *Murdochiella* spp., *Odoribacter* spp., *Peptostreptococcus* spp., *Ezakiella* spp.,

Prevotella spp., and Clostridiales Family XI were significantly more abundant compared to their healthy skin control samples. The prevalence of *Corynebacterium* spp., Ruminococcaceae UCG-005, Christensenellaceae R-7 group, *Eubacterium coprostanoligenes*, and Lachnospiraceae NK3A20 group was significantly higher in the healthy skin samples compared to the SU lesion ones (Figure 3.6).

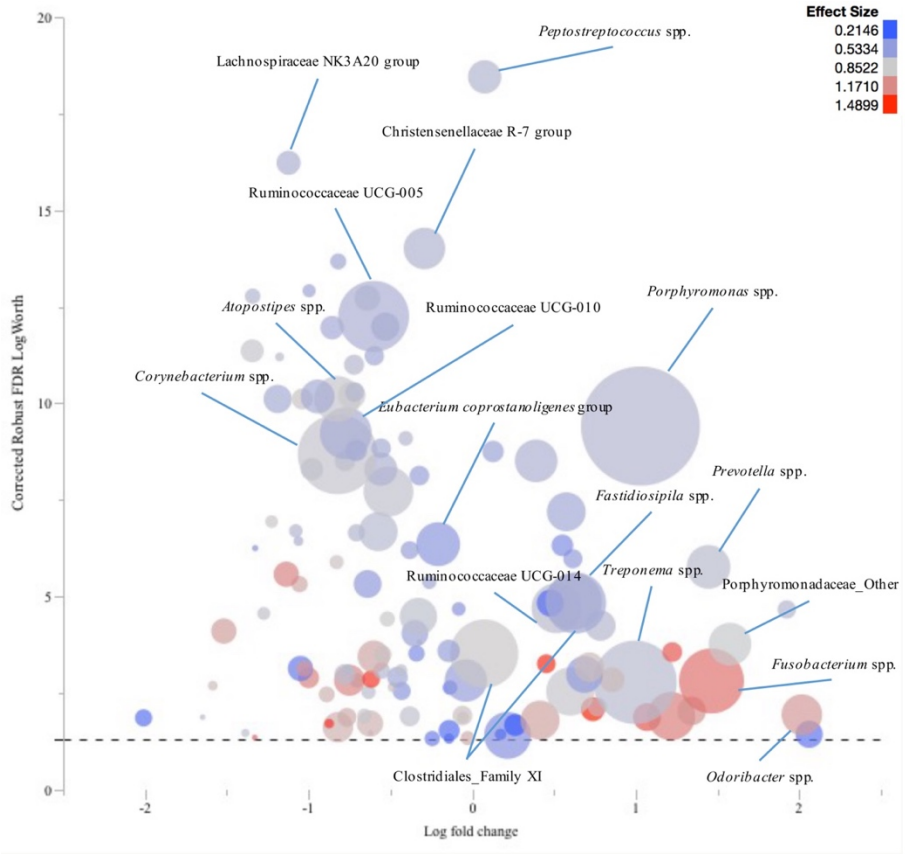

Figure 3.6. Comparison of microbiome profile from SU samples and their healthy skin control samples. Size of the circle represents the prevalence of each genera, and colour represents the effect size. The graph shows log fold change in 16S rRNA gene abundance in SU lesion relative to their healthy skin control samples versus corrected robust false discovery rate (FDR) logWorth (i.e. log10P). The dashed line shows the P-values (0.05) adjusted for FDR.

In TN lesion samples, *Fusobacterium* spp., *Treponema* spp., *Fastidiosipila* spp., *Odoribacter* spp., *Filifactor* spp., Ruminococcaceae UCG-014 and other genera of the family Porphyromonadaceae were significantly more prevalent compared to their healthy skin control samples. *Corynebacterium* spp., *Dietzia* spp., *Facklamia* spp., *Romboutsia* spp., *Atopobium* spp., Ruminococcaceae UCG-005 and other

genera of the family Corynebacteriaceae were more prevalent in the respective healthy skin control samples compared to TN lesion samples (Figure 3.7).

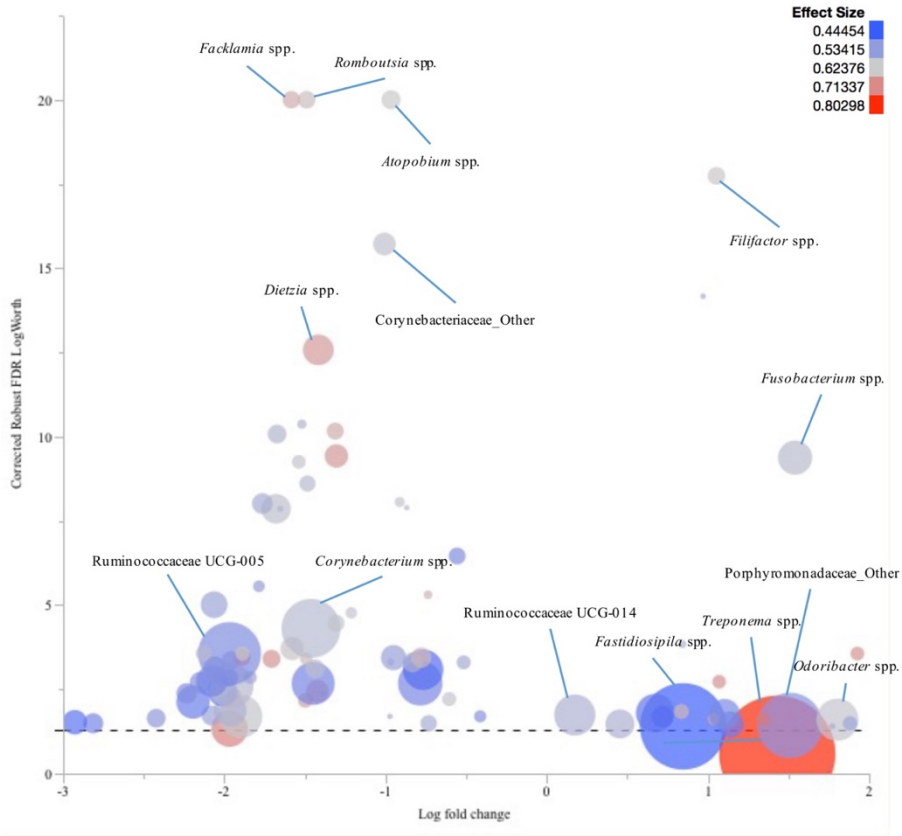

Figure 3.7. Comparison of microbiome profile from TN samples and their healthy skin control samples. Size of the circle represents the prevalence of each genera, and colour represents the effect size. The graph shows log fold change in 16S rRNA gene abundance in TN lesion relative to their healthy skin control samples versus corrected robust false discovery rate (FDR) logWorth (i.e. log10P). The dashed line shows the P-values (0.05) adjusted for FDR.

In WLD lesion samples, *Porphyromonas* spp., *Murdochiella* spp., *Fastidiosipila* spp., Clostridiales Family XI, and other genera of the family Porphyromonadaceae, were significantly more prevalent compared to their healthy skin control samples, while the prevalence of *Corynebacterium* spp., *Atopostipes* spp., Ruminococcaceae UCG-005, Ruminococcaceae UCG-010, Christensenellaceae R-7 group, *Eubacterium coprostanoligenes* group, and Rikenellaceae RC9 gut group

were higher in healthy skin control samples compared to WLD lesion samples. (Figure 3.8).

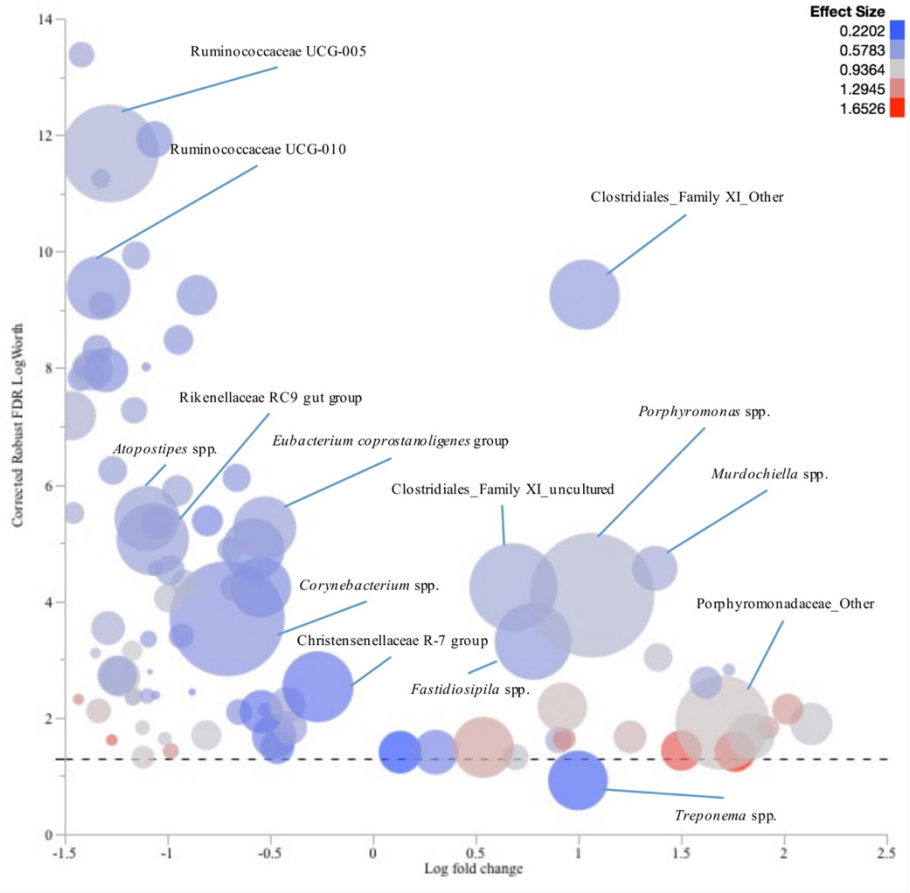

Figure 3.8. Comparison of microbiome profile from WLD samples and their healthy skin control samples. Size of the circle represents the prevalence of each genera, and colour represents the effect size. The graph shows log fold change in 16S rRNA gene abundance in WLD lesion relative to their healthy skin control samples versus corrected robust false discovery rate (FDR) logWorth (i.e. log10P). The dashed line shows the P-values (0.05) adjusted for FDR.

Analysis of weighted UniFrac distances suggested that the microbial communities of complicated CHDL and IP lesions were similar. For this reason, response screening analysis was also performed for all the complicated CHDL and IP lesions together comparing them to all their healthy skin control samples. Several genera showed significant difference between lesions and control samples, therefore only the genera with mean relative abundance higher than 0.01 were charted in order to decrease complexity. *Porphyromonas* spp., *Fusobacterium* spp., *Treponema* spp.,

Clostridiales Family XI, *Fastidiosipila* spp., *Peptoniphilus* spp., Ruminococcaceae UCG-014, uncultured Bacteroidetes bacterium, and other genera of the family Porphyromonadaceae were found to be significantly more prevalent in lesion samples compared to healthy skin control samples. On the other hand, the prevalence of *Corynebacterium* spp., *Atopostipes* spp., *Acinetobacter* spp., Ruminococcaceae UCG-005, Ruminococcaceae UCG-010, Christensenellaceae R-7 group, *Eubacterium coprostanoligenes* group, Rikenellaceae RC9 gut group, and other genera of the family Lachnospiraceae were significantly higher in healthy skin control samples compared to lesion samples (Figure 3.9).

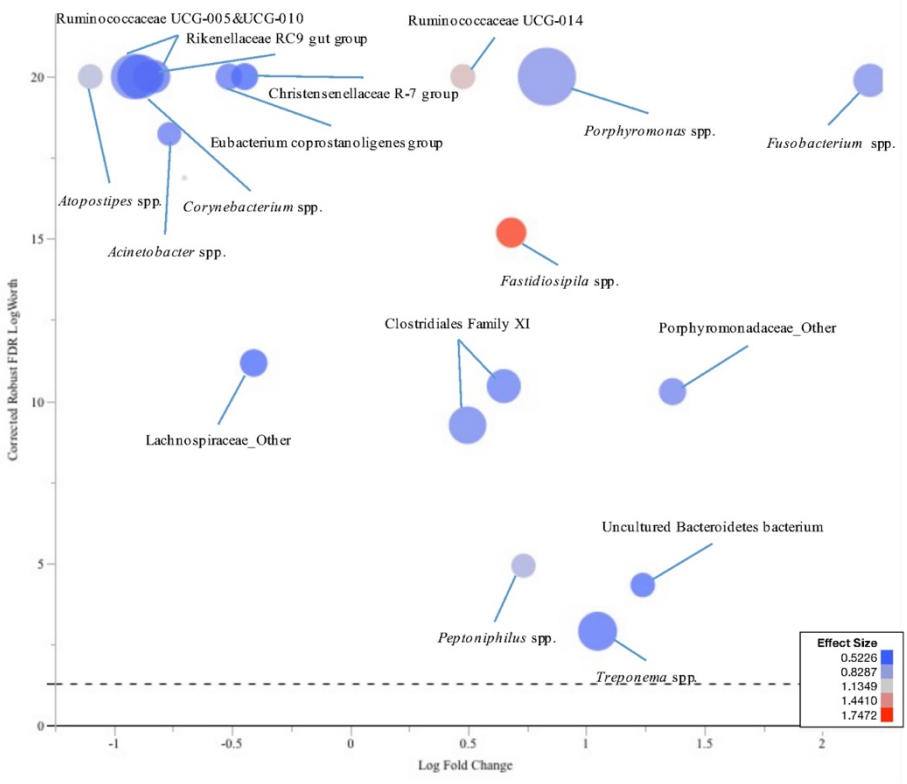

Figure 3.9. Comparison of microbiome profile from IP, SU, TN, and WLD lesion samples and their healthy skin control samples. Size of the circle represents the prevalence of each genera, and colour represents the effect size. The graph shows log fold change in 16S rRNA gene abundance in IP, SU, TN, and WLD lesions relative to their healthy skin control samples versus corrected robust false discovery rate (FDR) logWorth (i.e. log10P). The dashed line shows the P-values (0.05) adjusted for FDR.

In IH lesions, *Erysipelothrix* spp., *Guggenheimella* spp., *Peptococcus* spp.,

Petrimonas spp., Clostridiales Family XI, and other genera of the family Porphyromonadaceae were significantly more prevalent compared to their healthy skin control samples. However, their relative abundance was found to be low. The prevalence of *Alistipes* spp., *Bacteroides* spp., Christensenellaceae R-7 group, *Eubacterium coprostanoligenes*, and Clostridiales Family XIII AD3011 group were significantly higher in healthy skin control samples compared to IH lesion samples. Lastly, *Peptoniphilus* spp. were significantly more prevalent in IIH lesions than their healthy control samples.

3.5 Discussion

The microbiota composition of complicated CHDL, IP, and IH samples was investigated and compared to their healthy skin control samples. 16S rRNA gene sequencing revealed that all lesions are of polymicrobial nature rather than being associated with single taxa. Microbiota profiles of the healthy skin control samples were significantly different from lesion samples (with the exception of IH and IIH samples). In addition, healthy skin samples displayed an increased diversity compared to samples obtained from lesions. It might be caused by dysbiosis which is defined as proliferation of one or a few dominant bacteria and suppressed proliferation of other bacteria (Roberfroid et al. 2010). Recent studies on bovine mastitis have shown similar findings (Ganda et al. 2016). As shown in previous studies, *Treponema* spp. appear to play an important role in the aetiology of some of these lesions (Evans et al. 2011; Sykora et al. 2015); however, other anaerobic bacteria such as *Fusobacterium necrophorum, Fastidiosipila* spp. and *Porphyromonas* spp. were also found to be highly prevalent in most of the studied lesions. Complicated CHDL were previously shown to be associated with DD *Treponema* spp. using species-specific PCR primers (Evans et al. 2011). Here, the use of universal primers allowed the detection of other bacteria that could potentially be associated with the progression of these lesions. The presented results indicate that *Treponema* spp. were statistically significantly more prevalent in complicated SU lesions comparing to their corresponding healthy skin control samples. This was not the case for complicated WLD lesions.

The cross-sectional design of this study only allows us to describe a snapshot of the differences in microbiota profiles and therefore assumptions cannot be made regarding the importance of different taxa at different time points of disease progression. In some cases, a specific pathogen (e.g. *Fusobacterium* spp. in IP) could

have been primarily responsible for the lesion, which was then also colonized by other opportunistic pathogens. CHDLs are known to be of non – infectious aetiology and what is described here is most likely the secondary invasion of the exposed corium by a number of different opportunistic bacteria. Further, larger scale, longitudinal studies could better elucidate these diseases' aetiopathogenesis including the progression of the diseases. A shotgun metagenomics approach could also be employed and would allow for a more in-depth investigation of the studied lesions' microbial communities.

Spirochetes of the genus *Treponema* were previously described as the predominant bacteria in lameness associated DD lesions (Demirkan et al. 1999; Murray et al. 2002; Klitgaard et al. 2008). In this study, they were found to have significantly higher relative abundance in SU and TN samples compared to their healthy skin control samples. *T. medium*, *Treponema* phylotype 18, and other *Treponema* spp. were significantly prevalent in SU and TN lesions. *Treponema* phylotype 18 was shown to be sharing 95% sequence identity with recognized *Treponema* species (*T. putidum* ATCC 700334, *T. pedis* T3552B, and *T. denticola* ATCC 35405) (Klitgaard et al. 2013). TN lesions were also populated by *T. denticola*, and *Treponema* canine oral taxon 233. The detrimental effects of *T. medium*, and *T. denticola* were previously described in human oral lesions (Sela 2001; Siqueira and Rôças 2004), and contagious ovine digital dermatitis (CODD) (Sayers et al. 2009). However, it should be noted that *Treponema* spp. were only significantly prevalent in a small number of the TN and WLD samples; in many of them it was not possible to detect *Treponema* spp. sequences, and this would suggest that they are acting opportunistically in these cases that can also be complicated by other pathogens.

Porphyromonas spp. were formerly isolated from different human and animal infections (Finegold and Jousimies-somer 1997; Walter and Morck 2002). In the present study, all lesions were mainly dominated by *P. levii* which has the ability to synthesize anti-IgG_2 protease, and reduce macrophage activity (Elad et al. 2004). *P. levii* is a well-known opportunistic pathogen and is also commonly found in the vaginal discharge of metritic cows (Machado et al. 2012). Complicated SU, TN, and WLD samples were also harbouring *Odoribacter denticanis,* which belongs to the same family (Porphyromonadaceae) and was also previously associated with DD (Marcatili et al. 2016). *Fusobacterium* spp., another well-known opportunistic

anaerobic pathogen, were previously found to be associated with lameness, particularly with DD and IP lesions (Berg and Loan 1975; Moe et al. 2010). The results of the present study confirm its important role in the development of IP, but also indicated the significant presence of *Fusobacterium necrophorum* in all the other studied lesions. Invasion and colonization of tissues by *Fusobacterium necrophorum* is mediated by its virulence factors such as endotoxin, leukotoxin, and secreted proteases (Kolenbrander et al. 1995; Bennett et al. 2009; Tadepalli et al. 2009). Its role in the aetiopathogenesis of dairy cattle metritis is also well known (Santos et al. 2011; Bicalho et al. 2012).

Several types of bacteria in the Clostridiales order of the Firmicutes phylum were shown to have significant prevalence in both lesions and healthy skin control samples. One of these; *Fastidiosipila* spp. was previously reported in human osteitis (Beauruelle et al. 2014) and also in DD lesions as indicated in the second chapter, and was found here in higher relative abundance in SU, TN, and WLD lesions (compared to healthy skin samples). Therefore, *Fastidiosipila* spp. could have a potentially important role in the aetiology of these lesions. Moreover, Clostridiales Family XI was significantly more abundant in SU, and WLD lesions (comparing to respective control samples); *Helcococcus* spp., and *Parvimonas* spp. from the same family were significantly more abundant in IP lesions. *Murdochiella* spp. were significantly more prevalent in WLD lesions and also belong to the Clostridiales family which is known to be associated with many human and animal diseases (Price et al. 2009; Peng et al. 2013).

Complicated CHDL and IP samples displayed similar microbiota profiles. Therefore, all these lesions were analysed together and compared to all control samples. Similar to individual analysis of these lesions, the prevalence of *Fusobacterium necrophorum*, *Porphyromonas spp.*, *Fastidiosipila* spp., and *Treponema* spp. was significantly higher in lesion samples compared to the prevalence of these bacteria in healthy skin control samples. In addition, the prevalence of *Corynebacterium* spp., *Atopostipes* spp., *Acinetobacter* spp., Ruminococcaceae UCG-005, Ruminococcaceae UCG-010, Christensenellaceae R-7 group, *Eubacterium coprostanoligenes* group, and Rikenellaceae RC9 were significantly higher in healthy skin control samples compared to lesion samples.

Conversely, weighted UniFrac analysis suggested that IH and IIH samples were grouped together with healthy skin control samples. Therefore, these lesions might not be a result of bacterial infection. Alternatively, the causative bacteria in these lesions might be mainly located deep inside the lesions, and thus biopsy samples would have been more appropriate in order to identify them. Viral infections could also be implicated. *Erysipelothrix* spp. observed in these lesions, are known to cause erysipelas in pigs and are associated with acute septicaemia, cutaneous lesions, abortions, or chronic infection causing endocarditis and arthritis (McNeil et al. 2017). Moreover, *Guggenheimella* spp. were previously reported in DD lesions (Schlafer et al. 2008), but for other lesions in this study (IP, TN, WLD samples), *Guggenheimella* spp. were observed in healthy skin control samples. A notable presence of *Porphyromonas* spp., and *Treponema* spp. was also observed in IH and IIH samples, but the difference between lesions and healthy skin control samples was not statistically significant.

Healthy skin control samples were dominated by *Corynebacterium* spp, a well-known bacterial colonizer of the skin (Grice and Segre 2011) that was previously shown to be associated with healthy skin (Smeekens et al. 2014). The well-known gut bacterial family Christensenellaceae (Mancabelli et al. 2017) was also significantly more prevalent in all the healthy skin control samples compared to the lesion samples.

3.6 Conclusion

The aim of this study was to characterize the microbiota profile in the skin of cow feet with complicated CHDLs, IH, and IP. Therefore, the microbiota profiles of six different lameness causing lesions were characterized using 16S rRNA gene amplicon sequencing. Multivariate analysis approaches were used to analyse the data and significant differences between lesions and their control samples were described. The results showed that most of these lesions were associated with a similar range of pathogens, which are most likely acting opportunistically. *Porphyromonas* spp. were more prevalent in IP, SU, and WLD lesions, *Fusobacterium* spp. were more prevalent in IP, and SU lesions, and *Treponema* spp. were more prevalent in SU samples (compared to their respective healthy skin control samples). *Fastidiosipila* spp., a pathogen not previously associated with lameness causing lesions in cattle, showed a noteworthy prevalence in SU, TN, and WLD lesions. These findings could contribute towards future treatment and control strategies for these disorders.

CHAPTER IV

4 Quantitative trait loci mapping for lameness associated phenotypes in Holstein Friesian dairy cattle

4.1 Summary

Lameness represents a significant challenge for the dairy cattle industry, resulting in economic losses and reduced animal health and welfare. The existence of underlying genomic variation for lameness associated traits has the potential to improve selection strategies by using genomic markers. Therefore, the aim of this study was to identify genomic regions and potential candidate genes associated with lameness traits.

Lameness related lesions and digital cushion thickness (DCT) were studied using records collected by our research team, farm records and a combination of both. Genome wide association analyses (GWA) were performed to identify significant genomic effects and a combination of single SNP association analysis and regional heritability mapping (RHM) was used to identify associated genomic regions.

Significant genomic effects were identified for several lameness related traits: Two genomic regions were identified on chromosome 3 associated with digital dermatitis (DD) and interdigital hyperplasia (IH), one genomic region on chromosome 23 associated with IH, and one genomic region on chromosome 2 associated with sole haemorrhage (SH). Candidate genes in those regions are mainly related to immune response and fibroblast proliferation.

Quantitative trait loci (QTL) identified in this study could enlighten the understanding of lameness pathogenesis, providing an opportunity to improve health and welfare in dairy cattle with the addition of these regions into selection programs.

4.2 Introduction

Lameness is a complex trait defined as an abnormal stance or gait of the animal that results from disorders of the locomotor system. In dairy cattle, lameness is one of the most important health conditions together with impaired fertility and mastitis (Green et al. 2002; Buitenhuis et al. 2007; Cha et al. 2010), and causes important economic losses and reduced animal health and welfare (Huxley 2013).

Many lameness cases are associated to various infectious and non-infectious diseases (Green et al. 2002; van der Waaij et al. 2005; Bicalho and Oikonomou 2013) resulting in painful foot lesions such as ulcers, white line lesions, haemorrhages, hyperplasia and others. Previous studies have shown that both animal genetic and management factors contribute to the development of these diseases (Olmos et al. 2009; van der Linde et al. 2010; Swalve et al. 2014). The existence of genetic variation underlying lameness-associated traits has been previously demonstrated using pedigree data analyses, with heritability estimates ranging from 0.06 to 0.52 (Boettcher et al. 1998; Zwald et al. 2004; Koenig et al. 2005; Laursen et al. 2009; Schöpke et al. 2015). Furthermore, susceptibility to non-infectious foot lesions is also associated with morphological hoof traits such as the thickness of the digital cushion, a complex, force dissipating, subcutaneous tissue located under the distal phalanx (Bicalho et al. 2009).

While reducing the incidence of lameness is one of the main objectives for the dairy cattle industry, current means are based on observational scores such as claw health status data, lameness and mobility scores, conformational traits, and data collected by automated sensors (Heringstad et al. 2018). All this information is obtained once the animal has started to show symptoms of lameness, thus are not available early in life and also show relatively low heritabilities (Buitenhuis et al. 2007). Therefore, the identification of genomic regions and genes associated to lameness and lameness associated traits could strongly improve selection strategies, providing genomic information available early in life and potentially informing more accurate genomic-based selection programmes.

Few studies have addressed traits associated with lameness using a genomic approach. The largest study (Malchiodi et al. 2018) grouped lameness-associated lesions into two categories, infectious and non-infectious, and used SNP data to identify several genomic regions with candidate genes linked to immune system,

morphogenesis and cell proliferation. Some studies used microsatellite data (Buitenhuis et al. 2007) to identify lameness-associated regions and SNP data (Scholey et al. 2012; Swalve et al. 2014) to identify regions associated to digital dermatitis (DD) and sole haemorrhage (SH). The relatively high number of identified regions together with the complex aetiology of lameness seems to support a potential polygenic architecture with many genes influencing the different biological factors involved. Therefore, it is necessary to study the different types of lesions separately in order to identify particular and common genomic regions that contribute to the main condition phenotype.

The objective of the present study was to perform genome-wide analyses to identify regions and candidate genes and understand the genetic basis of a wide range of lameness-related traits. This knowledge may inform genetic improvement schemes aiming to reduce prevalence of dairy cattle lameness. Digital cushion thickness (DCT) measurements at different times are studied here for the first time from a genomic perspective.

4.3 Materials and Methods

4.3.1 Animals and phenotypes

Ethical approval for the study was granted by the University of Liverpool Research Ethics Committee. ASPA regulated procedures were conducted under a Home Office Project License (Reference Number: PPL 70/8330).

The study included a total of 554 Holstein-Friesian cows in lactation 0-8 from three different farms and two different record sources: research and farm records. These records were also combined as a different data set. The recorded lameness causing foot lesions were DD, sole ulcer (SU), white line disease (WLD), SH and IH. Cases were defined following the ICAR Claw Health Atlas definitions (Egger-Danner et al. 2015).

Farm phenotypic records for presence (1) or absence (0) of these lesions were extracted from the farm database for all these animals (single record per animal) from May 2006 to October 2017 using TotalVet software (Sum-IT). In addition, 475 cows were individually monitored for the same lesions by a research team led by an experienced veterinarian during three separate time intervals between December 2014 and October 2017. DCT measurements were taken using ultrasonography between December 2014 and January 2016 (1st research interval), and between

October 2016 and August 2017 (2nd research interval). A number of cows were also followed during the period between August 2017 and October 2017 but no DCT measurements were obtained (3rd research interval). Lameness lesions were recorded for 88 animals between December 2014 and January 2016, and for 337 animals between October 2016 and October 2017; 50 cows had records from both these two different time intervals. DCT measurements and recording of lameness causing lesions were performed by the research team at three time points around animals' calving: 3-4 weeks before the expected calving, the first week after calving, and approximately 8 weeks after calving. 79 animals only had lameness lesion records obtained from the farms' records. Research and farm records were analysed both separately and combined. For the latter, animals were considered as affected when at least one of the available records (research or farm) indicated presence of the lesion.

Cows were restrained in a foot trimming crush for the measurement of DCT and the recording of lameness causing foot lesions. Measurement of DCT was performed using an Easi-Scan ultrasound machine (sonographic B-mode, BCFTM Technology, UK) equipped with a linear probe 5-8 MHz. All measurements of DCT were undertaken at the midline, on the lateral claw of the hind left foot. To measure the DCT the foot was cleaned and loose horn was removed with a hoof knife. Sole contact with the transducer was made using ultrasound gel (Ultrasound Gel, Henry Schein) and a gel standoff (Flexi gel standoff, BCFTM Technology, UK). After freezing the image on the ultrasound monitor (Easi-Scan Ultrasound Remote Display, BCFTM Technology, UK), measurements were taken to the nearest millimetre. The DCT was measured just cranial to the tuberculum flexorum of the pedal bone at the typical SU site. The distance from the inner margin of the sole (identified as a thin echogenic line) to the distal edge of the pedal bone (identified as a thick echogenic line) was assessed.

4.3.2 DNA sampling, extraction and genotyping

Blood samples were collected from the tail vein of each cow in vacutainer tubes containing EDTA. Genomic DNA was extracted from buffy coat samples using QIAamp DNA Blood MiniKit from Qiagen. Extracted DNA samples were quantified using a NanoDrop and stored at -20°C. Initially, 266 cows were genotyped using the Affymetrix Axiome bovine 54K SNP array. The Illumina BovineSNP50 bead chip containing 53,714 SNP was used to genotype the rest of the animals. Genotype data

obtained from the Affymetrix array were converted to the Illumina chip format before further analysed.

4.3.3 Sample and genotype quality control

Quality control was performed using PLINK (Purcell et al. 2007) in order to assess both sample and marker quality. A genotype call rate threshold of 95% was applied, removing SNPs with low genotyping quality. Further quality control on the markers removed those with low minor allele frequency (MAF<0.01) and showing strong deviations from Hardy-Weinberg equilibrium (threshold of 1.45E-6 Bonferroni corrected). Additional quality control of samples was performed by removing individuals with poor genotype quality (sample call rate lower than 95%). All these quality control procedures resulted in a final dataset of 549 animals genotyped for 34,658 SNPs with positions assigned according to the UMD 3.1 assembly.

4.3.4 Population structure

Principal component analyses of the genotyped animals showed a relatively light population structure not explained by a single descriptive factor (e.g.: farm, parity number, lactation, etc). Therefore, the genomic relationship matrix among animals was fitted in all ensuing statistical models of analysis as a random polygenic effect to account for any potential inflation effects caused by population structure.

4.3.5 Estimation of variance components

Estimates of the variance components for each trait were obtained by fitting the following model using REACTA (Cebamanos et al. 2014):

$$\mathbf{y}=\mathbf{W}\boldsymbol{\alpha}+\mathbf{Z}\mathbf{u}+\boldsymbol{\varepsilon} \qquad [1]$$

where **y** represents the vector of phenotypes, **W** is an incidence matrix, α is the vector of associated fixed effects, **Z** is the design matrix for the vector **u** of random polygenic effects (distributed as a multivariate normal distribution MVN$(0,V_g\mathbf{G})$ with **G** being the genomic relationship matrix (GRM) and V_g the genetic variance of the trait), and **ε** represents the vector of residual errors (distributed as MVN$(0,V_e\mathbf{I})$ with **I** being the identity matrix and V_e the residual variance). The significance of the genomic (polygenic) effect ($P = 0.05$) was assessed using the likelihood ratio test statistic to compare a model that fits the effect against the base model that excludes it.

Fixed effects used in model [1] were tested previously using Wald tests in ASReml 4 (Gilmour et al. 2009), fitting a logit model for disease traits and a linear model for DCT records, and following a backward elimination approach. After performing analyses for all traits, concordant models were chosen incorporating as fixed effects: i) for the disease research records: farm (3 levels), parity number at recording (3 levels, 1, 2 and ≥3) and research interval (5 levels, grouped as 1= interval 1, 2= interval 2, 3= interval 3, 4= intervals 1 and 2, 5= intervals 1 and 3); ii) for the farm and combined disease records: farm (as before), lactation number at the end of study (4 levels 0, 1, 2 and >3), and interval (as before); and iii) for the DCT records: farm (as before), parity number (as before), and assessor (6 levels).

4.3.6 Genome-wide association analysis (GWA)

Individual SNP association analyses were performed in those traits with a significant genomic effect from model [1] using GEMMA (Zhou and Stephens 2012). The linear mixed model was:

$$\mathbf{y}=\mathbf{W}\boldsymbol{\alpha} + \mathbf{x}\beta + \mathbf{Z}\mathbf{u} + \boldsymbol{\varepsilon} \qquad [2]$$

where $\boldsymbol{x}$ represents the vector of genotypes (coded as 0/1/2) and β is the regression coefficient of the phenotype on the genotypes; all other effects are as described in model [1]. The statistical significance of the regression coefficient was assessed using a Wald test. When determining the significant thresholds, a Bonferroni correction was performed for multiple testing due to the number of markers, but not for multiple traits. This resulted in a genome-wide significant threshold ($P = 0.05$) defined at $P = 1.44\text{E}{-6}$ ($-\log10(P) = 5.84$) and a suggestive threshold (one false positive per genome scan) defined at $P = 2.89\text{E-}5$ ($-\log10\,(P) = 4.54$).

Despite including the polygenic effect in the model, genotyping errors or other artefacts such as cryptic population structure may inflate test statistics. Therefore, to account for any potential remaining inflation, the ratio of the median of the empirically observed distribution of the test statistic to the expected median (inflation factor λ) was used for correction, following the method described by Amin *et al*. (Amin et al. 2007), which assumes that the inflation is constant across the genome.

4.3.7 Regional Heritability Mapping (RHM)

Under the RHM approach, the genome was divided into non-overlapping windows of 20 consecutive SNPs. The following model was used in REACTA (Cebamanos et al. 2014):

$$\mathbf{y}=\mathbf{W}\boldsymbol{\alpha}+\mathbf{X}\mathbf{u}_{(i)}+\mathbf{Z}\mathbf{u}_{(-i)}+\boldsymbol{\varepsilon} \qquad [3]$$

where $\mathbf{X}$ and $\mathbf{Z}$ are the corresponding design matrices for the effects $\mathbf{u}_{(i)}$ of the corresponding region *i* (distributed as $MVN(0,V_{g(i)}\mathbf{G}_{(i)})$ with $V_{g(i)}$ and $\mathbf{G}_{(i)}$ being the genomic variance and the GRM corresponding to the SNPs in the i^{th} region, respectively) and $\mathbf{u}_{(-i)}$ of the genome (polygenic effect) excluding the region *i* (distributed as $MVN(0,V_{g(-i)}\mathbf{G}_{(-i)})$ with $V_{g(-i)}$ and $\mathbf{G}_{(-i)}$ being the genetic variance and the GRM corresponding to all SNPs other than those on the region *i*, respectively).

The significance of the region effect was assessed using the likelihood ratio test statistic. A total of 1733 regions were analysed, leading to a genome-wide significant threshold ($P = 0.05$) defined at $P = 2.89E{-}5$ with Bonferroni correction for multiple regions ($-\log10(P) = 4.54$) and a suggestive threshold (one false positive per genome scan) defined at $P = 5.77E\text{-}4$ ($-\log10(P) = 3.24$). As with the GWA analyses, a correction by the inflation factor λ was applied to account for any remaining inflation after fitting the polygenic effect in the model.

4.4 Results

4.4.1 Population structure

Figure 4.1A shows the eigenvalues corresponding to the principal component analysis performed on the GRM of the genotyped animals. The first seven principal components accounted for about 10% of the total variance, with the first three components explaining 2.19%, 1.83% and 1.50%, respectively. Figure 4.1B shows a light population structure mainly due to the first principal component. No population attributes were available that could explain the light population structure present. Therefore, a polygenic effect (GRM) was fitted to account for this light population structure.

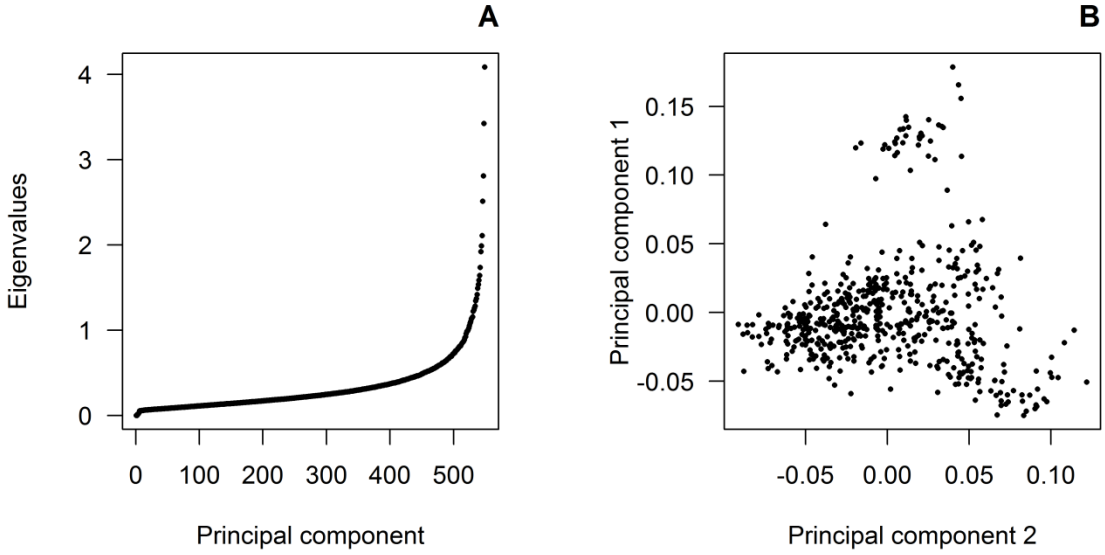

Figure 4.1: Principal component analyses: Figure 1A shows the eigenvalues corresponding to the decomposition of the genomic relationship matrix for each of the principal components. Figure 1B shows the population structure according to the first and second principal components.

4.4.2 Full genomic variance analysis

Table 4.1 shows the variance component estimates for those traits with a significant genomic effect (P<0.05) based on the likelihood ratio test. Heritabilities for the disease traits are provided in the observed scale (0 or 1), ranging from 0.129 to 0.516 and corresponding to genomic variances in the range of 0.008 to 0.067. Heritability for DCT at calving was moderate (0.228), corresponding to a genetic variance of 0.549.

Table 4.1: Estimates of heritability and variance components for traits with a significant (P<0.05) genomic effect

	Trait	h^2	Vg	Ve	*P*	N
DCT records	**DCT_fresh**	0.23 ± 0.12	0.549 ± 0.298	1.854 ± 0.290	6.69E-3	360
Research records	**DD**	0.19 ± 0.09	0.043 ± 0.022	0.189 ± 0.023	5.55E-3	469
	IH	0.52 ± 0.11	0.067 ± 0.016	0.063 ± 0.012	1.05E-8	469
	SH	0.20 ± 0.10	0.035 ± 0.017	0.135 ± 0.017	1.25E-2	469
Farm records	**DD**	0.20 ± 0.09	0.023 ± 0.011	0.094 ± 0.011	5.89E-3	549
	SU	0.29 ± 0.09	0.037 ± 0.013	0.092 ± 0.011	6.29E-5	549
	IH	0.13 ± 0.08	0.008 ± 0.005	0.054 ± 0.005	1.85E-2	549
Combined records	**DD**	0.20 ± 0.08	0.046 ± 0.019	0.183 ± 0.019	2.56E-4	549
	SU	0.35 ± 0.10	0.057 ± 0.017	0.106 ± 0.015	8.24E-6	549
	WLD	0.13 ± 0.08	0.020 ± 0.013	0.135 ± 0.014	2.83E-2	549
	IH	0.37 ± 0.09	0.047 ± 0.013	0.081 ± 0.011	4.42E-8	549
	SH	0.14 ± 0.08	0.024 ± 0.014	0.142 ± 0.015	3.19E-2	549

Genomic heritabilities (h^2), genomic (*Vg*) and residual variances (*Ve*) estimated together with their standard errors. P-values (*P*) for the significance of the genomic effect and the number of total records (*N*). Digital cushion thickness at calving (DCT_fresh), digital dermatitis (DD), interdigital hyperplasia (IH), sole haemorrhage (SH), sole ulcer (SU) and white line disease (WLD).

4.4.3 Genome-wide association analysis (GWA)

Two genome-wide significant SNPs and 19 genome-wide suggestive SNPs were detected in the GWA analyses (Table 4.2). After performing the correction by the inflation factor, all λ estimates ranged from 1.002 to 1.033, thus implying the absence of any significant inflation in the test estimates.

Minor allele frequencies ranged from 0.020 to 0.479 and most substitution effects were positive (with the exception of WLD in the combined records), thus implying a positive effect of the minor allele against the disease. However, due to the Beavis effect (Xu 2003), the provided effect sizes are expected to be slightly overestimated.

DCT at calving and SU in the combined records did not provide any significant or suggestive SNP despite showing a significant genomic effect in the previous analyses.

Table 4.2: Significant SNP from the genome-wide association analyses

	Trait	BTA	Position (BP)	Beta coef.	MAF	P
Research records	**DD**	3	70931186	0.380 ± 0.086	0.038	1.23E-5
		20	30216498	0.166 ± 0.037	0.276	7.74E-6
	IH	11	99952182	0.180 ± 0.042	0.098	2.54E-5
	SH	2	4958110	0.170 ± 0.038	0.171	1.00E-5
Farm records	**DD**	3	70931186	0.275 ± 0.056	0.038	1.32E-6
		7	28258117	0.322 ± 0.074	0.020	1.97E-5
		19	10140328	0.264 ± 0.062	0.027	2.91E-5
		24	37354445	0.137 ± 0.031	0.147	1.32E-5
	SU	12	12612422	0.185 ± 0.043	0.084	2.82E-5
	IH	23	44153826	0.266 ± 0.048	0.027	4.83E-8
Combined records	**DD**	3	70931186	0.383 ± 0.080	0.038	2.17E-6
		3	90367814	0.224 ± 0.047	0.123	2.22E-6
		3	90523019	0.239 ± 0.053	0.096	7.42E-6
		20	30216498	0.165 ± 0.035	0.270	2.30E-6
	WLD	5	94496854	-0.106 ± 0.025	0.479	2.31E-5
		7	75190535	-0.122 ± 0.027	0.339	4.99E-6
		14	5883219	-0.148 ± 0.034	0.152	1.60E-5
	IH	2	23628756	0.157 ± 0.037	0.106	2.07E-5
		3	23764339	0.160 ± 0.037	0.119	1.61E-5
	SH	2	4958110	0.152 ± 0.035	0.165	1.93E-5
		21	46018333	0.262 ± 0.057	0.052	4.94E-6

Chromosome (BTA) and base pair position follow the UMD 3.1 assembly. Beta coefficient (minor allele substitution effect) and standard error, minor allele frequency (*MAF*) and P-value (*P*) for the beta coefficient. Digital dermatitis (DD), interdigital hyperplasia (IH), sole haemorrhage (SH), sole ulcer (SU) and white line disease (WLD).

4.4.4 Regional Heritability Mapping (RHM) and concordant regions

A significant region for IH was detected based on farm records, and 10 suggestive regions were detected for other traits using the RHM approach (Table 4.3). The significant region detected on chromosome 3 for DD using research records was also detected as suggestive for IH using the combined records.

Four of the detected regions were concordant with suggestive/significant SNPs detected in the GWA analyses (Table 4.3 and Appendix Figure C.1), two of them detected from the farm records and another two from the combined records. In the farm records, the concordant regions detected both by RHM and GWA explained 32.90% and 43.90% of the total genomic variance of DD and IH, respectively. In the combined records, the concordant regions explained 10.53% and 11.29% of the total genomic variance of IH and SH, respectively. Caution must be exercised while assessing these findings because of possible overestimation due to the Beavis effect which is inflation in the estimated effects caused by small sample size (Xu 2003).

Table 4.3: Significant genomic regions from the regional heritability mapping analyses

	Trait	BTA	Position (BP)	P
Research records	**DD**	22	34216267-36047120	4.14E-4
		22	31087678-32538832	4.36E-4
Farm records	**DD**	3	70077512-71882823	3.40E-4
		25	34887253-35853810	2.61E-4
	SU	25	3126438-4354023	8.03E-5
	IH	23	43151282-44458259	2.28E-6
Combined records	**SU**	25	3126438-4354023	1.86E-4
	WLD	14	6850767-7718808	5.43E-4
	IH	3	22069239-23764339	1.35E-4
		3	70077512-71882823	5.94E-5
	SH	2	4587203-5640288	3.35E-4

Chromosome (BTA) and base pair position follow the UMD 3.1 assembly. P-value (*P*) for the region effect. Digital dermatitis (DD), interdigital hyperplasia (IH), sole haemorrhage (SH), sole ulcer (SU) and white line disease (WLD).

4.5 Discussion

In the present study, two genome-wide association approaches were used to identify QTL affecting lameness related traits. Comparison of the results provided by individual SNP association analyses (GWA) and RHM was performed to strengthen the evidence of the identified regions. QTLs were detected for DD, IH and SH.

Three types of records (research, farm, and combined) were used in this study in order to identify genomic regions for lameness related diseases. Although using only research-confirmed records is expected to provide more accurate phenotypes than using farm records, the number of observations available was smaller, thus reducing the power to detect significant genomic effects. Similarly, using farm records provided a larger number of records spanning animals' whole lifetime; these records however are potentially less accurate, thus leading to a low detection power and increase the chances of introducing misclassification bias. It has been shown previously that farm records could seriously under-record certain lesions (Heringstad et al. 2018) and this has also been the case with the present dataset. The most convenient dataset available in the present study was the combination of farm and research records, which provided a larger number of records than research alone but more accurate compared to farm, thus leading to significant estimates of genomic effects for more traits.

Heritabilities in the observed scale for some of these traits have been previously estimated using pedigree data (Koenig et al. 2005; van der Waaij et al. 2005; Gernand et al. 2012; Oberbauer et al. 2013; van der Spek et al. 2013; Malchiodi et al. 2017). Such estimates range from 0.07 to 0.4 for DD, 0.10 to 0.39 for IH and 0.04 to 0.17 for SH. The heritability estimates in this study were generally in concordance within these ranges, particularly considering the estimates obtained using the combined records. It has to be recognised that heritability estimates are presented in the observed scale (0-1) and, therefore, population parameters are dependent on the disease prevalence (Lee et al. 2011). However, it is expected that estimates will not vary widely when transformed to the liability scale (normal distribution). In the case of DD, the heritability observed for the combined records was 0.20, resulting in heritabilities between 0.21 to 0.30 on the liability scale when assuming a disease prevalence from 10% to 30% (Holzhauer et al. 2006). Similarly, the heritability observed for IH was 0.37, resulting in a heritability of 0.39 on the liability scale when assuming a prevalence of 1.3% (Solano et al. 2015).

Although GWA and RHM revealed several QTLs independently, 4 QTLs were commonly reported by both approaches (Tables 4.2 and 4.3 and Appendix Figure C.1). On chromosome 3, a suggestive region was associated with DD in farm records, explaining 32.90% of the total genomic variance and being also suggestive of IH in the combined records. Two potential gene candidates are contained within this region: i) *FPGT* (fucose-1-phosphate guanylyl transferase) part of the L-fucose pathway, a key sugar in complex carbohydrates involved in cell-to-cell recognition, inflammation and immune processes (Becker and Lowe 2003); and ii) *TNNI3K* (serine/threonine-protein kinase TNNI3K), also associated with inflammation mechanisms (Wiltshire et al. 2011). Based on this function, it was surmised that a candidate gene for lameness resistance may be found within this QTL.

On chromosome 23, a significant region was associated with IH in farm records, explaining 43.90% of the total genomic variance. IH, also known as interdigital fibroma (Atkinson 2013), results in a thickening of interdigital connective tissue causing fibroid tumours. Thus, a potential candidate gene found within this region is *EDN1* (endothelin-1), a vasoconstrictor associated with several cardiovascular diseases and inflammatory and fibrotic processes (Matsushima et al. 2004), acting as fibroblast mitogen in systemic sclerosis (Vancheeswaran et al. 1994), pulmonary fibrosis (Hocher et al. 2000) and hepatic fibrosis (Rockey and Chung 1996).

On chromosome 3, another suggestive region was associated with IH using combined records, explaining 10.53% of the total genomic variance. This region includes several potential candidate genes, particularly *PHGDH* (D-3-phosphoglycerate dehydrogenase), an oxidoreductase that has been associated previously with pulmonary fibrosis (Hamanaka et al. 2017).

On chromosome 2, a suggestive region was associated with SH from the combined records, explaining 11.29% of the total genomic variance. With SH being related to impaired vascular system and cellular inflammatory reactions (Ossent and Lischer 1998), a potential candidate gene within this region is *GPR17* (uracil nucleotide/cysteinyl leukotriene receptor). This gene is as a sensor molecule involved in traumatic, vascular and inflammatory pathologies in the central nervous system (Boda et al. 2011), and is also related to vascular permeability and

inflammatory processes as a regulator of the cysteinyl leukotriene 1 receptor response (Maekawa et al. 2009).

Most candidate genes within the detected genomic regions are related either to inflammatory processes or fibroblast proliferation, as expected due to the nature of the analysed traits. However, these are not independent processes, but linked networks where fibroblasts present complex biosynthetic pathways, playing a role in pathogenesis and mediating inflammatory processes through their proliferation (Smith 2005). Thus, the analysed traits are expected to present a complex genomic architecture with several genes and pathways involved in their phenotypic expression. This is concordant with the overestimates observed for the SNP and regional effects due to the Beavis effect (Xu 2003) as well as with the lack of consistency across QTLs detected in several studies (Buitenhuis et al. 2007; Scholey et al. 2012; Swalve et al. 2014; Malchiodi et al. 2018). Therefore, it is expected that increasing the sample size and using accurate records will increase the number of identified regions, providing also more accurate estimates of their effects.

DCT is a novel trait analysed using genomic data for the first time in the present study. Previous studies have shown an association between lameness-related diseases such as SU and WLD with a thinner digital cushion, indicating also a potential change in the tissue composition of the cushion (Bicalho et al. 2009). In the present study, given the relatively small number of samples available, no significant or suggestive markers were identified. However, a significant genomic effect was detected for DCT at calving, providing a moderate genomic heritability of 0.23 ± 0.12. When compared with the heritability of 0.33 obtained in a previous pedigree-based study (Oikonomou et al. 2014b), the estimate in this study was smaller but within the standard error boundaries.

As with the lameness-associated lesions, the genomic architecture of DCT traits is expected to be polygenic, being particularly related with body fatty acid and lipid metabolism. Further studies with an increased sample size will refine the heritability estimates and provide some potential candidate genes associated with this structure.

4.6 Conclusion

The aim of this study was to investigate the bovine genomic regions that are linked to lameness associated traits. Four genomic regions were identified for DD,

IH and SH, harbouring genes involved in inflammatory and fibroblastic processes. These traits are moderately heritable and potentially associated with a polygenic architecture. Therefore, the identification of associated regions may be useful to inform genomic selection programmes against lameness and to increase our knowledge of the underlying pathology.

In addition, addressing DCT is a novelty of this study from a genomic perspective, showing a moderate genomic heritability for this structure during the period of calving. The genomic architecture of this trait warrants further research attention.

CHAPTER V

5 Host Genotype – Foot Skin Microbiome Associations

5.1 Summary

All higher organisms including humans are populated by microbes. The microbiome in specific body parts can affect the phenotype of the host, and host genetics can shape the microbiome content. Digital dermatitis (DD) has a polymicrobial nature and has been shown to be associated with the host's genetic background. The aim of this study was to investigate for the first time the possible links between bacteria associated with DD and the bovine genome.

Foot skin microbiota profiles of 242 cows in 3 different farms were characterized and these cows were also genotyped with a 50K SNP chip. Genome wide association analyses (GWA) and regional heritability mapping (RHM) were performed to identify genomic regions associated with alpha diversity indexes and relative abundances of DD associated genera.

Two genomic regions on chromosome 1, one genomic region on chromosome 16 and one genomic region on chromosome 17 were found to be significantly associated with the relative abundance of *Treponema* spp., and one genomic region on chromosome 19 with the relative abundance of *Peptoclostridium* spp. Candidate genes in those regions are mainly related to lipid metabolism, immune response and skin disorders.

Genomic regions identified in this study could elucidate the associations between foot microbiome and host genome regarding DD. Thus, it could provide a prospect to improve animal health and welfare with the addition of these genomic regions into breeding programs.

5.2 Introduction

All higher eukaryotes are inhabited by diverse microbial communities which provide a great contribution to the diversity of life by contributing to the phenotypes of their hosts (Bosch and McFall-Ngai 2011; Ross et al. 2013). The human body is resided by at least 10 times more bacteria than human cells (Sender et al. 2016) with 100 times more microbial genes than human genes (Ezenwa et al. 2012). The human gut microbiota plays important roles in human metabolism, such as harvesting the foods' energetic values (Cummings and Macfarlane 1997; Turnbaugh et al. 2006), the synthesis of amino acids and vitamins (Gill et al. 2006), the metabolism of xenobiotics and other metabolic phenotypes (Sidhu et al. 2001; Martin et al. 2007), maturation and activity of innate and adaptive immune systems (Mazmanian et al. 2005). The initial colonization of new-born infants' gastrointestinal tract was shown to be started with selected transmission from maternal gut microbiota (Korpela et al. 2018). After that, early colonizers can create a favourable environment for themselves and latter colonizers, and prevent the growth of other bacteria by altering the gene expression of the host (Hooper et al. 2001). The colonization pattern is affected by the type of birth, diet, and environmental conditions (Knight et al. 2003).

Different body parts of each individual harbour certain microbial communities which may be more similar for family members compared to unrelated individuals (Eckburg et al. 2006; Turnbaugh et al. 2008). This can be explained as the result of shared environment and also the result of similar host genetics in related individuals. However, the relationship between host genome and microbiota profiles is still uncertain (Spor et al. 2011).

Benson et al. (2010) investigated the interaction between the host genotype and the gut microbiota composition in mice using an inter-cross murine line (n=645) and 530 characterized SNP markers. They identified 18 host QTL associated with relative abundances of some specific microbial taxa indicating the co-segregation of host genome and microbiota as quantitative traits (Benson et al. 2010). In addition, another study revealed the interaction between host genome and microbiome using a BXD mouse line, which is well characterized at molecular and phenotypic level, and faecal bacteria samples of 30 BXD strains. Immunity-related QTL regions on chromosome 4, chromosome 15, and chromosome 12 were shown to be associated with *Bacteroides* spp., Rikenellaceae population, and Prevotellaceae population, respectively (McKnite et al. 2012).

In a genome-wide association study in human, Goodrich et al. (2014) investigated the effect of host genome on gut microbiome using faecal samples of 977 individuals from the TwinsUK population. They revealed that monozygotic twins have more similar microbiota than dizygotic twins. They also showed significant associations between the family Christensenellaceae and low BMI (Goodrich et al. 2014). In another genome-wide study, Davenport et al. (2015) investigated the genomic associations between an isolated community, Hutterites, and faecal microbiome samples from 93, 91, and 57 individuals collected in winter, summer, and in both seasons, respectively. They found at least 8 taxa associated with at least one SNP in the host genome in each season. Specifically, relative abundance of *Akkermansia* spp. was shown to be associated with 5 SNPs near *PLD1* gene on chromosome 3 which was previously associated with BMI (Davenport et al. 2015) In addition, Blekhman et al. (2015) used Human Microbiome Project (HMP) data which includes catalogued microbial taxa in more than ten body sites and the human genome data from contaminant reads of 93 individuals. They found out SNPs on the immune-related genes associated with relative abundances of *Selenomonas* spp. and *Lautropia* spp. in the throat and tongue dorsum, respectively. They also showed an association between the SNPs on *LCT* gene, that produce lactase, and relative abundance of lactose metabolizing *Bifidobacterium* spp. in the GI tract (Blekhman et al. 2015).

Similar to the gut, the skin also harbours complex bacterial communities (Grice et al. 2008) that vary between individuals that suggest the contribution of host genetics to this variation (Belheouane et al. 2017). Srinivas et al. (2013) investigated the contribution of host genetics on the skin microbiota using a fourth generation of advanced intercross mouse line with 1199 informative SNPs. They demonstrated 3 significant and 6 suggestive QTLs associated with 9 OTUs. However, using a relatively low number of SNPs limited the detailed characterization of the individual genes associated with the skin microbiota profile (Srinivas et al. 2013). Furthermore, using the 15th generation of the same mouse line and increasing the SNP number to 53203, Belheouane et al. (2017) investigated the effect of host genetics on skin microbiota. They described 21 significant SNP-skin microbiota associations. Many genes related to skin inflammation and cancer were identified in this study (Belheouane et al. 2017).

Digital dermatitis (DD) lesions have been shown to have polymicrobial characteristics (Cruz et al. 2005; Zinicola et al. 2015a). However, the association of the foot skin microbiota and DD related bacteria with the host genomic variation remains unclear. Therefore, it might be useful to investigate the associations between host genome and DD related bacteria to better understand the aetiopathogenesis of DD.

The aim of the present study was to perform genome-wide association analyses to identify QTLs in the bovine genome associated with presence of DD-related bacteria in the animal's foot.

5.3 Materials and Methods

5.3.1 Ethical Statement and Data Collection

Ethical approval for the study was granted by the University of Liverpool Research Ethics Committee. ASPA regulated procedures were conducted under a Home Office Project License (Reference Number: PPL 70/8330). Foot microbiome profile data of 242 Holstein Friesian cows in the second chapter and genome data of the corresponding cows in the fourth chapter were used for the analyses.

5.3.2 Traits used in the analyses

The phenotypic traits (10 traits) analysed with the genomic association models were three different alpha diversity indexes; Chao1, Shannon, Simpson indexes, and relative abundances of seven genera; *Porphyromonas* spp., Clostridiales Family XI, *Fastidiosipila* spp., *Peptoclostridium* spp., *Macrococcus* spp., *Treponema* spp., and other genera of the family Bacteroidetes for each sample. The relative abundances of the first four species were significantly higher in HtIn animals, which were healthy at initial check point but developed DD lesions during the study, compared to the HtHt animals, which did not have DD throughout the study. *Macrococcus* spp. were significantly more prevalent in HtHt animals compared to HtIn animals. *Treponema* spp. were significantly more prevalent in InIn samples compared to HtHt samples and are known to be highly associated with DD aetiopathogenesis.

5.3.3 Quality control and model assessment

Quality control (QC) of animal genotypes was performed as explained in the fourth chapter, resulting in 549 animals with genotypes including 34,658 SNPs.

Briefly, a genotype call rate threshold of 95%, minor allele frequency (MAF<0.01), Hardy-Weinberg equilibrium (threshold of 1.45E-6 Bonferroni corrected), sample call rate threshold of 95% were applied. The population size for this specific study was 236 out of 242 cows with foot microbiota records, because 6 cows were lost in QC steps.

Wald tests using ASReml software package (Gilmour et al. 2009) were used to determine statistically significant (*P*-value= 0.05) fixed effects to be included in the analysis as explained in the fourth chapter [1]. After performing this analysis for all foot microbiota phenotypic traits, concordant models were chosen including farm, parity and season as fixed effects.

$$\mathbf{y}=\mathbf{W}\boldsymbol{\alpha}+\mathbf{Z}\mathbf{u}+\boldsymbol{\varepsilon} \qquad [1]$$

where **y** represents the vector of phenotypes, **W** is an incidence matrix, $\boldsymbol{\alpha}$ is the vector of associated fixed effects, **Z** is the design matrix for the vector **u** of random polygenic effects (distributed as a multivariate normal distribution MVN$(0,V_g\mathbf{G})$ with **G** being the genomic relationship matrix (GRM) and V_g the genetic variance of the trait), and $\boldsymbol{\varepsilon}$ represents the vector of residual errors (distributed as MVN$(0,V_e\mathbf{I})$ with **I** being the identity matrix and V_e the residual variance). The significance of the genomic (polygenic) effect ($P = 0.05$) was assessed using the likelihood ratio test statistic to compare a model that fits the effect against the base model that excludes it.

5.3.4 Population structure

Genomic relationship matrix (GRM) was computed using GEMMA (Zhou and Stephens 2012) and principal component analysis (PCA) was used to find out any genetic structure of the cow population as described in the fourth chapter. This population structure was accounted for in GWA models by automatically fitting the GRM as part of the polygenic effect, whereas in RHM analysis the first 7 PCs were fitted to account for this structure with further correction for the inflation factor (λ) (Amin et al. 2007).

5.3.5 Genomic analyses

REACTA (Cebamanos et al. 2014) was first used to assess the full genomic variance for each trait with a general explanatory analysis.

5.3.6 Genome-wide association analysis (GWA)

GWA was performed as described in the fourth chapter using GEMMA (Zhou and Stephens 2012) using the following linear mixed model:

$$\mathbf{y}=\mathbf{W}\boldsymbol{\alpha} + \mathbf{x}\boldsymbol{\beta} + \mathbf{u} + \boldsymbol{\varepsilon}$$

where **y** represents the vector of foot microbiota phenotypes, **W** is covariate matrix, $\boldsymbol{\alpha}$ is the vector of associated fixed effects, **x** is the vector of genotypes which were coded as 0/1/2, $\boldsymbol{\beta}$ is phenotype's regression on genotypes, **u** is a vector of random polygenic effects (distributed as MVN$(0,V_g\mathbf{G})$, with **G** being the GRM matrix and V_g being the genetic variance), and $\boldsymbol{\varepsilon}$ represents the vector of residual errors (distributed as MVN$(0,V_e\mathbf{I})$, with **I** being the identity matrix and V_e being the residual variance). Further λ correction was applied to lower any potential inflation.

5.3.7 Regional Heritability Mapping (RHM)

The RHM was performed using the following model:

y (phenotype)= μ (Mean) + W*α (fixed effects + PCs)* + $\mathbf{u_{(i)}}$ (GRM for the region) + ε (error)

where y represents the vector for foot microbiota phenotypes, **W** is covariate matrix, $\boldsymbol{\alpha}$ is the vector of associated fixed effects (including the principal components), $\mathbf{u_{(i)}}$ is the effect for the corresponding region *i* (distributed as MVN$(0,V_{g(i)}\mathbf{G_{(i)}})$, with $V_{g(i)}$ and $\mathbf{G_{(i)}}$ being the genetic variance and the GRM corresponding to the SNPs in the i^{th} region, respectively), and $\boldsymbol{\varepsilon}$ is the vector of residual errors (distributed as MVN$(0,V_e\mathbf{I})$, with **I** being the identity matrix and V_e being the residual variance). λ correction was also applied to account for any additional cause of inflation.

The significance of the region effect was assessed using the likelihood ratio test statistic. A total of 1733 regions were analysed, leading to a genome-wide significant threshold ($P = 0.05$) defined at $P = 2.89\text{E}{-5}$ with Bonferroni correction for multiple regions ($-\log10(P) = 4.54$) and a suggestive threshold (one false positive per genome scan) defined at $P = 5.77\text{E-}4$ ($-\log10\,(P) = 3.24$). As with the GWA analyses, a correction by the inflation factor λ was applied to account for any remaining inflation after fitting the polygenic effect in the model.

GWA and RHM results were compared to determine common significant/suggestive regions, and the proportion of variance explained by each

region was worked out as a percentage of the total genomic variance as described in the fourth chapter.

5.4 Results

Table 5.1 shows the total genomic variance estimates for the traits relative abundances of *Peptoclostridium* spp. and *Treponema* spp., including heritabilities and standard errors. All other examined traits are not included due to total genomic variance estimates being non-significantly different from zero (Appendix Table D.1). The heritabilities for relative abundances of *Peptoclostridium* spp. and *Treponema* spp. were 0.59 ± 0.18 and 0.52 ± 0.00, respectively.

Table 5.1. Estimates of heritability and variance components for traits with a significant (P<0.05) genomic effect. Genomic heritabilities (h^2), genomic variance (*Vg*) estimated together with their standard errors. P-values (*P*) for the significance of the genomic effect and the number of total records (*N*).

Trait	Vg	h^2	*P*	N
Mean Relative abundance of *Peptoclostridium* spp.	0.000053 ± 0.000019	0.59 ± 0.18	0.0007	236
Mean Relative abundance of *Treponema* spp.	0.000129 ± 0.000055	0.52 ± 0.00	0.0070	236

Suggestive and significant SNPs associated with these two traits after GWA analyses are shown in Table 5.2, including their positions on corresponding chromosomes (BTAs) and significance level (*P*-value). One suggestive SNP on BTA6 and one significant SNP on BTA 19 were associated with relative abundance of *Peptoclostridium* spp. Four significant and two suggestive SNPs on BTA1, one suggestive and one significant SNP on both BTA9 and BTA17, one significant SNP on BTA16, and one suggestive SNP on BTA2, BTA6, BTA8, BTA19, BTA21 and BTA29 were associated with relative abundance of *Treponema* spp. Association between individual SNPs and relative abundances of *Peptoclostridium* spp. and *Treponema* spp. were shown using Manhattan plots, including a closer look at the SNPs on BTA1 associated with relative abundance of *Treponema* spp. (Figure 5.1).

Quantile-Quantile (QQ) plots of GWA analyses after λ correction are shown in the Appendix Figure D.1 for those traits.

Table 5.2: Summary of genome-wide suggestive and significant SNPs for the traits

Trait	BTA	Position (BP)	P-value	Significance
Mean Relative abundance of *Peptoclostridium* spp.	6	92217233	2.49E-05	Suggestive
	19	50478941	6.78E-09	Significant
Mean Relative abundance of *Treponema* spp.	1	112526671	3.65E-09	Significant
	1	112344219	3.65E-08	Significant
	1	115738119	2.28E-07	Significant
	1	110924093	1.06E-06	Significant
	1	113745976	1.49E-06	Suggestive
	1	116472073	1.03E-05	Suggestive
	2	64462072	1.74E-05	Suggestive
	6	20730690	5.32E-06	Suggestive
	8	54239367	3.67E-06	Suggestive
	9	99334002	5.22E-10	Significant
	9	90719582	2.26E-05	Suggestive
	16	79449472	3.93E-10	Significant
	17	7185597	8.88E-08	Significant
	17	7399427	2.65E-05	Suggestive
	19	50478941	1.14E-05	Suggestive
	21	34339514	2.28E-06	Suggestive
	29	23162838	4.97E-06	Suggestive

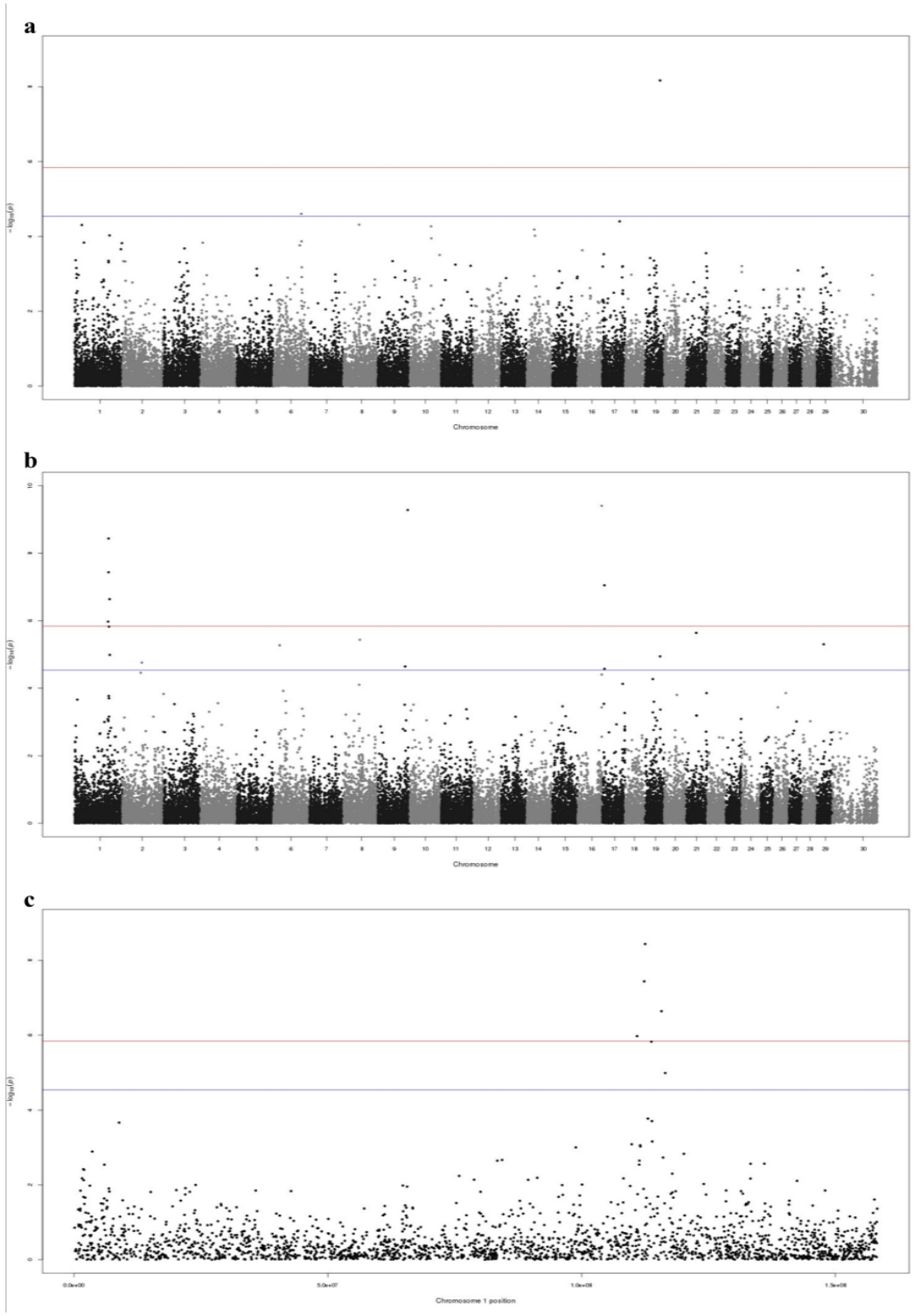

Figure 5.1. GWA analysis for **a)** relative abundance of *Peptoclostridium* spp., **b)** relative abundance of *Treponema* spp., **c)** a closer look at the SNPs on BTA1 associated with relative abundance of *Treponema* spp. Red line represents the genome-wide significance (Bonferroni correction for P = 0.05). Blue line represents the suggestive threshold.

The RHM analyses results are shown in Table 5.3 with the start and ending positions of each region on corresponding BTAs and *P*-values. This analysis identified 1 suggestive region on both BTA1 and BTA6 and 2 suggestive regions on BTA19 for the trait relative abundance of *Peptoclostridium* spp. For the trait relative abundance of *Treponema* spp. RHM results indicated 1 region on both BTA1 and BTA16 with genome-wide significance, besides 1 suggestive region on BTA1, BTA11, BTA17 and BTA19 (Figure 5.2). QQ plot of RHM analyses after λ correction for IHfarm was also shown in Appendix Figure D.2.

Table 5.3. Significant genomic regions from the regional heritability mapping analyses

Trait	BTA	Start Position (BP)	Ending position (BP)	P-value	Significance
Mean Relative abundance of *Peptoclostridium* spp.	1	23938895	25321721	4.22E-05	Suggestive
	6	92757120	94158559	7.38E-05	Suggestive
	19	30300498	31382204	0.000476	Suggestive
	19	49930085	50848144	0.000574	Suggestive
Mean Relative abundance of *Treponema* spp.	1	111955405	113215525	1.17E-05	Significant
	1	115499944	117585231	0.000279	Suggestive
	11	83637998	85153576	0.00029	Suggestive
	16	79135948	80351106	1.23E-06	Significant
	17	7185597	8690167	9.14E-05	Suggestive
	19	26135182	27326573	0.00021	Suggestive

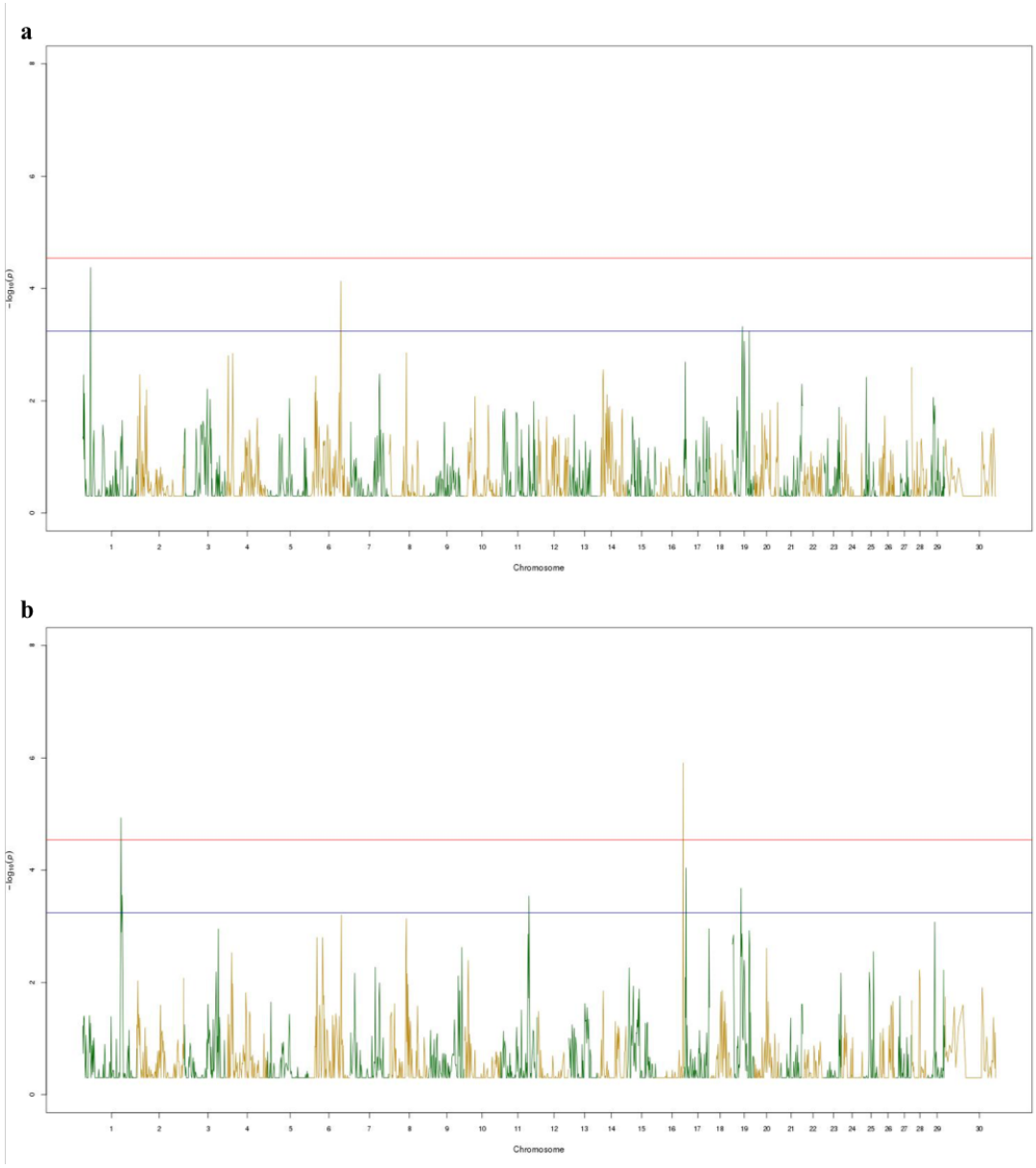

Figure 5.2. Manhattan plots displaying the results of RHM analyses for **a)** relative abundance of *Peptoclostridium* spp., **b)** relative abundance of *Treponema* spp. Red line represents the genome-wide significance, and blue line represents the suggestive threshold

The results of GWA and RHM analyses were compared, and a consensus table of genomic regions was created with the start and ending positions of each region on corresponding BTAs, the proportion of *Vg* explained by each region, and potential candidate genes neighbouring the regions (Table 5.4).

Table 5.4. Summary of the consensus genomic regions after GWA and RHM analyses including the proportion of genomic variance (V_g) explained by the detected region, and candidate genes in these regions.

Traits	BTA	Start Position (BP)	Ending position (BP)	*P*-value	Proportion of Vg explained	Candidate Genes
Mean Relative abundance of *Peptoclostridium* spp.	19	49930085	50848144	0.000574	28.07%	*ZNF750*
Mean Relative abundance of *Treponema* spp.	1	111955405	113215525	1.17E-05	9.88%	*GMPS* and *PLCH1*
	1	115499944	117585231	0.000279	1.97%	*MBNL1*
	16	79135948	80351106	1.23E-06	34.78%	*PTPRC*
	17	7185597	8690167	9.14E-05	7.11%	*LRBA*

5.5 Discussion

In the present study, genome-wide association of DD-linked microbiome traits were investigated using GWA and RHM approaches, leading to a first understanding of the genomic architecture of these traits. Traits with significant genomic variance were further analysed using GWA, and 4 SNPs on BTA1, 1 SNP on BTA9, BTA16, and BTA17 were found to be significant for the trait relative abundance of *Treponema* spp. In addition to that, 1 SNP on BTA19 was found to be significant for the trait relative abundance of *Peptoclostridium* spp. The RHM analyses revealed that one genomic region on BTA1 and one on BTA16 were significantly associated with relative abundance of *Treponema* spp. When these two approaches were compared, regarding relative abundance of *Treponema* spp.; one significant and one suggestive SNP on BTA1 in GWA results were found to be within the significant and suggestive genomic regions in RHM results, respectively. The significant SNP on BTA16 in GWA results was found to be within the significant genomic region in RHM results, and the significant SNP on BTA17 in GWA results was found to be within the suggestive genomic region in RHM results. Regarding relative abundance of *Peptoclostridium* spp.; the significant SNP on BTA19 in GWA results was found to be within the suggestive genomic region in RHM results, and the suggestive SNP on BTA6 in GWA results was found to be in close proximity to the suggestive genomic region in RHM results. The proportion of

the total genomic variance explained by the detected regions for each trait ranged from 1.97% to 34.78% suggest a partially oligogenic architecture, but this could be overestimated due to the Beavis effect (Xu 2003). The region on BTA1 indicated substantial associations with relative abundance of *Treponema* spp. supported by several SNPs. However, finer mapping with a higher SNP density would be needed to confirm potential candidates especially when one considers the small size of the population used here.

On BTA1, the region associated with relative abundance of *Treponema* spp. explains 9.88% of the total genomic variance and includes the genes *GMPS* and *PLCH1*. *GMPS* encodes guanine monophosphate synthetase which plays a role in *de novo* synthesis of guanine nucleotides; the cyclic form of GMP was shown to be associated with immune signalling pathways (O'Gorman et al. 2009; Wu et al. 2013). PLCH1 is a member of the phospholipase enzyme family that generates the secondary messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) by cleaving phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2). Phospholipases were shown to be involved in inflammation mechanisms (Lemos et al. 2016), especially the expression of *PLCH1* were shown to be downregulated by lipopolysaccharides (LPS) (Lo Vasco et al. 2013) which is found in the outer membrane of Gram-negative bacteria (Heumann and Roger 2002). These associations may explain the role of the genomic region on BTA1 in immune and inflammatory response against the Gram-negative *Treponema* (Holt 1978) infections. The second region on BTA1 explains 1.97% of the total genomic variance and includes the gene *MBNL1* encoding a member of muscleblind protein family which was shown to play role in adipogenesis (Labrecque et al. 2009). So, this genomic region might be associated with lipid metabolism that facilitate *Treponema* invasion and proliferation as in human periodontal disease (Plummer and Krull 2017).

The region associated with relative abundance of *Treponema* spp. on BTA16 explains 34.78% of the total genomic variance and includes the gene *PTPRC* encoding a transmembrane tyrosine phosphatase which was shown to be upregulated after administration of external bacteria to the intestine of mice (Nerstedt et al. 2007; Fink et al. 2012). So, the genomic region on BTA16 might be associated with immune signalling pathways.

On BTA17, the region associated with the relative abundance of *Treponema* spp. explains 7.11% of the total genomic variance and harbours the LPS-responsive beige-like anchor gene (*LRBA*) which is expressed in immune cells after stimulation by LPS (Wang et al. 2001). Mutations on *LRBA* gene were shown to be associated with immune system related disorders such as immunodeficiency, inflammatory bowel disease (Alangari et al. 2012), and autoimmunity (Revel-Vilk et al. 2015). Therefore, the genomic region on BTA17 might be associated with the immune response against the bacteria comprising LPS; e.g. *Treponema* spp.

On BTA19, the region associated with relative abundance of *Peptoclostridium* spp. explains 28.07% of the total genomic variance and harbours the gene *ZNF750* which is encoding a putative C2H2 zinc finger protein which was shown to be associated with the skin disorders Seborrhea-like dermatitis (Birnbaum et al. 2006) and familial psoriasis (Yang et al. 2008). In addition, increased dietary zinc was shown to be associated with reduced DD incidence in dairy cows (Nocek et al. 2000). Therefore, the genomic region on BTA19 might have potential associations with skin disorders such as DD.

A relatively small sample size was one of the main limitations of this study. On the other hand, it is the first study investigating the association between host genome and DD linked microbiome traits and performing large scale microbial research demands more time and funds. Microbiome sampling could be done at different stages of DD using larger sample size and significantly DD-associated bacteria could be analysed for the association with host genetics. Furthermore, inflated heritability estimates of the studied traits could be caused by small sample size. Another limitation was fitting PCs instead of polygenic effect since the software used could not perform the analysis fitting the GRM. Therefore, another software could be used to confirm these results.

5.6 Conclusion

In this study the aim was to show the associations between bovine foot skin microbiota profiles and host genetics with a focus on DD. Five genomic regions were associated with the relative abundance of *Treponema* spp. and *Peptoclostridium* spp., harbouring genes related to lipid metabolism, immune system, and skin disorders. The findings of this study suggest that these traits are moderately heritable and partially oligogenic. The genomic regions detected by both GWA and RHM

approaches are likely to explain the association between the host genome and foot microbiome profiles even though the sample size is relatively small. Therefore, further research with larger sample size is needed to verify these results.

CHAPTER VI

5 General Discussion

Lameness is indisputably a major problem for the dairy industry in terms of economic cost and animal welfare. Lameness and impaired mobility constitute one of the three most significant health issues together with reduced fertility and mastitis (Green et al. 2002; Buitenhuis et al. 2007; Cha et al. 2010; Bicalho and Oikonomou 2013; Huxley 2013). Lameness is associated with lesions of both infectious or a non-infectious aetiology (Green et al. 2002; van der Waaij et al. 2005; Bicalho and Oikonomou 2013). Lameness is also associated with the environmental conditions, diet and genetic factors (Olmos et al. 2009).

DD is one of the main disorders associated with lameness (Palmer and O'Connell 2015). In the present thesis, 16S rRNA gene amplicon sequencing was performed on DNA samples extracted from the foot skin swabs of 242 dairy cows from three different farms in England and Wales. The results suggest that *Treponema* spp., which has been strongly associated with DD in several studies, might act together with other pathogens such as *Porphyromonas* spp. in the aetiopathogenesis of DD lesions. These pathogens are mostly anaerobic, and oxygenation of the lesions could be useful to treat DD similar to the oxygenation therapies used to treat periodontal disease (Signoretto et al. 2007; Fernandez y Mostajo et al. 2014).

Additionally, healthy skin samples were shown to have higher relative abundance of *Macrococcus* spp. than the ones which developed or already had DD. Therefore, potential protective roles of *Macrococcus* spp. against DD could be further investigated. If the potential protective roles of these bacteria are confirmed by larger scale studies, it could lead to production of probiotic foot bathing solutions against DD similar to the probiotics used to treat periodontal disease (Shimauchi et al. 2008; Vicario et al. 2013).

In addition, cows in farms with different management practices had different foot microbiota profiles; nevertheless, they still shared a similar range of pathogens in DD lesions. The profound differences in the foot skin microbiota profiles between farms with different management suggest that the manipulation of the foot skin microbiota may be possible through specific management interventions. This is a very promising area for future research.

Complicated CHDLs (including SU, TN, and WLD), IH, and IP are important lameness associated foot lesions with multifactorial aetiologies (Clark et al. 1985; Evans et al. 2011; Kofler et al. 2011). 16S rRNA gene amplicon sequencing of swab samples obtained from 51 cattle across ten different dairy farms in the UK was used to explore the microbial profiles of these lesions. The results showed that microbial richness and diversity were lower in disease samples compared to their healthy skin control samples with the exception of IH. In addition, a more or less common array of opportunistic anaerobes were found to be associated with most of these lesions. As a novel finding, *Fastidiosipila* spp. which are also shown to be associated with lameness causing DD lesions in the second chapter, showed a significant prevalence in SU, TN, and WLD lesions. The role of these bacteria in human osteitis has been previously shown but this is the first time this emerging pathogen is implicated in the development of lameness causing foot lesions in dairy cattle.

These findings could further elucidate the aetiopathogenesis of complicated CHDLs and IP lesions and lead to the development of novel therapeutic approaches that could be applied to reduce the prevalence of these lesions. For instance, foot bathing with a mixture of appropriate probiotic bacteria or oxygenated solutions could be performed to combat anaerobes. Further, longitudinal, larger scale studies could identify the importance of these different pathogens at different stages of disease progression.

Culture-independent studies of mixed microbial communities rely either on target-specific 16S rRNA gene sequencing or shotgun metagenomic sequencing in which fragmented whole metagenomic DNA is sequenced (Shah et al. 2011; Addis et al. 2016). 16S amplicon sequencing has some advantages like being cost effective, having established pipelines for data analyses, and having large archived reference data, but whole genome sequencing approaches could provide a lot more information, including information on viruses or fungi and on the functional profiles of the studied microbial communities (Ranjan et al. 2016). In the microbiomics parts of this study, the V3-V4 region of 16S rRNA gene was amplified and analysed for characterization of microbial profiles. Different regions of 16S rRNA gene could have also been amplified and analysed for comparison purposes and for the detection of any potential primer biases. Moreover, the copy number of 16S rRNA gene per genome could be variable even in the same species (Klappenbach 2001; Acinas et al. 2004). Alternatively, a better conserved gene with less or single copies such as *cpn60*

(Schellenberg et al. 2011) or *rpoB* (Case et al. 2007) could be used or whole genome sequencing could be performed with the advent of more developed technologies. Another limitation of this study was using swabs that could not help describing bacteria present in the deeper skin and taking part in the disease development. To better describe these bacteria skin biopsy samples could be used instead of swabs. However, biopsy is more intrusive and will compromise the skin integrity and therefore make the skin more prone to infections.

Lameness associated lesions investigated in this study display polymicrobial profiles, but they might have primary aetiological agents. To further investigate this, candidate species such as *Treponema* spp. *Porphyromonas* spp. etc. could be studied by using different approaches to fulfil Koch's postulates which are;

1. The agent must be present in all individuals suffering from the disease.
2. The agent must be isolated from diseased individual and grown in pure culture.
3. The agent must cause disease when a healthy individual is infected with the agent and must be re-isolated from experimentally infected individual (Koch 1882; Thébaud and Abman 2007).

Alternatively, mixture of bacteria that were isolated from DD lesions could be used to induce DD in healthy animals.

Lameness associated traits were shown here to have significant genomic variation. Therefore, using genomic markers could provide a potential to improve lameness preventive breeding strategies (Olmos et al. 2009; Swalve et al. 2014). Genome-wide analyses were used to identify genomic regions which are significantly associated with lameness associated traits and DCT (n=554). The traits with significant genomic variations were found to be moderately heritable and potentially associated with a polygenic architecture. Four genomic regions with a significant effect were identified for DD, IH and SH, and these regions include the genes involved in immune and fibroblastic processes. DCT at calving displayed a moderate genomic heritability; however, no significant genomic region was identified for this trait. Due to presence of genetic variation, follow-up studies with larger datasets could be useful to identify significant genomic regions associated with DCT. Moreover, the present study showed that farm records had lower quality than research records. Therefore, dairy farmers and foot trimmers might be trained

and encouraged to increase the quality of routinely recorded claw health data. Alternatively, a reference population of both males and females with regular phenotyping and genotyping might be monitored to develop more accurate genomic predictions.

Microbiome profiles in various body niches have been shown to be associated with host genetics and contribute to the phenotype of the host (Ross et al. 2013). Here, the relationship of the foot skin microbiota and DD associated bacteria with the bovine genome was investigated for the first-time using genome wide analyses and microbiota profiles determined by 16S rRNA amplicon sequencing (n=242). The genomic regions identified in the present study harbour genes predominantly related to lipid metabolism, immune response, and skin disorders associated with the relative abundance of *Treponema* spp. and *Peptoclostridium* spp. Furthermore, these traits were found to be moderately heritable and partially oligogenic. Although the sample size is relatively small for a genomic association study, this study is still one of the largest genotype-microbiome interaction studies. As the cost of sequencing decrease, larger scale studies could be performed, and the genomic regions associated with specific microbiota profiles could be detected better.

Heritabilities of lameness associated traits investigated in this study were relatively higher compared to the similar studies. It might be caused by using only three farms which might lower the effect of the environment. Therefore, caution must be exercised while evaluating these results. Heritabilities might be verified by increasing the sample size and number of farms involved in the study.

To confirm the effect of detected SNPs, transcriptomic and proteomic analyses could be performed, and it could be observed whether these SNPs cause increased or reduced transcription and/or translation of nearby genes. After that, SNPs with true effects might be included into breeding programs. For instance, individuals having the SNPs associated with less DD or resistance to the growth of opportunistic anaerobes could be selected for future generations and more disease resistant individuals could be bred.

In summary, lameness associated lesions have polymicrobial profiles and alterations in the microbial diversity can be associated with the risk of disease. The results of the microbiomics part of this study could contribute towards future treatment and control strategies for these diseases. The findings of the genomics

studies could elucidate the pathogenesis of lameness and provide a prospect to improve health and welfare in dairy cattle with the addition of the significant genomic regions into cattle breeding programs or the manipulation of problematic genomic regions using contemporary technologies such as gene editing. Moreover, clarification of the relationship between host genome and microbiome with larger scale studies could bring a new perspective to genomics studies. Thus, new research questions could arise to investigate the animals in both genomic and microbiomics aspects.

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Appendices

Appendix A: Investigating the Role of The Foot Skin Microbiota in The Development of Digital Dermatitis Lesions in Dairy Cattle (Chapter II)

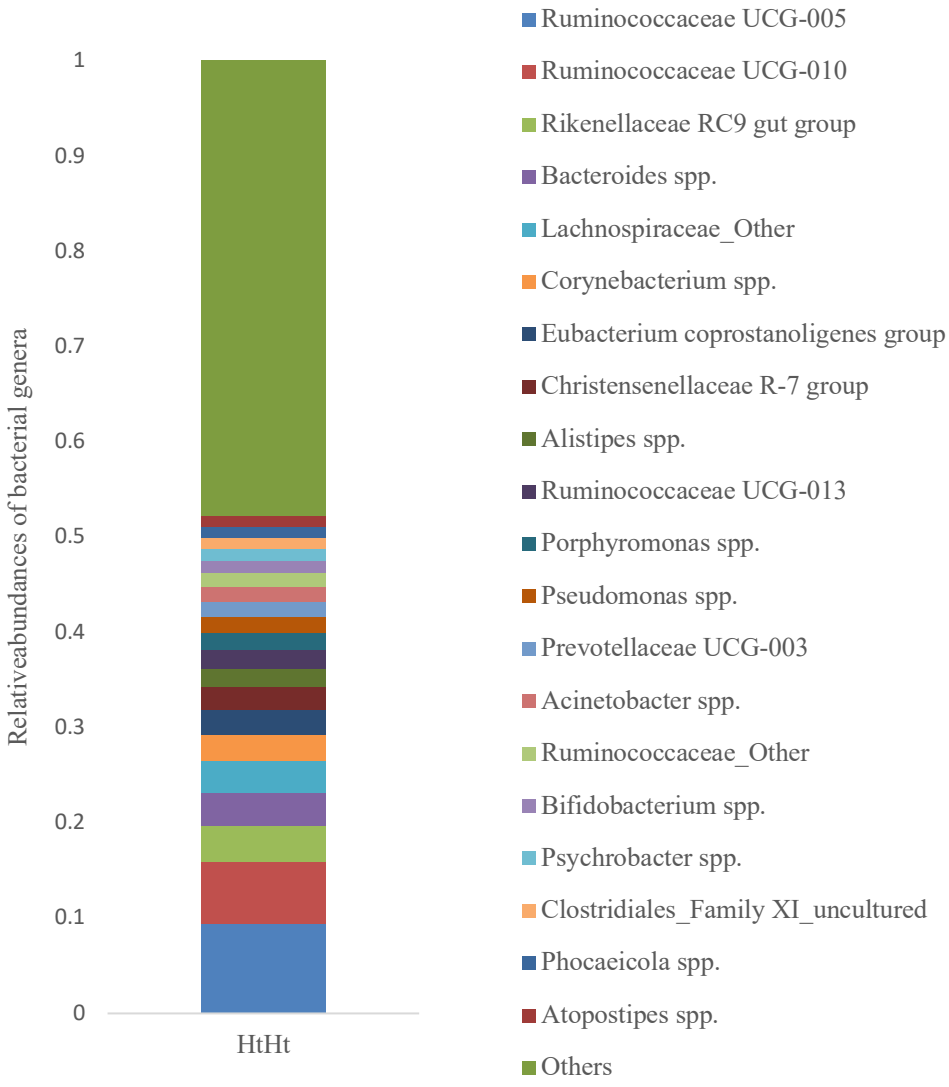

Figure A.1. Relative abundances of 20 most prevalent bacterial genera in HtHt samples in cow level (HtHt: The cows which were healthy during the study.)

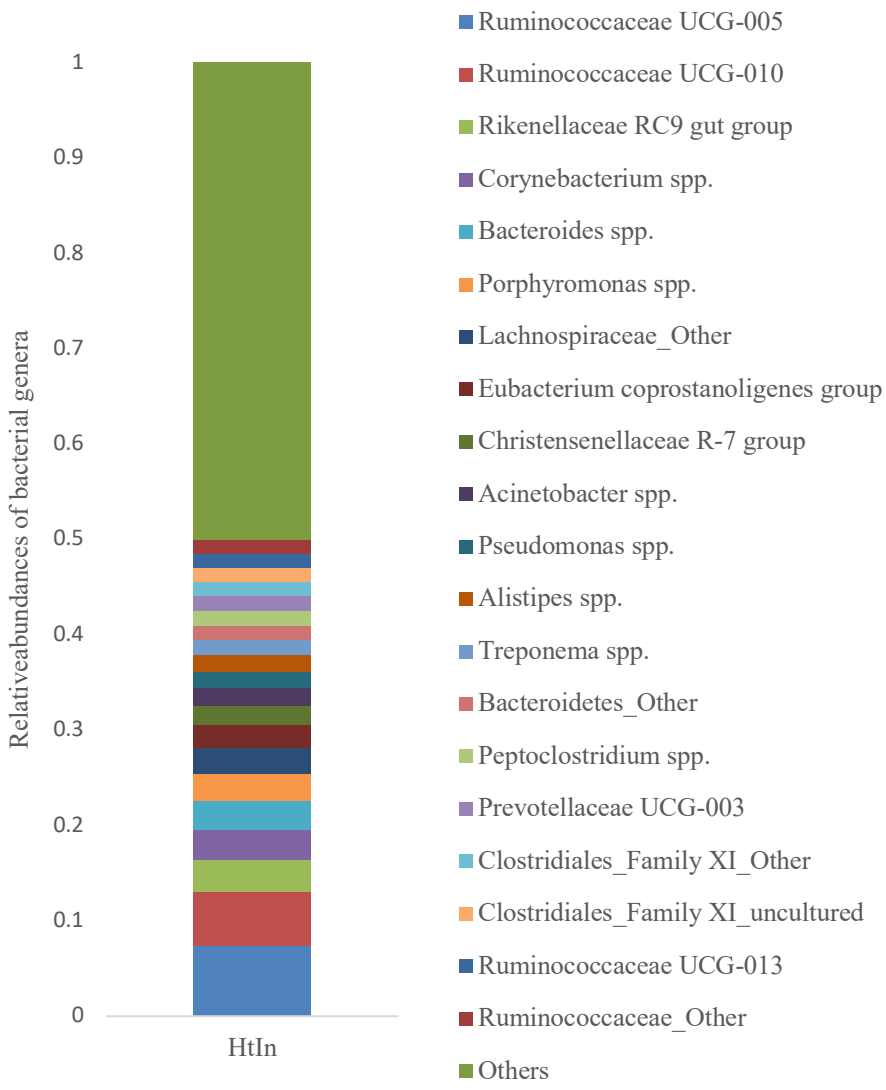

Figure A.2. Relative abundances of 20 most prevalent bacterial genera in HtIn samples (HtIn: The cows which were healthy at sampling, then developed DD.)

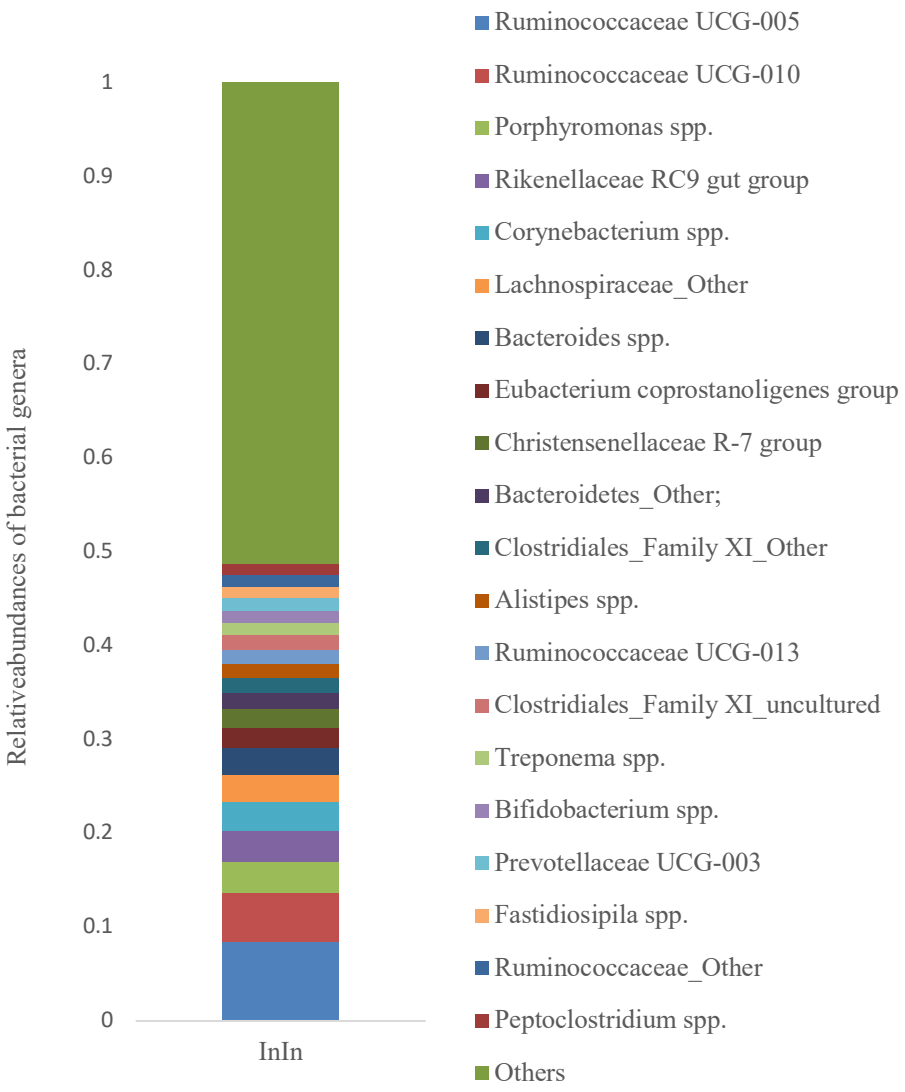

Figure A.3. Relative abundances of 20 most prevalent bacterial genera in InIn samples (InIn: The cows which had DD in all checkpoints)

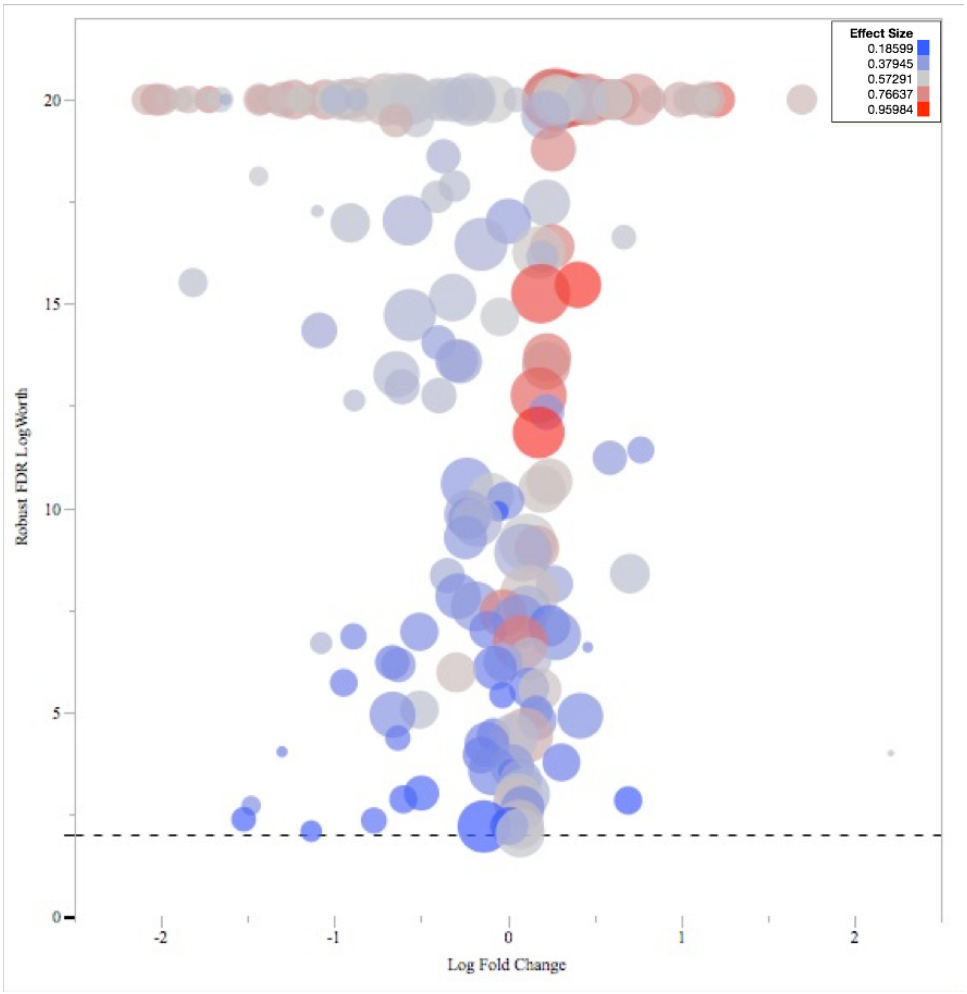

Figure A.4. Comparison of microbiota profile of farms 1 and 2 and farm 3 samples. Size of the circle represents the prevalence of each genera, and colour represents the effect size. The graph shows log fold change in 16S rRNA gene abundance in farm 3 samples relative to farms 1 and 2 samples versus corrected robust false discovery rate (FDR) logWorth (i.e. log10P). The dashed line shows the P-values (0.01) adjusted for FDR.

Appendix B: 16S rRNA amplicon sequencing reveals a polymicrobial nature of complicated claw horn disruption lesions and interdigital phlegmon in dairy cattle (Chapter III)

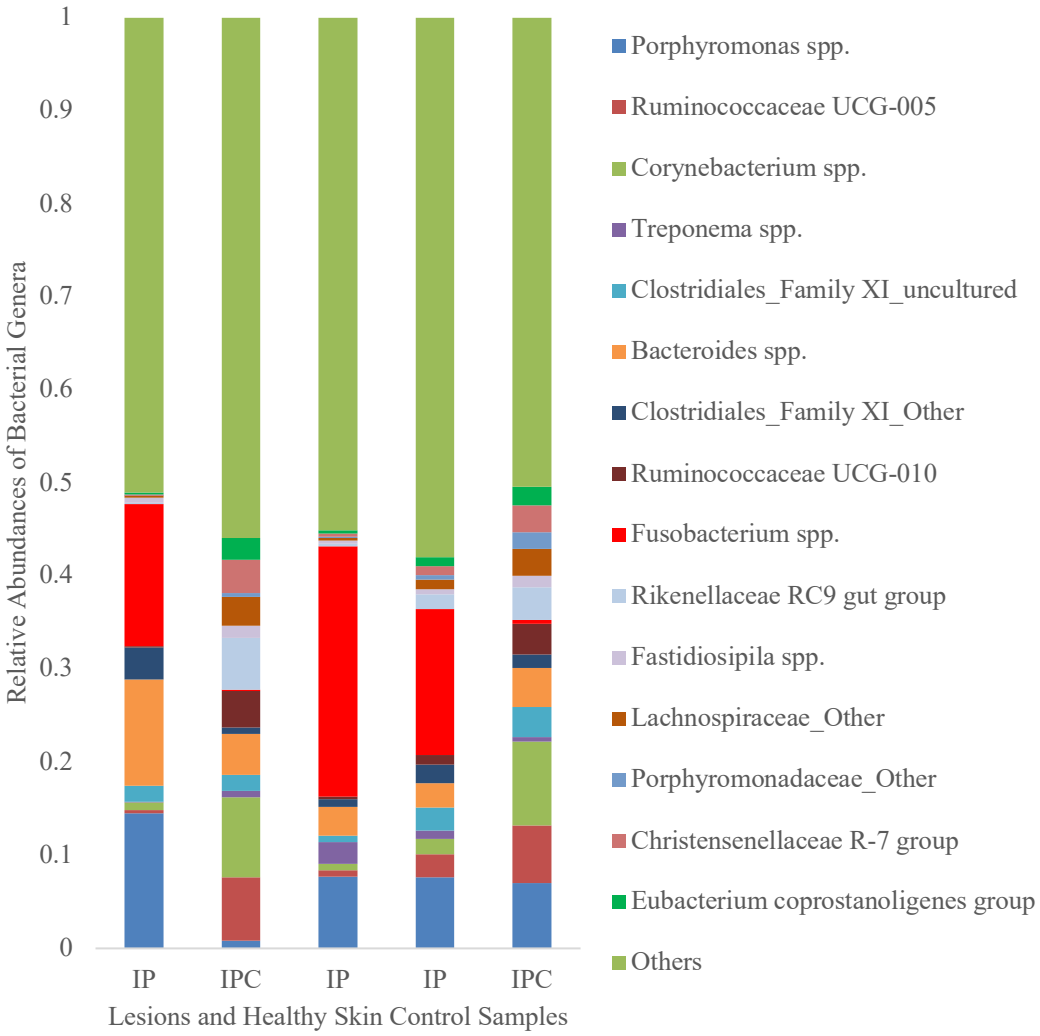

Figure B.1. Relative abundances of fifteen most prevalent bacterial genera in IP lesions and their healthy skin control samples. (IP: Interdigital Phlegmon, IPC: IP Control)

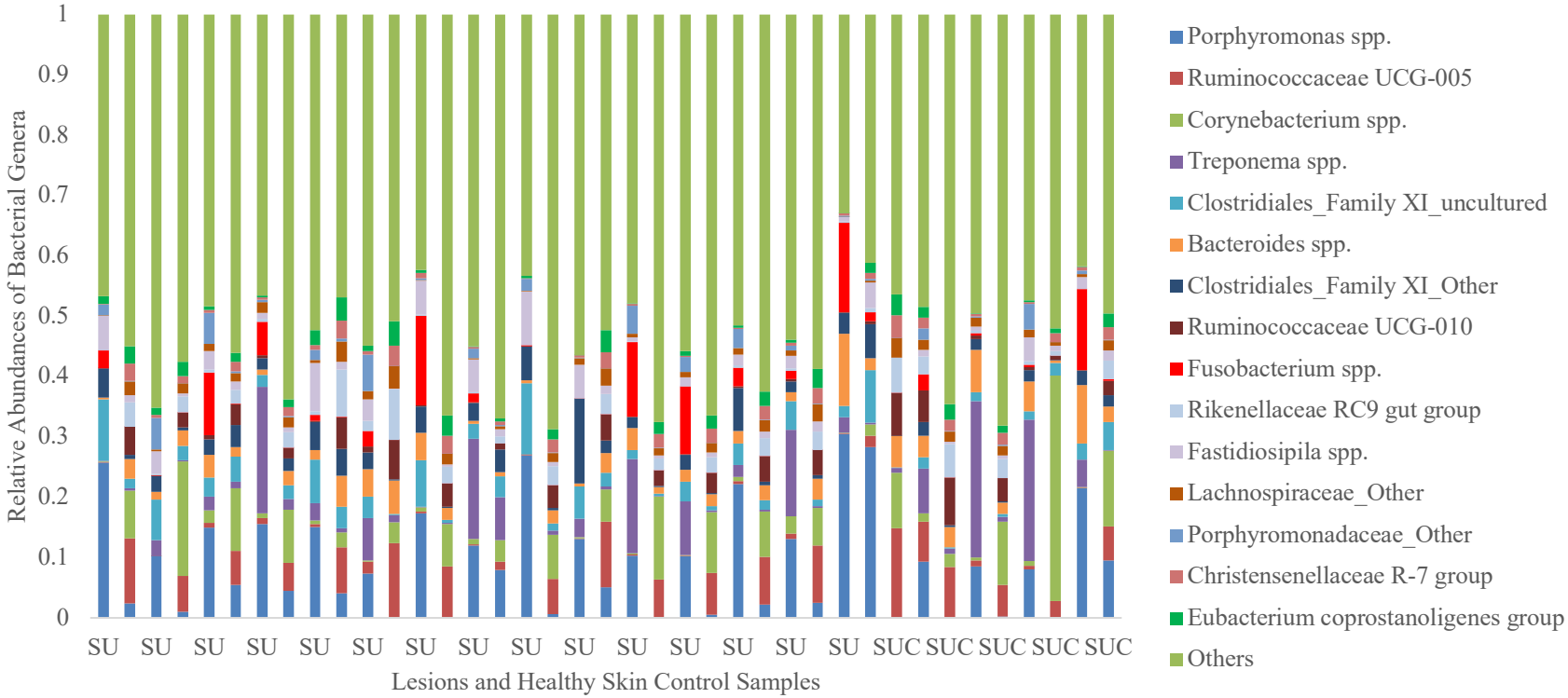

Figure B.2. Relative abundances of fifteen most prevalent bacterial genera in SU lesions and their healthy skin control samples. (SU: Sole Ulcer, SUC: SU Control)

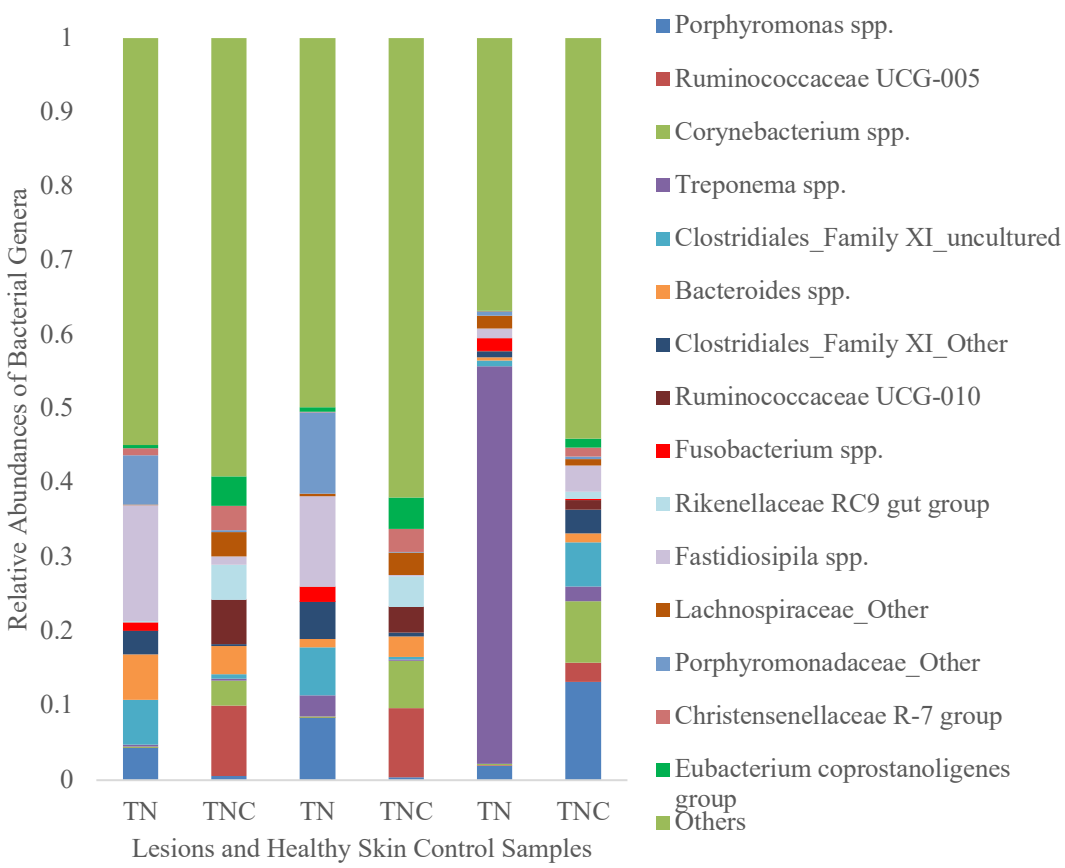

Figure B.3. Relative abundances of fifteen most prevalent bacterial genera in TN lesions and their healthy skin control samples. (TN: Toe Necrosis, TNC: TN Control)

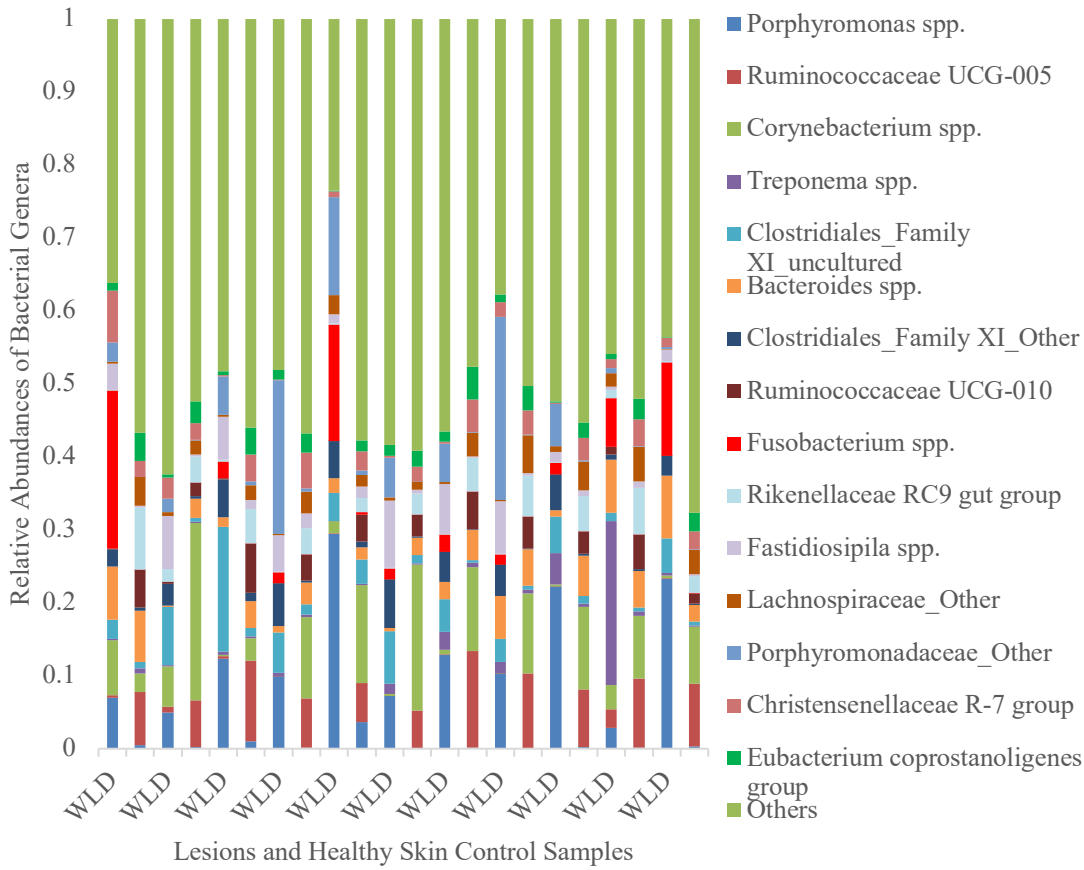

Figure B.4. Relative abundances of fifteen most prevalent bacterial genera in WLD lesions and their healthy skin control samples. (WLD: White Line Disease, WLDC: WLD Control)

Appendix C: Quantitative trait loci mapping for lameness associated phenotypes in Holstein Friesian dairy cattle (Chapter IV)

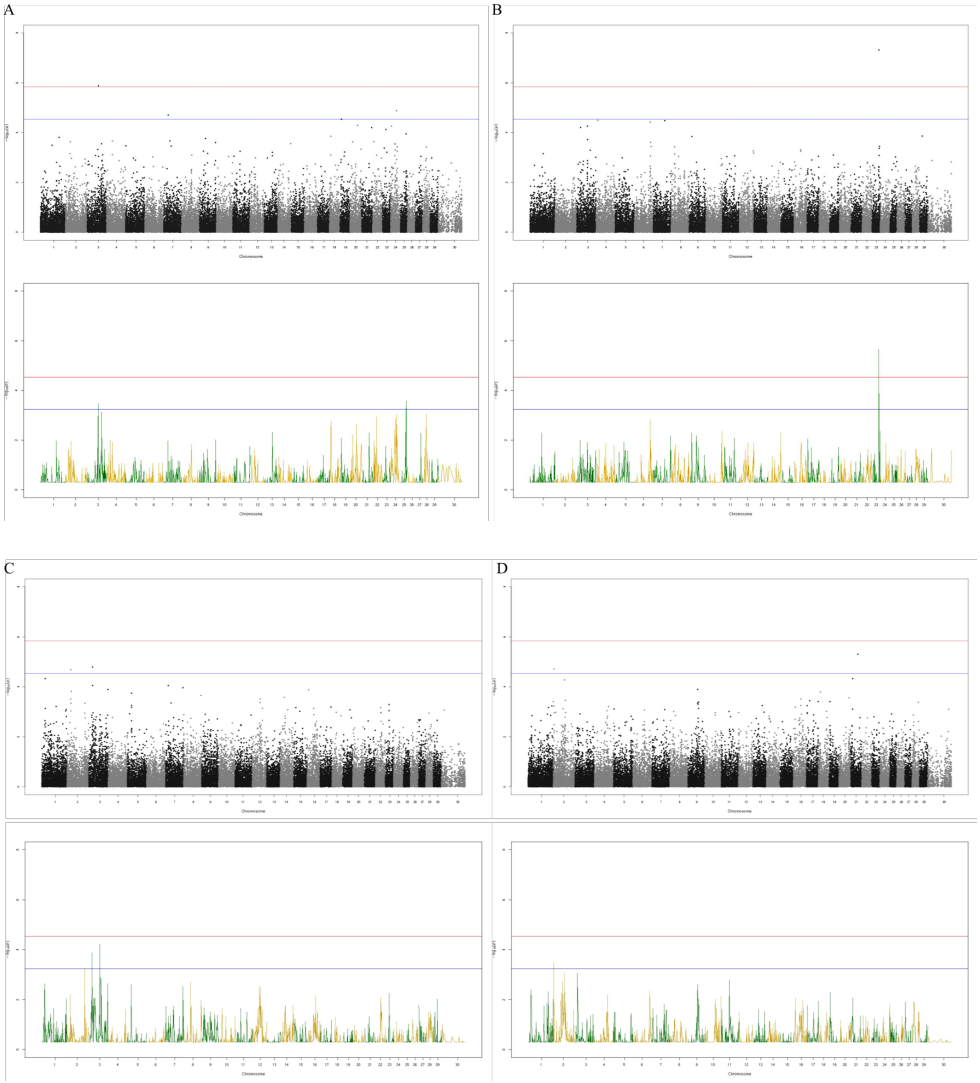

Figure C.1: Genome-wide association (GWA) and regional heritability mapping (RHM) results for traits with concordant regions: Figure shows the Manhattan plots for the GWA (upper) and RHM (lower) for each one of the traits where concordant regions between both approaches were observed (A: DD for farm records; B: IH for farm records; C: IH for combined records and; D: SH for combined records). Red lines correspond to the genome-wide significant threshold and blue lines correspond to the genome-wide suggestive threshold.

Appendix D: Host Genotype – Foot Skin Microbiome Associations (Chapter V)

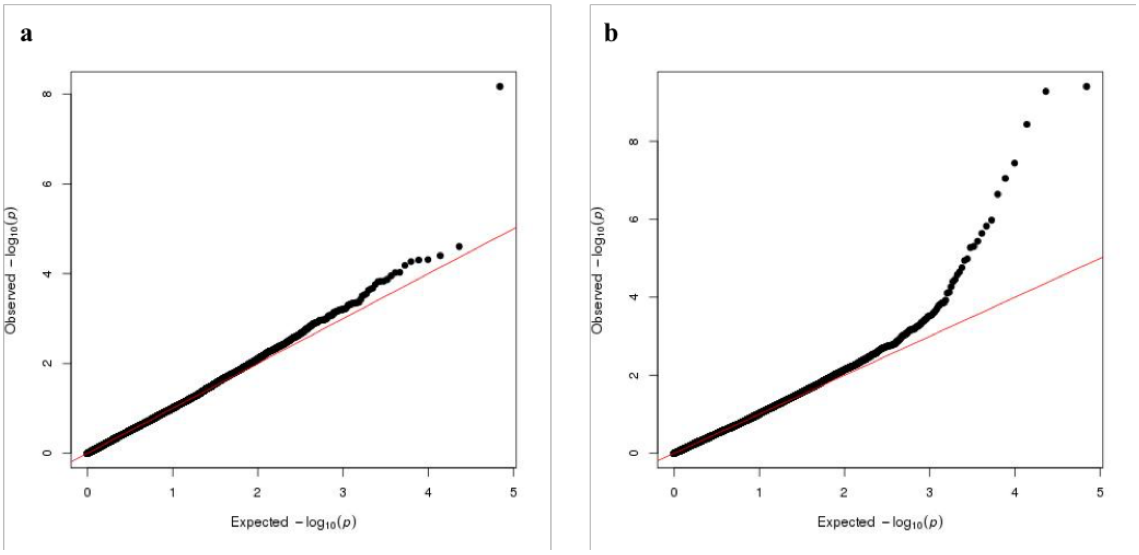

Figure D.1. QQ plots of observed vs. expected *P*-values from GWA analyses of **a)** relative abundance of *Peptoclostridium* spp., **b)** relative abundance of *Treponema* spp.

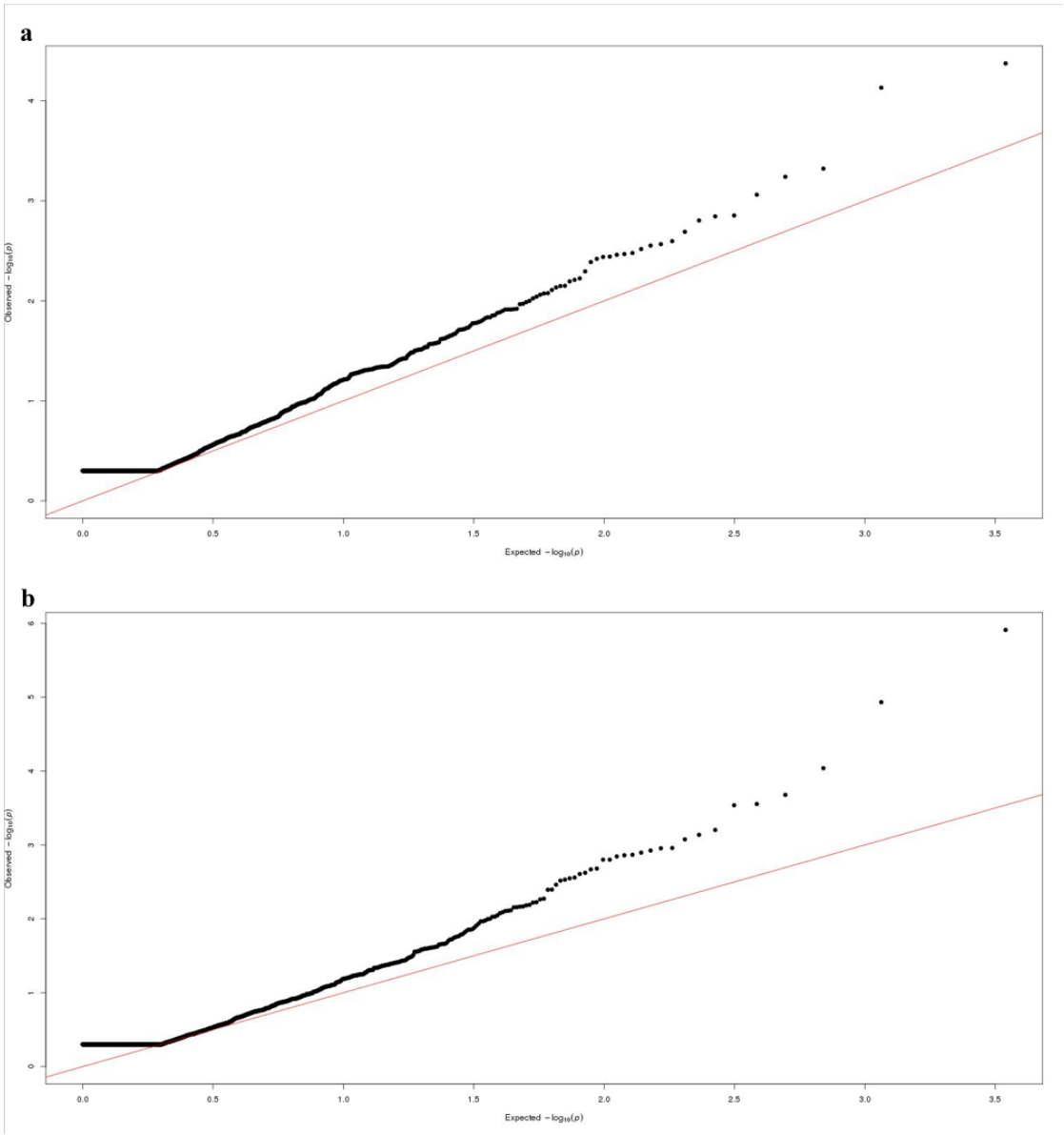

Figure D.2. QQ plot of observed vs. expected *P*-values from RHM analyses of **a)** relative abundance of *Peptoclostridium* spp., **b)** relative abundance of *Treponema* spp.

Table D.1. Estimates of heritability and variance components for all traits. Genomic heritabilities (h^2), genomic variance (Vg) estimated together with their standard errors (SE), and *P*-values for the significance of the genomic effect.

Trait	Vg	SE	h2	SE	*P*-value
Chao1 index	5.802635	580901	0	0.149274	0.5
Shannon index	0	0.089175	0	0.175109	0.5
Simpson index	0	0	0	0.14227	0.5
Relative abundance of *Porphyromonas* spp.	0	0.000175	0	0.186462	0.5
Relative abundance of Bacteroidetes_other	0	0.000091	0	0.159378	0.5
Relative abundance of *Fastidiosipila* spp.	0	0.000028	0	0.166199	0.184
Relative abundance of Clostridiales Family XI	0.000007	0.000023	0.05	0.157471	0.381
Relative abundance of *Peptoclostridium* spp.	0.000053	0.000019	0.59	0.179016	0.000717
Relative abundance of *Macrococcus* spp.	0	0.000001	0	0.110497	0.5
Relative abundance of *Treponema* spp.	0.000129	0.000055	0.52	0.000025	7.04E-03