The Bovine Foot Skin Microbiota is Associated with Host Genotype and the Development of Infectious Digital Dermatitis Lesions

V. Bay
Aegean Agricultural Research Institute
A. Gillespie
University of Liverpool
E.K. Ganda
Penn State: The Pennsylvania State University
Nicholas Evans
University of Liverpool
Stuart Carter
University of Liverpool
L. Lenzi
University of Liverpool
A. Lucaci
University of Liverpool
S. Haldenby
University of Liverpool
M. Barden
University of Liverpool
B.E. Griffiths
University of Liverpool
E. Sánchez-Molano
The University of Edinburgh The Roslin Institute
R. Bicalho
Cornell University
G. Banos
SRUC: Scotland's Rural College
A. Darby
University of Liverpool
Georgios Oikonomou (Georgios.Oikonomou@liverpool.ac.uk)
University of Liverpool https://orcid.org/0000-0002-4451-4199

Research

Keywords: Bovine Digital Dermatitis, dairy cows, Treponema spp. and Peptoclostridium spp

Posted Date: June 30th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-650860/v1

Abstract

Bovine Digital Dermatitis (BDD) is a prevalent infectious disease, causing painful foot skin lesions and lameness in cattle. The polymicrobial nature of this disease has led to the hypothesis that the foot skin microbiota may be associated with occurrence and progression of lesions. We describe herein the bovine foot skin microbiota using 16S rRNA gene amplicon and shotgun metagenomic sequencing on samples from 259 dairy cows from three UK dairy farms. We show differences in the foot skin microbiome profiles of clinically healthy animals that were associated with subsequent development of BDD. We also present the first co-occurrence analysis of the bovine foot skin microbiome showing ecological relationships among bacterial species. Taxonomical and functional differences together with alterations in ecological interactions between bacteria in the normal foot skin microbiome may predispose an animal to develop BDD lesions. Using genome-wide association and regional heritability mapping approaches, we provide first evidence for interactions between host genotype and the foot skin microbiota profiles. We show the existence of genetic variation in the relative abundance of *Treponema* spp. and *Peptoclostridium* spp. and identify regions in the bovine genome that explain a significant proportion of this variation.

Introduction

Bovine Digital Dermatitis (BDD) is a prevalent infectious disease, causing painful foot skin lesions. This results in cattle becoming lame which in turn compromises animal welfare and causes significant production losses [1]. Many BDD-associated pathogens are also considered commensals of the foot skin, gastrointestinal tract and faeces of ruminants, or ubiquitous to the farm environment. The polymicrobial nature of this disease has led to the hypothesis that the foot skin microbiota, and the relationships between its members, may affect occurrence and progression of lesions [2].

Changes in bacterial populations in bovine foot skin throughout the progression of BDD lesions have been investigated using both 16S rRNA gene and shotgun metagenomic sequencing. Analysis from lesion biopsies showed that temporal changes in the foot skin microbiota composition and diversity occur at each of the five morphologically distinct lesion stages. *Treponema* species, which are the most common pathogens associated with BDD, were found in low abundance in early lesions and became dominant in latter stage lesions [3]. The foot skin microbiota of clinically healthy cows and its potential role in disease development is yet to be investigated [2]. Functional differences in motility/chemotaxis, respiration, iron acquisition, phosphorus metabolism, cell division and cell cycle, and regulation and cell signalling, have been demonstrated between healthy and diseased foot skin; however, it is unknown if these differences are detectable prior to the appearance of visible BDD lesions [4].

Srinivas *et al*. [5] investigated the contribution of host genetics to the skin microbiota using a fourth generation of an advanced intercross mouse line with 1,199 informative SNPs. They demonstrated 3 significant and 6 suggestive quantitative trait loci (QTL) associated with 9 operational taxonomic units (OTUs). Using the 15th generation of the same mouse line and increasing the SNP number to 53,203, Belheouane *et al*. [6] also investigated the effect of host genetics on skin microbiota, describing 21 significant SNP-skin microbiota associations and identifying genes related to skin inflammation and cancer. However, the association of the bovine foot skin microbiota and BDD related bacteria with the host genetics remains unclear.

We describe herein the bovine foot skin microbiota using 16S rRNA amplicon and shotgun metagenomic sequencing on samples taken from dairy cows from three UK dairy farms. We show differences in the foot skin microbiome profiles of clinically healthy animals that were associated with subsequent development of BDD. We also present the first co-occurrence analysis of the bovine foot skin microbiome showing ecological relationships among bacterial species in the microbiome. We hypothesise that taxonomical and functional differences, and differences in ecological interactions between bacteria in the normal foot skin microbiome may predispose an animal to development of BDD lesions. Additionally, using genome-wide association and regional heritability mapping approaches, we provide first evidence for significant interactions between host genotype and bovine foot skin microbiota profiles.

Materials And Methods

Detailed description of methodology is provided in the Supplementary Information.

Ethics and overview of the study population

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

Sample collection and classification into foot-health groups

Primiparous and multiparous Holstein cows (259) from three farms (detailed description of the three farms is provided by Griffiths *et al.* [7]

ne 2017 and approximately 3–4 weeks prior to their expected calving. Animals were restrained in a

handling crush suitable for lifting feet for inspection. The back-left foot was lifted, and gross contamination removed using a clean paper towel. Sterile cotton swabs were used to sample the area of the foot most susceptible to developing BDD lesions, namely at the skin-horn junction of the heel bulbs [8]. Samples were initially kept on ice and were frozen at -80^{o}C within a few hours for use in 16S rRNA gene sequencing. All four feet were inspected, and lesions recorded of any of the five clinical BDD stages according to the established M-scoring system [9]. Feet were inspected on three further occasions: one week, four weeks and 8–10 weeks post-calving. This resulted in classification of the study population into four foot-health groups based on foot lesion data for all four feet: HtHt cows never had digital dermatitis, HtIn were healthy pre-calving, but subsequently developed BDD, InIn had BDD pre-calving and did not recover at any sampling point, and InHt had BDD pre-calving but recovered by the second sampling point. Shotgun metagenomic analysis was undertaken for five samples from each of the HtHt and HtIn groups (all from the same farm) to compare cows that developed BDD with those that did not with higher taxonomic resolution and to investigate differences in functional profiles.

In a genome-wide association study (GWAS), 554 cows from the three farms were studied to identify genomic regions and potential candidate genes associated with lameness traits [10]. This also allowed us to investigate associations between host genotype and the foot skin microbiota for 242 of these animals for which both foot skin microbiome and genomic data were available.

DNA extraction, 16S rRNA gene amplification, and sequencing

Microbial DNA was extracted from collected swabs using the PureLink™ Microbiome DNA Kit (Invitrogen, Carlsbad, CA, USA) which utilizes chemical, heat and bead-beating cell lysis prior to purification. Extracted DNA samples were stored at -20°C until amplification for sequencing. DNA was also extracted from two swabs that were not used to sample cows; these served as negative controls. Amplification of the V3-V4 hypervariable region of the 16S rRNA gene for sequencing was conducted using Illumina_16S_341F and Illumina_16S_805R universal primers with adapter sequences. Amplicons were sequenced using the Illumina® HiSeq 2500 platform (Illumina, San Diego, CA, USA) to generate 2 x 300 bp paired-end reads. 15% PhiX fragment library was added to increase sample diversity.

Quality control and filtering

PCR primer sequences and Illumina adapter sequences were trimmed using Cutadapt (version 1.2.1), sequencing errors were corrected using the SPAdes sequence assembler (version 3.1.0), and sequences outside the 200-750bp range were removed. Final sequences were analysed using a custom pipeline based on QIIME 1.9.0. Amplicon sequences in each sample were assigned to clusters, based on 97% similarity threshold, using the Silva database (release 123) and the VSEARCH 1.1.3 and SWARM clustering algorithms, merging the results. Potential chimeric sequences were discarded. In total, 48,991,273 analysed sequences were clustered in 75,643 different operational taxonomic units (OTUs).

Analysis of taxonomic composition

Taxonomic assignment was carried out using QIIME and the RDP classifier. OTUs were removed from the dataset if they appeared in fewer than ten samples. Samples were rarefied to 135,000 sequences per sample leading to exclusion of 17 samples; consequently, 242 samples remained in the final dataset.

Comparison of microbial diversity between different foot-health groups

Alpha diversity was assessed using the Shannon and Simpson diversity indices for species evenness and the Chao 1 index for species richness. Data were analysed using both the t-test and the Wilcoxon rank sum test to compare foot health groups to each other.

Beta diversity was assessed at the OTU level with unweighted and weighted UniFrac phylogenetic distances using QIIME (v2). Phylogenetic distance matrices were analysed using Principal Coordinates Analysis (PCoA) and plots were generated and visualized in EMPeror.

Beta diversity was further investigated using pairwise Permutational Multivariate Analysis of Variance (PERMANOVA) with 999 permutations at farm level after restricting the dataset to the HtHt and HtIn groups of primary interest. Differences in beta diversity between farms in the HtHt and HtIn foot health groups was also further investigated using DEICODE in QIIME (v2) to carry out Robust Aitchison Principal Coordinates Analysis [11]. Qualitative data generated was used to construct a biplot showing which OTUs were most influencing beta diversity [12].

Comparing the foot-skin microbiome between HtHt, HtIn and InIn foot-health groups.

For genus-level comparison between samples from cows with different DD status, the dataset was restricted to the twenty most prevalent phyla and genera with a minimum 0.5% mean relative abundance. Log fold changes (Log10) were calculated for each sample and mean relative abundances were logit transformed. Response screening was carried out in JMP Pro 12 (SAS Institute Inc., Cary, NC) to evaluate the differences in OTU (genus level assignments) relative abundance between the samples with different foot-health status. P-values were adjusted for False discovery rate (FDR) and presented as Robust FDR LogWorth. Log fold change of genera was plotted versus Robust FDR LogWorth using Y as correlation and X as circle size, and effect size as colouring.

Because of the limitations of response screening as a method for analysing compositional data [13], data were also analysed using the Songbird package for multinomial regression in QIIME 2 to rank differential abundance of OTUs in the foot-health groups of primary interest (HtHt and HtIn) for comparison.

Co-occurrence/ Network analysis

To identify the ecological interactions [14] among the microbial taxa in the samples, co-occurrence analysis for the previously identified OTUs was performed using the SParse InversE Covariance Estimation for Ecological ASsociation Inference tool (SPIECEASI, [15]). OTUs below 0.005% of the total frequency were excluded (as proposed by Bokulich *et al*, 2013 [16]). This approach reduced the number of OTUs from 75,643 to 3,039. Network analysis was carried out using Cytoscape Version 3.6.1 (USA). Comparisons were made between HtHt and HtIn groups to identify mutualistic or competitive interactions that differ between the two disease groups at phylum level, and therefore may influence development of BDD lesions. Network statistics were computed using NetworkAnalyzer in Cytoscape. Genus level analysis was restricted to the following genera (this was guided by results obtained from response screening analysis): *Succiniclasticum* spp., *Porphyromonas* spp., *Acholeplasma* spp., *Anaerococcus* spp., *Fastidiosipila* spp., *Prevotella* spp. and *Peptoclostridium* spp., which were found to be more prevalent in HtIn groups compared to HtHt groups, and *Brachybacterium* spp. and *Macrococcus* spp. which were found to be more prevalent in HtHt groups. *Treponema* spp, were also investigated as they were absent from the top 20 most prevalent genera in HtHt samples, but present in the top 20 for HtIn samples, and they are widely considered to be one of the key pathogens in BDD pathogenesis [1]. The network propagation algorithm “Diffusion” was used for each group of interest to give the top 10% most highly connected nodes according to network distance [17].

Shotgun metagenomic analysis

To maximise the chances of achieving sufficient sequencing depth, cows were selected at random from those whose previous 16SrRNA samples had a DNA content of > 5ng/µl after the initial DNA extraction, as measured using the Qubit™ dsDNA HS Assay Kit. Microbial DNA was extracted from a second set of swabs that had been collected parallel to those used in the marker gene analysis. The DNA extraction method was the same, using the PureLink™ Microbiome DNA Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Agarose gel electrophoresis was carried out using SYBR green as the nucleic acid stain (Thermo Fisher Scientific Fair Lawn, NJ, USA) to ensure presence of clear DNA bands. Library preparation was carried out on gDNA samples using the Nextera XT kit (Illumina). gDNA input was quantified using Qubit™ to ensure 1ng of each sample was submitted for tagmentation. The libraries were sequenced on an Illumina HiSeq 4000 platform using sequencing by synthesis (SBS) technology to generate 2 x 150 bp paired-end reads.

Quality control and filtering of shotgun metagenomic sequences

Data files were demultiplexed and converted to FASTQ format using Casava v.1.8.2 (Illumina). FASTQ files were trimmed using option _O3 Cutadapt version 1.2.1 [18] to exclude those matching Illumina adaptor sequences by $\geq$ 3bp at the 3’ end. Reads were further trimmed to remove low quality bases, using Sickle version 1.200 with a minimum window quality score of 20. After trimming, reads shorter than 20 bp were removed, and single reads were excluded as length distributions showed they were of poor quality. Host reads were removed following alignment against the host *Bos taurus* genome using Bowtie2 v2.2.6 [19]: read pairs where one or both reads aligned were removed. The remaining reads in pairs were merged using PEAR v0.9.11 [20] to form a single long read based on overlapping homology. Those that could not be merged in this way were concatenated with an intervening N-base. Resulting sequences underwent taxonomic assignment using Kraken v0.10.6 [21] and results were filtered using a confidence threshold of 0.1. Results were analysed using Linear discriminant analysis effect size (LefSe) [22] to determine taxa most likely to explain differences between the two classes HtHt and HtIn. The HUMAnN2 search strategy [23] was used to functionally annotate read data and abstracts to show biological pathway abundance and completeness. Finally, reads that did not align to their pangenomes using this strategy were submitted to a protein database (UniRef) for translated searching [23]. The gene families identified were further analysed using the MetaCyc database to reconstruct and quantify complete metabolic pathways [24].

Genome Wide Association and regional heritability mapping study of foot skin microbiota related traits

Animal sampling and genotyping are described by Sánchez-Molano *et al*. [10]. Phenotypic traits (n = 10) analysed here included three different alpha diversity indices; Chao1, Shannon, Simpson indices, and relative abundances of seven genera; *Porphyromonas* spp., Clostridiales Family XI, *Fastidiosipila* spp., *Peptoclostridium* spp., *Macrococcus* spp., *Treponema* spp., and genera of the family Bacteroidetes.

The Genomic relationship matrix (GRM) was computed using GEMMA [25] and principal component analysis (PCA) was used to find out any genetic structure of the cow population. 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 (RHM analyses failed to converge when the GRM was fitted); further correction for the inflation factor (λ) was applied as described by Amin *et al*. [26]. REACTA [27] was first used to assess the full genomic variance for each trait with a general explanatory analysis. GWA and RHM was performed using GEMMA [25].

Results

A summary of the number of samples included in each foot-health group, and the farm of origin, can be found in Supplementary Information Table 1.

Table 1
Alpha diversity metrics showing species richness and evenness. Statistically significant P-values are shown in bold (P < 0.05), including the differences with P < 0.1. (HtHt: The cows which remained healthy throughout the study, HtIn: The cows which were healthy at initial sampling, then developed DD, InIn: The cows which had DD at all inspection points, InHt: The cows which had DD at initial sampling then recovered, P-value: P value of t-test, SE: standard error, N_P_value: P-value of nonparametric Wilcoxon rank sum tests).

	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	0.1			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	12511.4	300.93			10.12	0.11			0.04	0.003		
HtHt	112	13412.2	190.76	0.6445	0.7079	10.61	0.06	**0.0166**	0.21	0.05	0.002	**0.0647**	**0.0452**
InHt	16	13162.6	504.69			10.2	0.16			0.04	0.005		
InIn	59	12511.4	404.08	0.459	0.3257	10.12	0.15	0.8114	0.7661	0.04	0.003	0.5691	0.4532
InHt	16	13162.6	775.94			10.2	0.29			0.04	0.005		

Taxonomic composition

Taxonomic composition was examined for HtHt and HtIn groups at phylum and genus level. Dominant phyla were Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria. For HtIn samples, Tenericutes and Spirochaetae also accounted for > 1% of bacteria identified. The most abundant family identified were Ruminococcaceae from the phyla Firmicutes. This was reflected at genus level, where the most abundant identified genera were *Ruminococcaceae* UCG-005 and UCG-010. The potential pathogens *Porphyromonas* spp. and *Treponema* spp. were identified at higher relative abundance in the HtIn group compared to the HtHt group (Supplementary Information Fig. 1).

Comparison of microbial diversity between foot-health groups

Differences in sample richness and evenness were identified between foot-health groups. Alpha-diversity metrics overall suggested that HtHt samples had significantly greater microbial diversity than InIn or InHt samples, and a tendency to greater microbial diversity than HtIn samples (Table 1).

Graphs showing PCoA of unweighted UniFrac distances displayed by farm and by foot-health group are shown in Figs. 1A and 1B. Farm three clusters away from farms one and two, suggesting the phylogeny of farm three samples differs from the other farms. Graphs showing PCoA of weighted UniFrac distances for farms and foot-health groups are available in Supplementary Information Fig. 2.

Because of these differences in beta-diversity found at farm level, PERMANOVA was used to test for significant differences in beta-diversity between HtHt and HtIn foot-health groups for each farm separately. Results show differences in farm one for both unweighted and weighted UniFrac distances ($P = 0.018$ and 0.005 respectively) and in farm three for unweighted UniFrac distances ($P = 0.001$). No significant differences were found for farm two, or for weighted UniFrac distance for farm three.

The biplot resulting from DEICODE analysis identified OTU32700 as most influential in causing farm three samples to cluster away from Farms 1 and 2 (Fig. 1C). This OTU was identified as coming from the Flavobacteriaceae family: specifically, *Aequorivita* spp.

Comparing the composition of the foot-skin microbiome of the HtHt foot-health group to the HtIn and InIn groups

Response screening showed that compared to HtHt samples, the HtIn samples showed higher prevalence of the genera *Succiniclasticum* spp., *Porphyromonas* spp., *Acholeplasma* spp., *Anaerococcus* spp., *Fastidiosipila* spp., *Peptoclostridium* spp. and *Prevotella* spp. HtIn samples showed lower prevalence of the genera *Macrococcus* spp. and *Brachybacterium* spp (Fig. 2A). InIn samples showed higher prevalence of the genera *Succiniclasticum* spp., *Porphyromonas* spp., *Treponema* spp., *Acholeplasma* spp., *Anaerococcus* spp., *Fastidiosipila* spp., *Peptoclostridium* spp., *Murdochiella* spp., *Ezakiella* spp. and *Peptoniphilus* spp. compared to HtHt samples, and lower prevalence of *Macrococcus* spp., *Moraxella* spp., *Kocuria* spp., *Jeotgalicoccus* spp., *Acinetobacter* spp., and the Ruminococcaceae NK4A214 group (Fig. 2B).

Songbird analysis identified the same genera that were found to be more abundant in HtIn samples compared to the HtHt samples in response screening to be highly associated with the HtIn group (except for *Anaerococcus* spp.). *Treponema* spp. were the fourth most highly associated taxa with HtIn samples; however, *Macrococcus* spp. and *Brachybacterium* spp. were not found in the top twenty-five taxa most associated with HtHt samples in this analysis. Further taxa strongly associated with either HtIn or HtHt groups are shown in Table 2.

Table 2
Twenty-five taxa most associated with the HtHt and HtIn foot health groups, as determined using Songbird analysis.

	HtHt	HtIn
1	Nosocomiicoccus	Acholeplasma
2	Flavobacterium	Mycoplasma
3	Oceanobacter	Family XIII AD3011 group
4	Ruminococcaceae UCG-010	Treponema 2
5	Nocardiodes	Murdochiella
6	Perlucidibaca	Fretibacterium
7	Salinococcus	uncultured Firmicutes bacterium
8	Bacteroides	Succiniclasticum
9	Prevotellaceae UCG-004	Fastidiosipila
10	Ruminococcaceae UCG-014	Catonella
11	Ruminococcaceae UCG-013	Peptoclostridium
12	Lachnoclostridium	Prevotella
13	Erysipelotrichaceae	Campylobacter
14	Christensenellaceae	Roseburia
15	Oceanobacillus	Peptostreptococcus
16	Clostridium sensu stricto	Ruminococcaceae UCG-014
17	Phocaeicola	Uncultured Bacteroidetes bacterium
18	Corynebacterium 1	Arcanobacterium
19	Chryseobacterium	Parvimonas
20	Ruminococcus 2	Lachnospiraceae AC2044 group
21	Epulopiscium	Prevotella
22	Psychrobacter	Porphyromonas
23	Ruminiclostridium	Cellvibrio
24	Marmoricola	Corynebacterium 1
25	Nocardioides	Fusibacter

Table 3
A. Significant ($P < 0.05$) estimates of heritability and variance. Genomic heritabilities (h^2), genomic variance (Vg) estimated together with their standard errors and number of records (N).

Trait	Vg	h^2	*P value*	N
Mean Relative abundance of Peptoclostridium **spp.**	0.000053 ± 0.000019	0.59 ± 0.18	7E-04	236
Mean Relative abundance of Treponema **spp.**	0.000129 ± 0.000055	0.52 ± 0.00	0.007	236

Table 3B: Summary of genome-wide suggestive and significant SNPs for the traits Mean Relative Abundance of *Peptoclostridium* and *Treponema* spp., including their positions on corresponding chromosomes (BTAs) and significance level (*P*-value).

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

Table 3C. Summary of the consensus genomic regions identified in the 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	5E+07	5.1E+07	0.00057	28.07%	*ZNF750*
Mean Relative abundance of *Treponema* spp.	1	1.1E+08	1.1E+08	1.17E-05	9.88%	*GMPS* and *PLCH1*
	1	1.2E+08	1.2E+08	0.00028	1.97%	*MBNL1*
	16	7.9E+07	8E+07	1.23E-06	34.78%	*PTPRC*
	17	7185597	8690167	9.14E-05	7.11%	*LRBA*

Co-occurrence analysis

Both HtHt and HtIn sample groups had low network density and network centralisation with no hub nodes identified. Network heterogeneity was slightly lower for HtIn groups with a higher average number of neighbours. HtIn groups had fewer connected components, shorter characteristic path length and smaller network diameter despite larger number of nodes, showing stronger connectivity and shorter expected distances between nodes.

When the genera that were known to differ in relative abundance or overall presence between groups were selected and examined alongside their 10% most highly connected neighbours, it was noted that more negative interactions existed in HtIn groups.

Shotgun metagenomic analysis

Shotgun metagenomic analysis showed some differences in taxa present in HtHt compared to HtIn samples. HtHt samples had increased relative abundance of many gram positive bacteria from the phylum Actinobacteria which would be expected to be part of the healthy foot skin flora (Fig. 3A) [4, 28]. *Corynebacteriaceae* were previously found to be over-represented in healthy skin samples [3]. HtIn samples showed biologically relevant increases in wall-less bacteria from the *Tenericutes* phyla, which has previously been found to be more abundant in DD lesions [28]. Taxonomic assignment of reads was low and therefore the significance of these findings is uncertain; however, there may be some agreement with the 16S rRNA sequencing analysis in finding increased *Acholeplasma* spp. in HtIn samples and increased *Brachybacterium sp.* in HtHt samples.

Ten functional pathways were identified as significantly more abundant in the HtHt group (Fig. 3B). All were metabolic pathways for synthesis or degradation of amino acids or fatty acids, or pathways involved in nucleotide synthesis. Eight functional pathways were identified as significantly more abundant in the HtIn group. Three of these pathways were associated with degradation of nucleotides and one indicated production of 4-deoxy-L-*threo*-hex-4enopyranuronate, which is a uronic acid resulting from the degradation of many polymers. These include plant polymers such as pectin and gellan, but also important components of connective tissue such as heparin, heparin sulfate, hyaluronan and chondroitin sulfate [29] (Fig. 3B). Despite detection of some differences in individual functional pathways, no overall differences in abundance for gene families in the GO slim categories of biological processes, cellular components, or molecular functions were detected (Supplementary Information Fig. 3A, B and C respectively).

Genome-Wide Association study and regional heritability mapping of foot skin microbiota related traits

Table 3A shows the total genomic variance and heritability estimates for the relative abundance of *Peptoclostridium* spp. and *Treponema* spp. All other examined traits are not included due to total genomic variance estimates being non-significantly different from zero. The heritabilities for the relative abundances of *Peptoclostridium* spp. and *Treponema* spp. were 0.59 ± 0.18 and 0.52 ± 0.00, respectively.

Suggestive and significant SNPs associated with these two traits after GWA analyses are shown in Table 3B. The association between individual SNPs and relative abundances of *Peptoclostridium* spp. and *Treponema* spp. are also shown in Manhattan plots (Fig. 4).

The RHM analysis identified one suggestive region on both BTA1 and BTA6 and two suggestive regions on BTA19 for the trait relative abundance of *Peptoclostridium* spp. For the trait relative abundance of *Treponema* spp. RHM results indicated one region on both BTA1 and BTA16 with genome-wide significance (Fig. 5a), besides suggestive regions on BTA1, BTA11, BTA17 and BTA19 (Fig. 5b).

The results of GWA and RHM analyses were combined, and a consensus table of genomic regions was created with the start and ending positions of each region on corresponding BTAs, the proportion of genomic variance explained by each region, and potential candidate genes neighbouring the regions (Table 3C). Interestingly, some of the identified regions explained a substantial proportion of the genomic variance with the region in BTA19 explaining 28.07% of the genomic variance for the relative abundance of *Peptoclostridium* spp. and the region in BTA16 explaining 34.78% of the genomic variance for the relative abundance of *Treponema* spp.

Discussion

We show differences in the commensal microbiota of the foot skin between dairy cows that remained healthy and those that went on to develop BDD. Additionally, we identify for the first time genomic regions associated with the relative abundance of bacterial genera important in the development of BDD lesions.

Although HtHt samples had greater species richness and evenness, statistically significant differences in alpha diversity were observed only when comparing samples from feet that were healthy and those that were infected at the time of sampling. Low bacterial diversity has been associated with skin inflammation in ovine footrot [30] and has been observed in bovine mastitis cases [31]. We did not detect this in advance of the appearance of morphological BDD lesions, indicating that microbiome diversity changes were occurring within the 11-14-week sampling interval. The use of probiotics to modify the gut microbiota has become an accepted concept for improving intestinal health in people [32]. Although similar research pertaining to the skin microbiome is in the early stages, there is evidence that topical application of Lactobacillus bacteria and ammonia-oxidizing and nitrifying bacteria may help to maintain a healthy skin microbiome [33]. There is also some indication that probiotics may be useful for treatment of atopic dermatitis in children, and there may be mechanisms by which using probiotics to influence the gut microbiome may exert beneficial effects on the skin [34]. It is possible that a similar concept for preventative treatment targeting the maintenance of microbial diversity in the bovine foot-skin may be successful in halting the development of BDD lesions and is a promising area for future research.

Differences in unweighted UniFrac distances were noted between farm three and the other two farms. Unweighted Unifrac distance provides a qualitative measure of differences in phylogeny between two habitats [35], suggesting that the foot-skin habitat of cows on farm three differs from those on the other two farms. These differences in the foot-skin microenvironment might be caused by differences in management systems, which have been previously shown to explain a proportion of the variation in the bovine faecal microbiota (Hagey 2019), members of which comprised the majority of identified bacteria in the present study. However, DEICODE analysis showed differences to be driven by *Flavobacterium* spp., which was identified by the Songbird analysis as associated with HtHt samples. This could reflect the fact that a greater proportion of farm three samples belonged to the HtHt foot-health group than on the other two farms, or an association of a rare species with farm three only.

PERMANOVA analysis of unweighted UniFrac distances on farm three also showed that beta diversity of HtHt samples differed from HtIn samples, suggesting that there may be differences in the skin micro-environment between the foot-health groups driven by different founding populations of microbes [35]. Farm one samples also showed this pattern as well as a statistically significant difference in weighted UniFrac distances, showing quantitative differences in relative taxon abundance between HtHt and HtIn groups. This difference in the quantitative measure of beta-diversity can be caused by the effects of more transient factors such as nutrient availability [35]. This would certainly fit with the paradigm that the pathogenesis of bovine digital dermatitis involves skin damage which creates an anaerobic environment favourable to lesion-associated pathogens.

The Songbird analysis identified *Mycoplasma* spp., *Acholeplasma* spp. and *Treponema* spp. in association with the HtIn foot health group (Table 2). *Mycoplasma* spp. have been previously identified as associated with bovine digital dermatitis [1, 36], as have *Acholeplasma* spp. [1]. Songbird analysis also identified *Murdochiella* spp., which have been previously associated with wounds and bacterial vaginosis in people, but are previously unreported for BDD [37, 38].

In the microbiome network analysis including the genera known to differ in abundance in HtIn compared to HtHt groups, the increase in negative interactions in the HtIn group may indicate a more competitive environment in the foot skin microbiome of these cows. This could be attributable to overgrowth of these genera relative to others [39]. It is not known whether this corresponds to dysbiosis and could identify these genera as pathogens that contribute to BDD lesion development.

Shotgun metagenomic analysis showed differences in taxa present in HtHt compared to HtIn samples. LEfSe analysis reveals biologically relevant differences between microbial communities rather than only statistical differences in features; in this project differences in relative abundance between clades and functional pathways. The scores assigned can be interpreted as the degree of consistent difference identified in the two classes that explain the greatest differences between the communities [22]. Functional differences in the microbiome, for example increases in genes for flagellar motility and zinc and copper resistance, have been previously reported in biopsies taken from DD lesions compared to healthy skin [4]. Our data may suggest an increase in pathways relating to degradation of connective tissues; however, overall significant differences in the abundance of gene families responsible were not detected. Either functional differences in the skin microbiome do not materialise before development of morphological lesions or are undetectable from our data, perhaps due to small sample sizes and a large percentage of unassigned sequences.

We show for the first time here that certain regions in the bovine genome may harbour genes associated with the relative abundance of members of the foot skin microbial communities. The proportion of the total genomic variance explained by the detected regions for each trait ranged from 1.97–34.78% suggesting a partially oligogenic architecture; however this could be overestimated due to the Beavis effect [40]. The region associated with relative abundance of *Treponema* spp. on BTA1 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 [41, 42]. *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 [43], especially the expression of *PLCH1* which was shown to be downregulated by lipopolysaccharides (LPS) [44] which are found in the outer membrane of Gram-negative bacteria [45]. 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 [46, 47]. 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 [48]. Mutations on the *LRBA* gene were shown to be associated with immune system related disorders such as immunodeficiency, inflammatory bowel disease [49], and autoimmunity [50]. 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 encodes a putative C2H2 zinc finger protein which was shown to be associated with the skin disorders Seborrhoea-like dermatitis [51] and familial psoriasis [52]. In addition, increased dietary zinc was shown to be associated with reduced BDD incidence in dairy cows [53]. Admittedly, the above proportions of genomic regions may be somewhat inflated and more research is needed to refine estimates of the

collective impact of the identified regions. Nevertheless, our results provide definite evidence of host genetic control of the foot skin microbiota profile, which, combined with the association of the latter with BDD lesion development offer new insights into a complex relationship that can be exploited in selective breeding programmes aiming to enhance bovine foot health.

Conclusion

We have shown for the first time that the bovine foot-skin microbiota is associated with host genotype and the development of BDD lesions. 16S rRNA gene sequencing analysis of swabs taken from morphologically normal foot-skin surfaces identified taxa associated with future development of BDD lesions and taxa which appeared protective. Although differences in microbial diversity on the skin surface were not statistically significant before appearance of morphological lesions, a more competitive environment was detected using network analysis in cows that went on to develop BDD, which could correspond to dysbiosis. Shotgun metagenomic analysis corroborated *Acholeplasma* spp. as detrimental, and *Brachybacterium* spp. as protective, and identified higher abundance of genes that could be associated with collagen degradation in samples from cows that subsequently developed BDD lesions. Finally, we identified regions of the bovine genome associated with relative abundance of *Treponema* spp. and *Peptoclostridium* spp., two of the genera identified by 16S rRNA sequencing as associated with future development of BDD lesions. Collectively this work shows the relevance of the bovine foot-skin microbiota to BDD.

Declarations

Data availability

Sequences are available on the MG-RAST metagenomics analysis server at https://www.mg-rast.org/linkin.cgi?project=mgp91792. The 16S rRNA gene amplicon sequences have been deposited at the NCBI BioProject database (BioProject ID PRJNA702425). The genotype data has been uploaded to a public repository hosted by the University of Edinburgh. Genotypes are therefore publicly available and can be obtained from: Edinburgh DataShare (University of Edinburgh), https://datashare.is.ed.ac.uk/handle/10283/3409.

Acknowledgements

This study was funded by the Academy of Medical Sciences. VB acknowledges support from The Turkish Ministry of Education, and Ministry of Food, Agriculture and Livestock. GO gratefully acknowledges support from the Wellcome Trust (Wellcome Trust ISSF non-clinical Fellowship). Work conducted at the Centre for Genomic Research was part-funded by the Technology Directorate Strategy Group, University of Liverpool. Samuel Higgs, Craig Scarisbrick, Nikolaos Kakatsidis and Yunfei Huang are gratefully acknowledged for assisting on farm sample collection. Imogen Grant and Catherine Gill are gratefully acknowledged for assisting laboratory analyses.

Authors' contribution

VB collected the samples, performed laboratory work, performed data analysis, and wrote part of the first draft of the manuscript. AG performed laboratory work, performed data analysis, and wrote part of the first draft of the manuscript. EKG performed bioinformatics and data analyses. NE, SC, supervised laboratory work. LL performed bioinformatics and data analyses. AL performed sequencing work. SH performed bioinformatics and data analyses. MB performed data analyses. BG organised the farm visits, assisted sample collection and performed foot lesions identification. ESM performed GWAS and RHM analyses. RCB assisted with study design and data analyses. GB supervised GWAS and RHM analyses. AD supervised laboratory work and assisted data analyses. GO (corresponding author) designed the study, provided funding, supervised the study, assisted data analysis and critically evaluated the manuscript. All authors read and approved the final version of the manuscript.

Competing Interests

The authors declare no competing financial interests or other competing interests.

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Figures

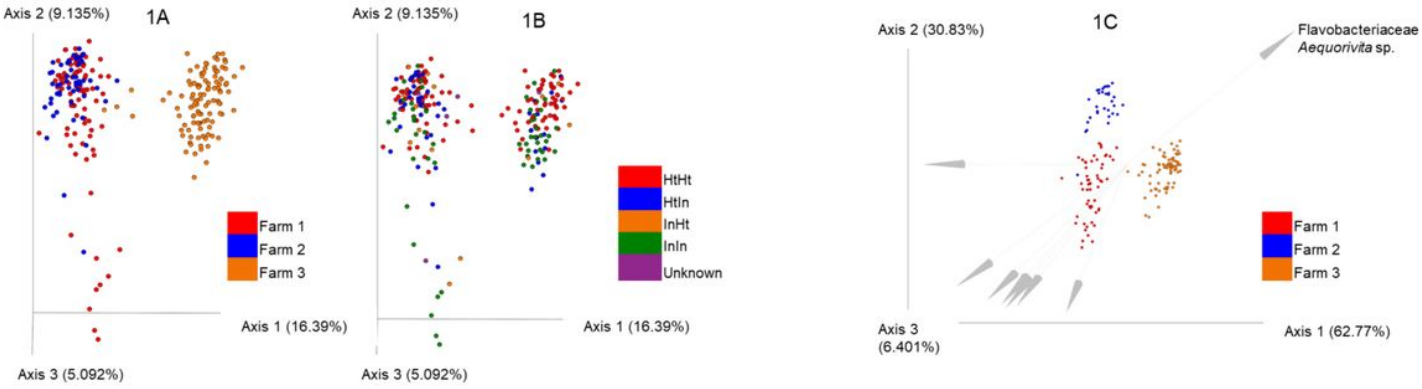

Figure 1

Unweighted unifrac distances showing beta diversity (A) by farm, and (B) by foot health group, with (C) the results from DEICODE analysis identifying the taxon most responsible for Farm 3 clustering away from Farms 1 and 2.

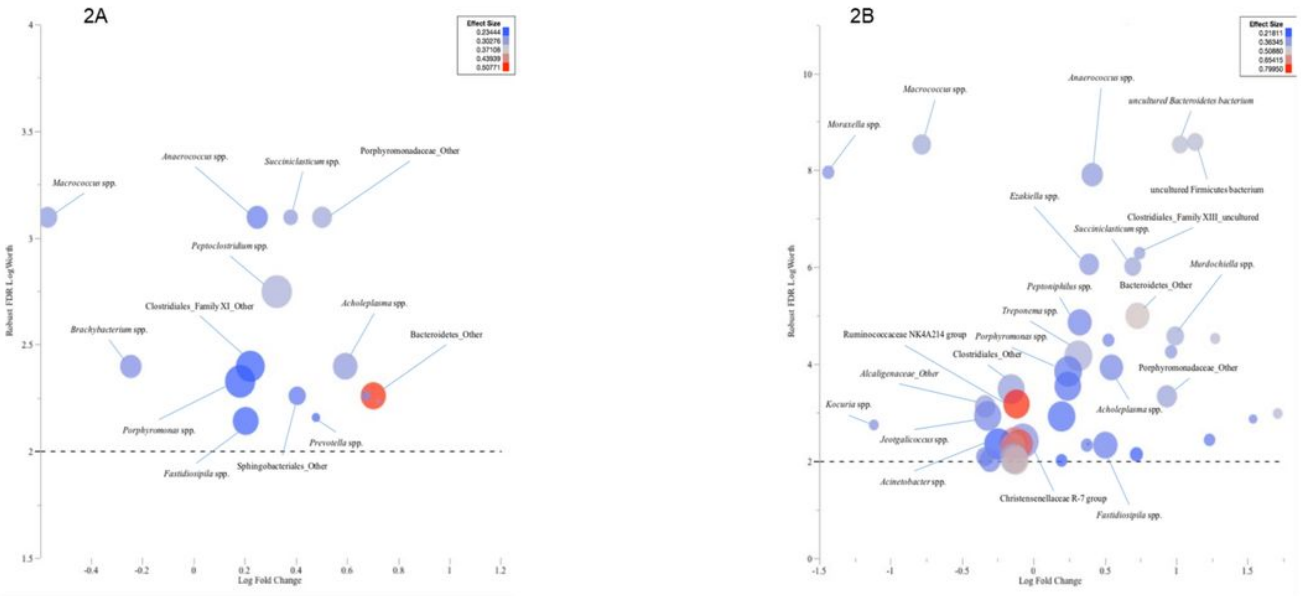

Figure 2

Results of response screening comparing the microbiota profile at genus-level of HtIn samples (2A) and InIn samples (2B) relative to HtHt samples versus corrected robust false discovery rate (FDR) logWorth (i.e. log10P). The dashed line shows the P-values (0.01) adjusted for FDR. The size of circles represents mean relative abundance of each genus, and colour represents the effect size.

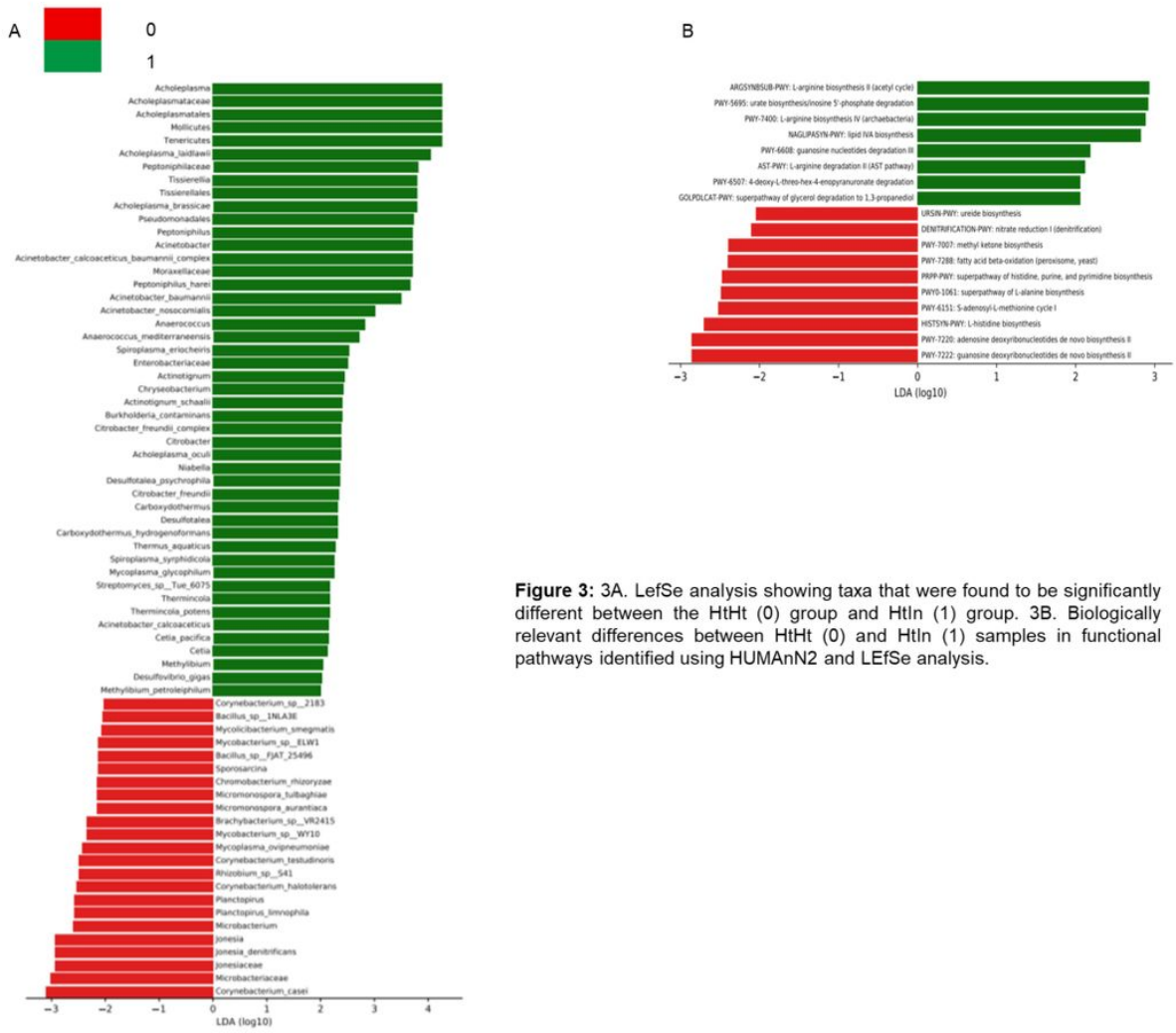

Figure 3: 3A. LefSe analysis showing taxa that were found to be significantly different between the HtHt (0) group and HtIn (1) group. 3B. Biologically relevant differences between HtHt (0) and HtIn (1) samples in functional pathways identified using HUMAnN2 and LEfSe analysis.

Figure 3

3A. LefSe analysis showing taxa that were found to be significantly different between the HtHt (0) group and HtIn (1) group. 3B. Biologically relevant differences between HtHt (0) and HtIn (1) samples in functional pathways identified using HUMAnN2 and LEfSe analysis.

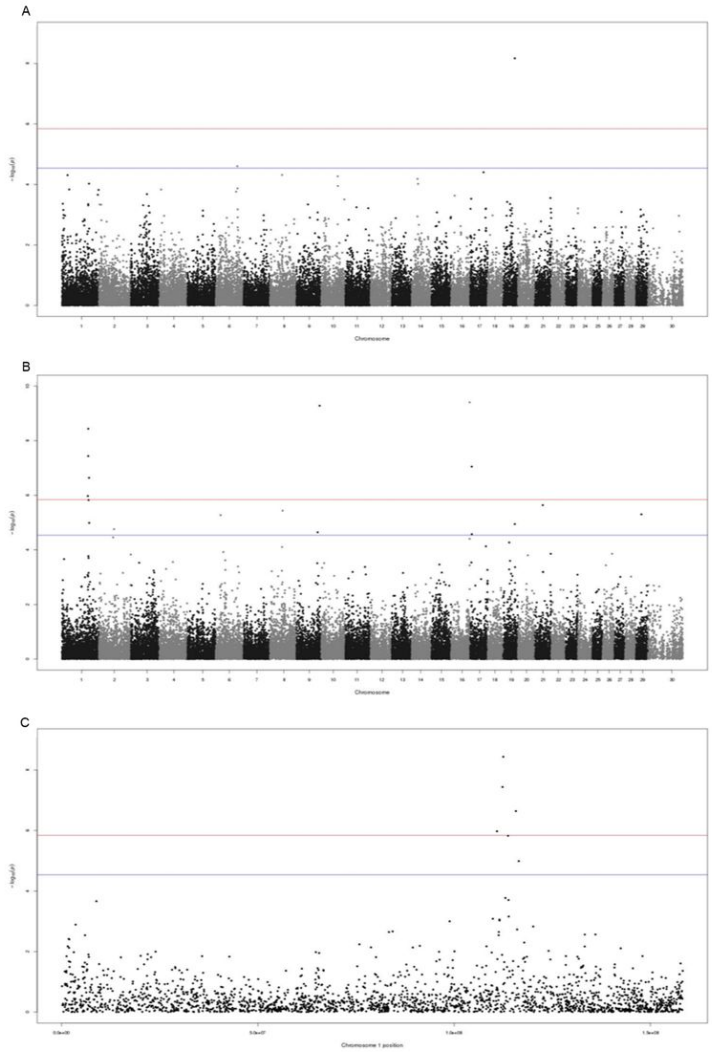

Figure 4

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.

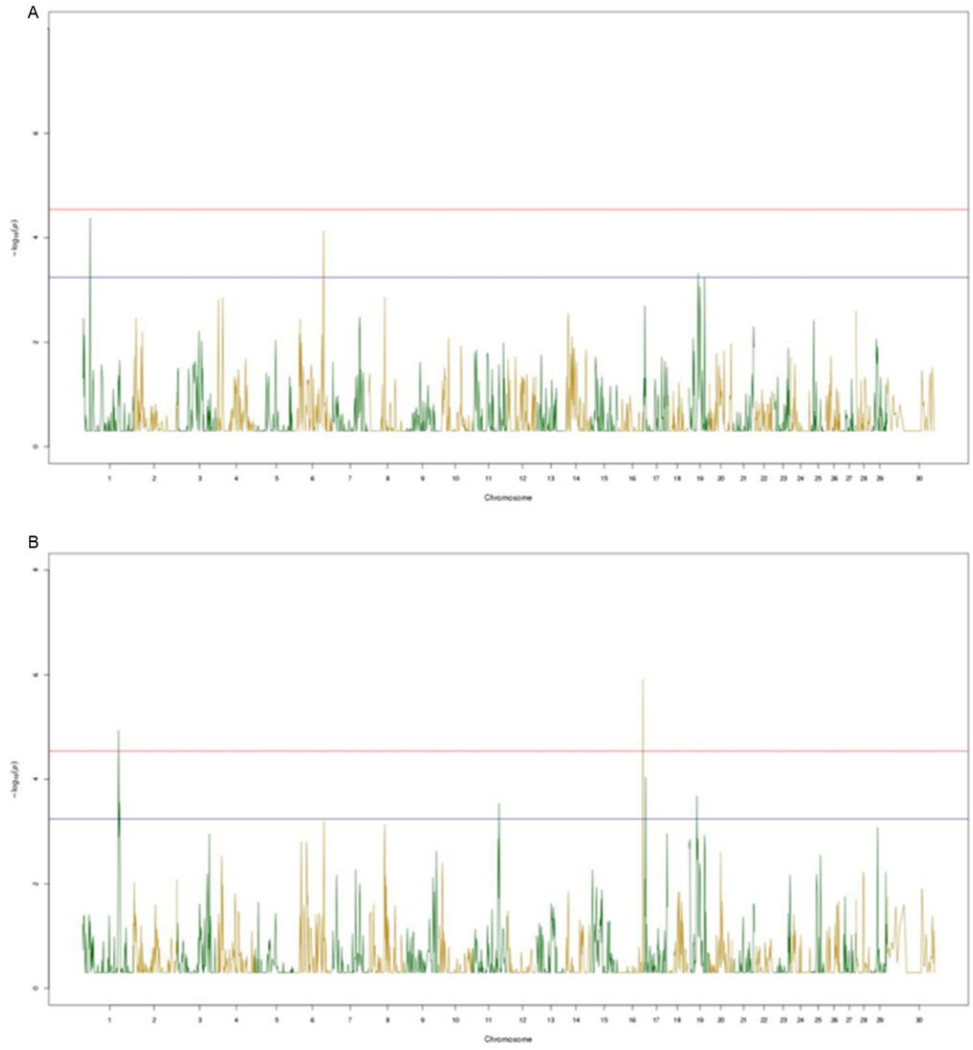

Figure 5

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.

Supplementary Files

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- SupplementaryInformation.pdf