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Metagenomic characterisation of canine skin reveals a core healthy skin microbiome

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Furthering our knowledge of the skin microbiome is essential to understand health and disease in canines. To date, studies into the canine skin microbiome have focused on 16S rRNA high throughput sequencing however, these lack the granularity of species and strain level taxonomic characterisation and their associated functions. The aim of this study was to provide a comprehensive assessment of the skin microbiome by analysing the skin microbiome of 72 healthy adult colony dogs, across four distinct skin sites and four breeds, using metagenomic sequencing. Our analysis revealed that breed and skin site are drivers of variation, and a core group of taxa and genes are present within the skin microbiome of healthy dogs, comprising 230 taxa and 1219 gene families. We identified 15 species within the core microbiome that are represented by more than one strain. The biosynthesis of secondary metabolites pathway was enriched in the core microbiome suggesting the skin microbiome may play a role in colonisation resistance and protection from invading pathogens. Additionally, we uncovered the novelty of the canine skin microbiome and show that further investigation is required to increase the suitability of current databases for metagenomic sequencing of canine skin samples.

The skin provides an interface between the external environment and an individual. It acts as a physical, immunological, microbial barrier; a sensory organ; plays an important role in body temperature regulation and protects the body from dehydration¹. The skin microbiome is the collection of microorganisms living on the skin and recent research has demonstrated across a range of hosts that it is important for maintaining health, such as through modulating the innate immune response², preventing colonisation from pathogens, and ensuring optimal skin function. Many factors influence the microbial composition of the skin, such as host genetic variation, lifestyle, hygiene, and the environment³. In both humans and dogs, alterations in the skin microbial composition have been associated with skin conditions, such as psoriasis and atopic dermatitis, although it remains unclear whether these changes are cause or effect⁴. However, it is thought that there may be links between factors such as altered epidermal barrier function and dysbiosis of the normal skin microbiome⁵.

In dogs, skin sites can be categorised into two groups based on physiology, haired skin sites and mucosal surfaces/mucocutaneous junctions⁶. However, as with the skin of most animals, the canine skin is covered mostly by dense fur with apocrine glands distributed throughout their bodies. The eccrine glands which produce sweat are found only on a dog's paw and, in comparison to humans, dogs have a more even distribution of sebaceous glands throughout their body⁶. Sebaceous glands produce lipids, such as cholesterol, which are involved in hair coat sheen and softness⁷.

To date, studies of the canine skin microbiota have focused on 16S rRNA high-throughput sequencing to describe the taxonomic profile of healthy dogs and those with skin conditions, such as atopic dermatitis^{1,8–19}. Across these studies, the most dominant phyla observed across different skin sites are the Proteobacteria, with Firmicutes, Actinobacteria and Bacteroidota observed to a lesser extent. Studies have described differences in the skin microbiota across skin sites including interdigital region of the paw, axilla, concave pinna, ear canal, dorsal lumber, conjunctiva, groin, perianal skin, chin, nasal and abdomen, and between individuals^{8–10}.

A limited number of studies have investigated factors influencing shifts in the microbial composition of the skin of healthy dogs. Seasonal changes and cohabitation with other dogs have been shown to significantly alter skin bacterial communities with dogs living together, in the same household, having a more similar bacterial profile than that of dogs living separately¹. In addition, geographical origin, hygiene status of living environment, access to outdoor environments, life stage²⁰ and diet¹⁹ have been shown to shape the skin microbiota of dogs^{19,20}.

Studies to date have given us a good overview of the taxonomic profile of the canine skin. However, the function and structure of the skin microbiome is still unknown, despite this being critical to understanding the

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role of the microbiome in skin health. The aim of this study was to provide a comprehensive assessment of the skin microbiome using metagenomic sequencing that gives clear insights into the species and strains present on the canine skin, as well as the associated functions that they are fulfilling. In total, samples were collected from four distinct skin sites across 72 dogs comprising four breeds and an age range of nine years and sequenced to a mean depth of 28 million reads per sample. The resulting data was used to give a comprehensive assessment of the structure and function of the canine skin microbiome and its role in health.

Results

Overview of the healthy canine skin microbiome

We analysed the skin microbiome from four distinct skin sites; right ear canal, interdigital region of the left forepaw, dorsal lumbar and right groin, named A, B, C and D respectively (Fig. 1a). After data filtering, a total of 162 samples across breeds and skin sites were analysed (Table S1). We identified a total of 2687 strains that could be identified as 2137 species, 624 genera, 172 families, 35 classes and 26 phyla. The canine skin microbiome is dominated by three phyla: Proteobacteria, Bacteroidota and Actinobacteriota, with these phyla accounting for 85% of the total count. At lower abundances (1–7%), Firmicutes, Firmicutes A and Fusobacteriota were detected. Taxa that were mapped to the Kingdom Bacteria but unassigned at phylum level were termed “unresolved.” Approximately 2% of all reads across sites were unresolved and represent potential novel phyla. At the phylum level the microbial profile was consistent across skin sites with the three most abundant phyla in the same order across all sites; Proteobacteria accounting for 36–40% of the total microbial composition, Bacteroidota accounting for 27–31% and Actinobacteriota for 16–23% (Fig. 1b). At the family level, Porphyromonadaceae, Moraxellaceae and Neisseriaceae were identified as the most abundant across all samples (Fig. 1c).

Breed and skin site are the main drivers for variation in the canine skin microbiome

To understand the main drivers of variation within the canine skin microbiome, we investigated whether breed, skin site or sex were explaining any variation in the skin microbiome using Bray–Curtis dissimilarity visualised using nMDS. We found breed (Fig. 2a) and skin site (Fig. 2b) showed separation on nMDS whereas sex (Fig. 2c) had no effect. PERMANOVA showed statistically significant differences in the microbial profile between three skin site comparisons (interdigital/groin; interdigital/dorsal lumbar; groin/dorsal lumbar; $\text{adj}P \leq 0.01$). All comparisons between ear canal and other skin site showed no statistical significance (supplementary material table S2). The interdigital and groin sites were most different and were driving 4.7% of the variation ($R^2 = 0.047$, $\text{adj}P = 0.006$). The comparisons between groin and dorsal lumbar sites ($R^2 = 0.037$, $\text{adj}P = 0.006$) and between

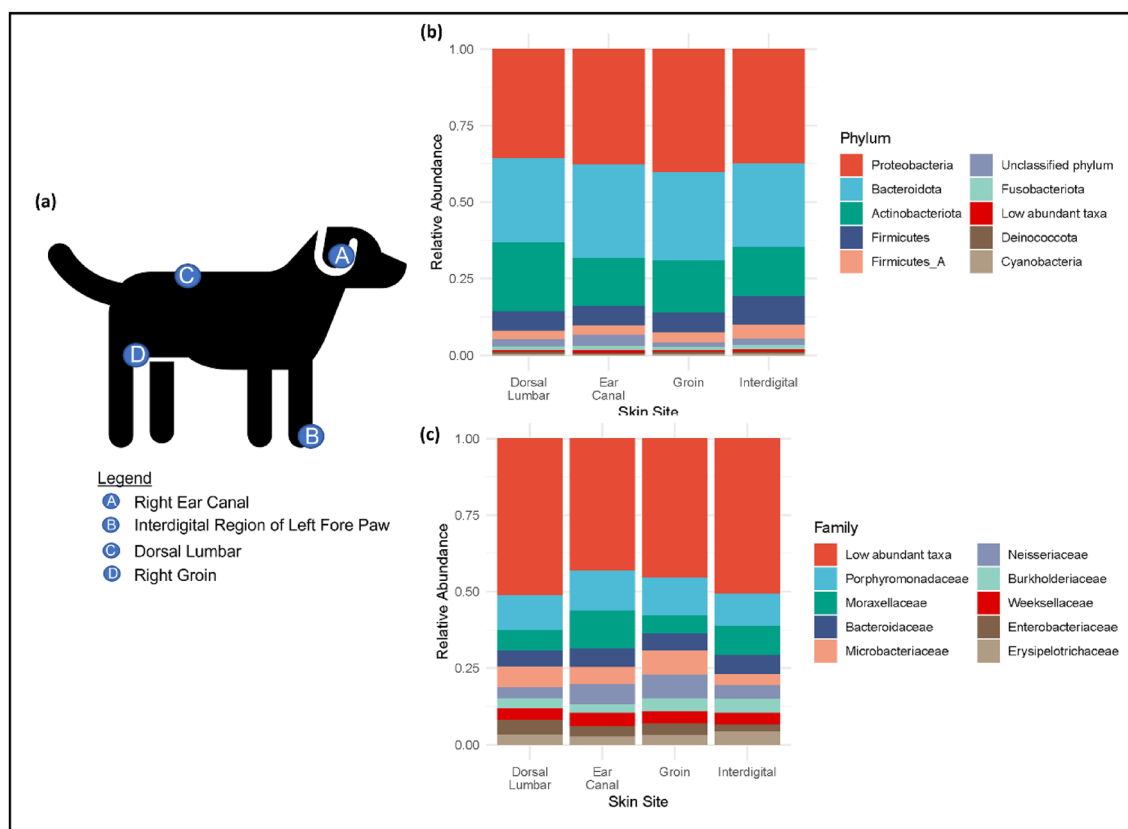


Figure 1. (a) Skin microbiome sampling sites, (b) composition of healthy canine skin at phylum level across skin sites, (c) composition of healthy canine skin at family level across skin sites, families with a mean relative abundance of >0.01 across all samples are plotted.

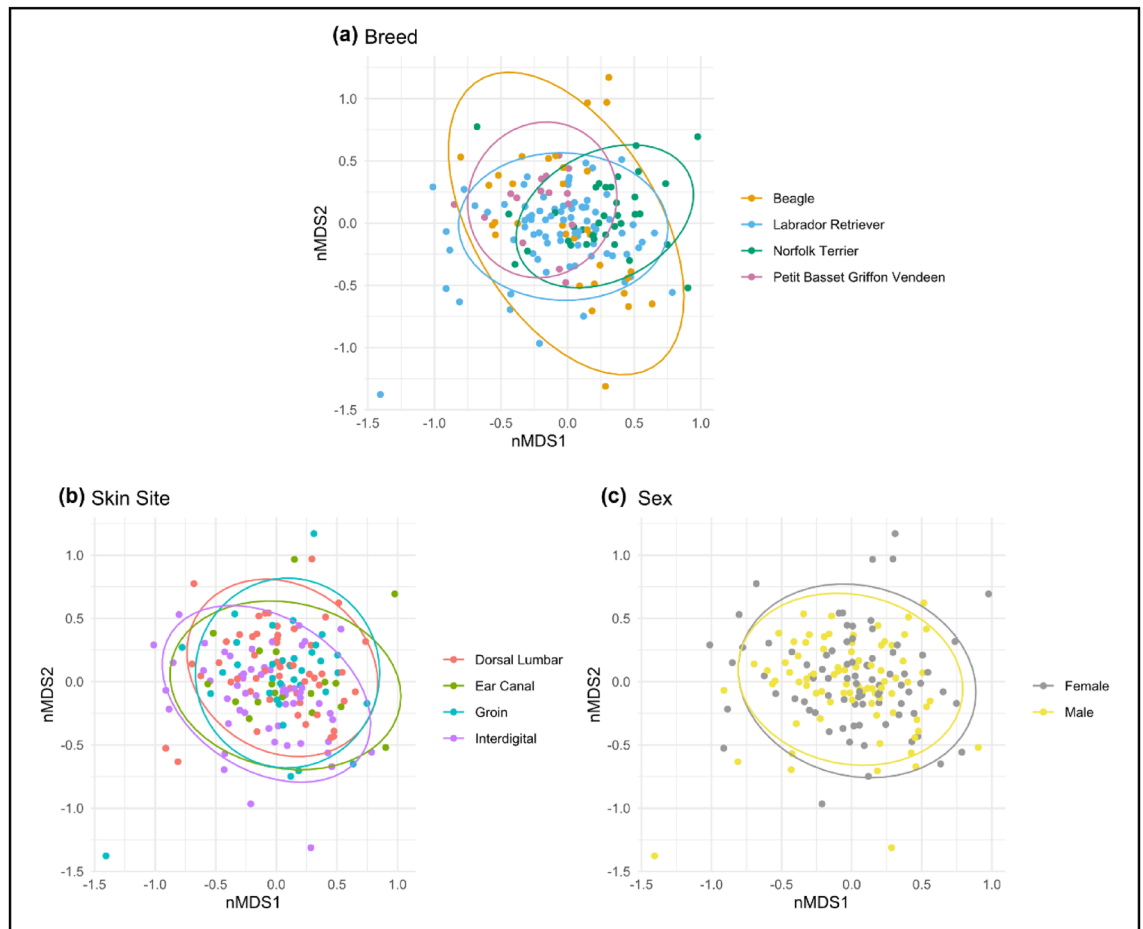


Figure 2. Bray–Curtis dissimilarity matrix plotting using non-metric multidimensional scaling (NMDS) with co-variables (a) breed, (b) skin site, (c) sex, showing breed and skin site as drivers of variation within the skin microbiome.

interdigital and dorsal lumbar ($R^2=0.030$, $\text{adj}P=0.006$) were the second and third biggest drivers for variation in the skin microbiome sites. We discovered that breed was the biggest driver for variation across the skin microbiome using Bray–Curtis dissimilarity and all comparisons showed statistical significance when tested using PERMANOVA ($\text{adj}P \leq 0.05$, supplementary material Table S2). The biggest variation was observed between Beagles and Norfolk Terriers, which were driving 6.5% of the variation in the skin microbiome ($R^2=0.065$, $\text{adj}P=0.006$). Additionally, over 5% of the variation of the skin microbiome can be explained by the differences between Beagles and Petit Basset Griffon Vendéen ($R^2=0.052$, $\text{adj}P=0.042$), and between Labrador Retrievers and Norfolk Terriers ($R^2=0.052$, $\text{adj}P=0.006$). Additionally, a dispersion test was conducted for each comparison within breed, skin site and sex (supplementary material table S3). Skin site and sex were not statistically significantly dispersed. For breed, Beagles were more dispersed than Norfolk Terriers ($\text{adj}P=0.017$) and Petit Basset Griffon Vendéen ($\text{adj}P=0.008$).

Alpha diversity was assessed for skin site and breed using Shannon and Species Richness metrics. The Shannon diversity of the canine skin microbiome ranged between 4.56 and 5.64 across sites and breeds. The species richness of the canine skin microbiome ranged between 574 and 885 across sites and breeds. There was no observed statistical significance in Shannon diversity between skin sites (Fig. 3a) however, the ear canal and interdigital sites were statistically significantly different when measured using species richness ($p \leq 0.05$, Fig. 3b). No other skin sites showed statistical significance in species richness. There were no statistically significant differences in alpha diversity between breeds by either metric (Fig. 3c, d).

Core canine skin microbiome

Due to the interface between the skin and the environment, we wanted to explore whether a core microbiome existed that was present across most dogs, and an accessory microbiome was present that only occurred across a small percentage of the population. To do this we assessed the prevalence of taxa identified on each canine skin site. The prevalence of a taxa was determined by assessing the proportion of samples within which that taxon appeared (Fig. 4a). Subsequently, taxa prevalent at defined thresholds between 10 and 100% across the four sites were overlaid to determine those taxa consistent across all sites (Fig. 4b). Based on cumulative taxa curves, the majority of bacterial diversity occurred in less than 25% of dogs. Furthermore, when overlaying taxa

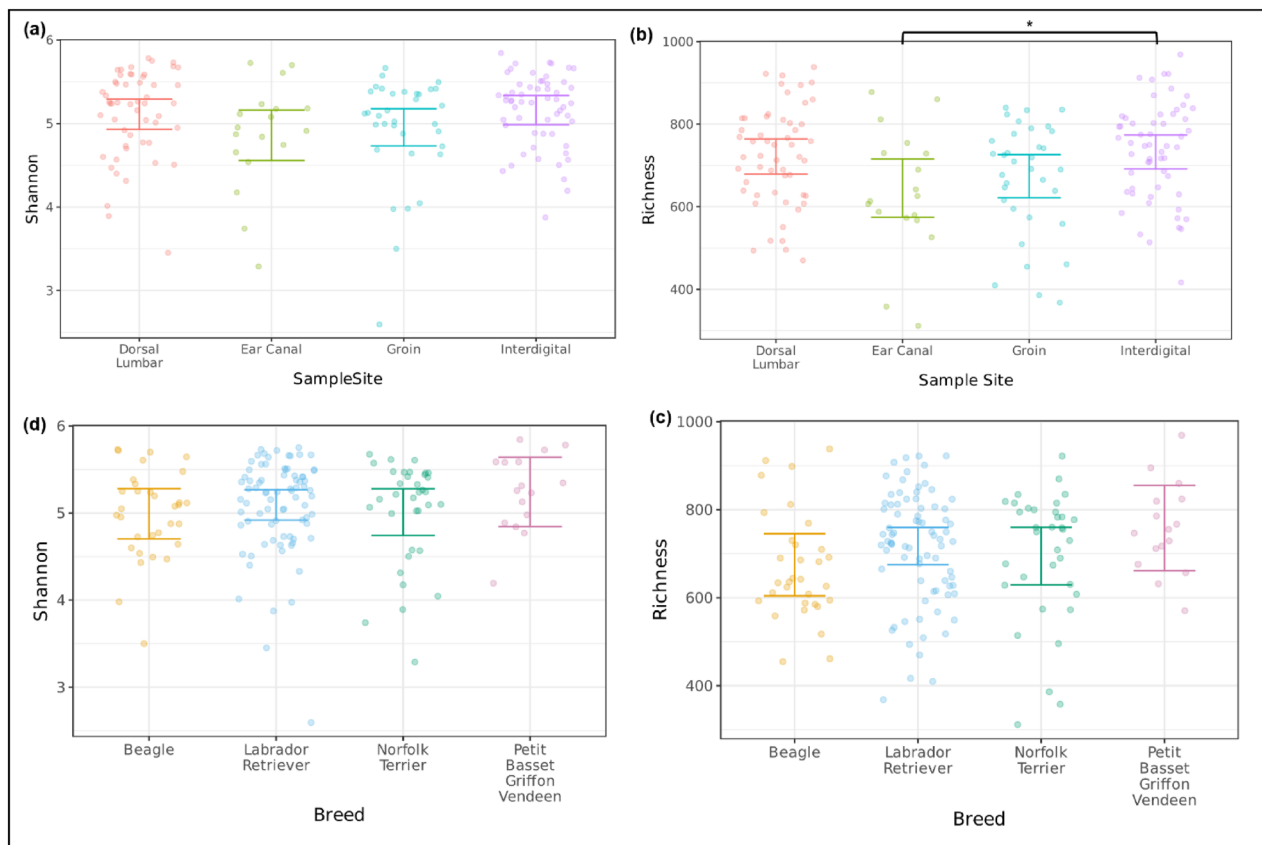


Figure 3. Alpha diversity of the canine skin microbiome; clockwise from top left (a) Shannon diversity across skin sites, (b) species richness of skin microbiome across skin sites, showing statistical difference ($p \leq 0.05$) between the ear canal and interdigital sites, (c) species richness of skin microbiome across breeds, (d) Shannon diversity across breeds.

shared across sites, there was a consistent proportion of taxa shared between 25 and 80% of dogs (59–61%), which decreased sharply when setting a higher threshold than 80% prevalence across dogs. Based on this, we used a prevalence threshold of 80% to define core. At this threshold a total of 375 taxa were detected across all dogs, with 230 of these identified across all sites and termed core (Fig. 4b). All other taxa were termed accessory (3241 taxa). The top three phyla represented in the core and accessory skin microbiome remained the same; Proteobacteria, Bacteroidota and Actinobacteriota however, of note the proportion of Bacteroidota appeared greater in the core microbiome than the accessory. Additionally, a higher proportion of “unresolved” taxa (taxa that were mapped to the kingdom bacteria but not assigned to taxonomy at phyla level) were identified in the core microbiome in comparison to the accessory (Fig. 4c). To further investigate the differences within the core and accessory at phyla level we investigated the fold change of the relative abundance of each phylum between the core and accessory microbiomes at each skin site (Fig. 5). Bacteroidota was highly abundant within the core microbiome in comparison to the accessory (fold change > 1) across all skin sites. Additionally, Fusobacteriota was observed to be more highly abundant in the core microbiome rather than the accessory at the dorsal lumbar site, whereas in the ear canal, groin, and interdigital region of the paw it was more highly abundant in the accessory microbiome. Within the accessory microbiome Proteobacteria, Firmicutes (including Firmicutes A and C), Deinococcota and Cyanobacteria were identified as more highly abundant (fold change < 1) than in the core microbiome. Other phyla were noted as being completely absent from the core microbiome and only present in the accessory as shown by phyla with a fold change of zero in Fig. 5. These included Spirochaetota, Myxococcota, Methanobacteriota, Firmicutes B, Desulfobacterota, Deferribacterota, Chlamydiota and Campylobacterota which were present in the accessory microbiome in all skin sites and absent from the core, showing that the core and accessory microbiomes are mutually exclusive at phylum level.

Within the core canine skin microbiome, we identified a total of 113 strains that could be classified as 136 species, 93 genera, 44 families, 28 orders, 11 classes and 10 phyla. A list of the 230 taxa in the core microbiome can be found in supplementary material Table S4. The most represented families within the core microbiome are represented by members of *Microbacteriaceae*, *Porphyromonadaceae*, *Flavobacteriaceae*, *Bacteroidaceae*, *Moraxellaceae*, *Lachnospiraceae* and *Spingomonadaceae*. The most represented genera within the core microbiome were *Capnocytophaga*, *Porphyromonas*, *Sphingomonas*, *Methylobacterium* and *Porphyromonas A*, where greater than five species belonging to each genus were identified. *Porphyromonas A* was the most abundant genera within the canine skin microbiome with an average abundance of 5.7–7.2% across all sites. *Cutibacterium* (1.2–5.0%), *Spingomonas* (1.3–3.4%) and *Psychrobacter* (1.0–2.1%) were also within the most abundant genera of the canine

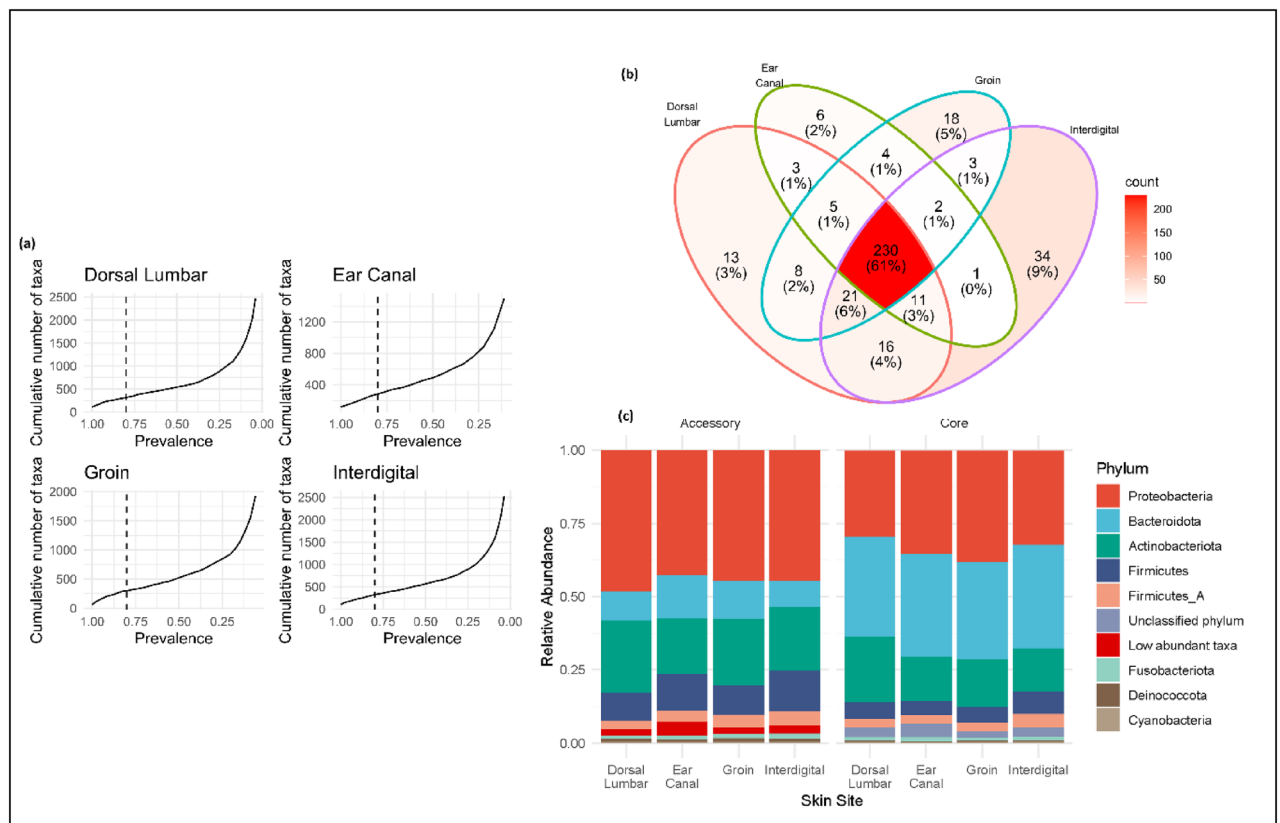


Figure 4. (a) Prevalence of skin microbiome taxa across sites (clockwise from top left; dorsal lumbar, ear canal, interdigital, groin). The dashed line represents a threshold of 80% prevalence, (b) Venn diagram showing the presence of 375 taxa across skin sites and the overlap of 230 core taxa with greater than 80% prevalence across all sites, (c) microbial composition of the accessory and core skin microbiome at each skin site at phylum taxonomic level.

skin microbiome across all skin sites (Fig. 6). A total of 15 species were detected in the core microbiome where more than one strain belonging to the species was present, a list of these species can be found in Table 1. Of particular interest, *Capnocytophaga canimorsus* and *Porphyromonas gulae* were represented by five strains each, and *Capnocytophaga canis*, *Paracoccus marcusii* and *Sphingomonas aerolata* were represented by four strains each.

Core function of the canine skin microbiome

We analysed the gene family metagenomic data to understand the core function of the canine skin microbiome. To establish the core function, we undertook a similar approach to that employed for the taxonomic core microbiome firstly by assessing the prevalence of gene families identified on each canine skin site (Fig. 7a). The cumulative number of genes decreased rapidly after 90% prevalence in dogs and increased rapidly after 12.5% of dogs. Furthermore, between thresholds of 10 and 90%, the proportion of gene families shared across sites was consistent, varying between 80 and 86%. After this point, the proportion of shared gene families decreased as the threshold was increased. Based on this a prevalence threshold of 90% was selected for further study where a total of 1538 gene families were detected. From the 1538, 1219 gene families were identified across all sites and were deemed core (Fig. 7b). All other gene families were classified as the accessory microbiome (total number). A full list of the core gene families identified within this cohort can be found supplementary material Table S5.

The most abundant functions within the core microbiome were functions involved in DNA, energy and intermediary metabolism, such as basic replication machinery genes such as *gyrA* and *gyrB*; and genes involved in biosynthesis of nucleotides such as *nrdE* and *nrdE* (Fig. 7c). Additionally, genes involved in lipid metabolism, such as *fadD*, and RNA metabolism, *rpoB* and *rpoC*, were highly abundant. More interestingly, genes associated with the active transport of large receptor molecules were discovered within the top 20 most abundant genes within the core; *cirA*, *cfrA*, *hmuR* (Fig. 7c). These genes encode outer membrane receptors associated with the transport of ferrienterobactin and colicins. Ferrienterochelin is also known as ferrienterochelin which is iron siderophore that contains an enterobactin, and colicin is a type of bacteriocin. Other genes encoding enterobactin exporter systems were also identified, such as *entS*. Upon delving deeper into the genes present in the core functional microbiome, the structural gene for lantibiotic Pep5 was detected; *pepA*. Other genes from the Pep5 gene cluster were also identified, *pepT*, *pepN*, *pepP*, *pepD*, which include the genes encoding the transport protein for PepA (*pepT*) and the serine protease for proteolytic processing of PepA (*pepP*).

Other commonly occurring genes of interest within the core functional microbiome were genes involved in multidrug resistance, such as proteins belonging to the Resistance Nodulation Cell Division (RND) superfamily.

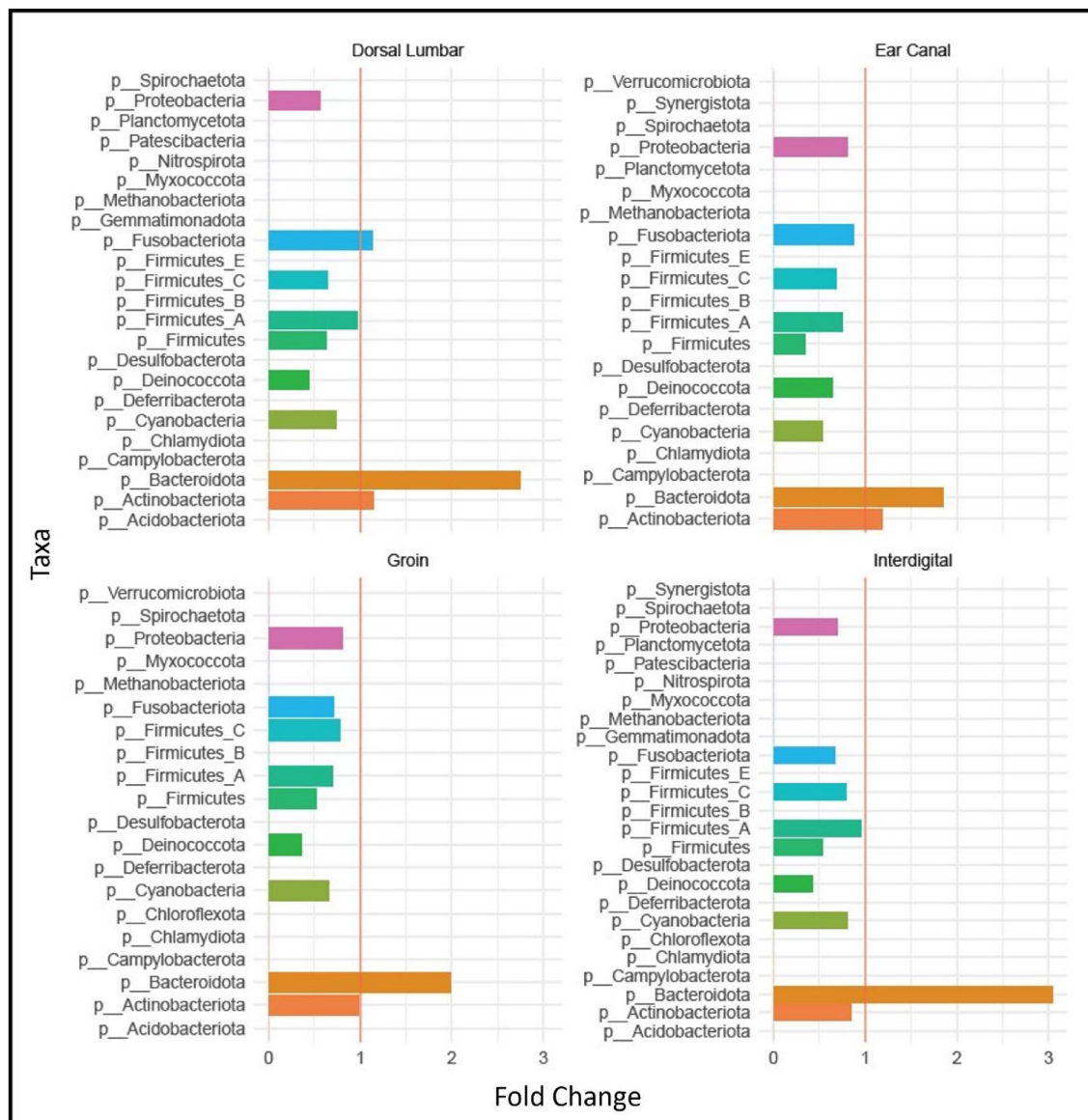


Figure 5. The core and accessory canine skin microbiomes at phyla level at four different skin sites. Clockwise from top left; dorsal lumbar; ear canal; interdigital region of paw; groin. The fold change of the relative abundance of each phylum detected in the canine skin microbiome was calculated and plotted to show which phyla are more highly abundant in the core microbiome (those with a fold change greater than one), and which are more highly abundant in the accessory microbiome (those with a fold change less than one). Phyla which are plotted with a fold change of zero are present within the accessory microbiome but absent from the core microbiome.

Genes encoding multidrug and toxic compound extrusion (MATE) transporters and hydrophobic/amphiphilic exporters (HAE) were identified. Although RND systems are involved in maintaining cell homeostasis, they are also known to broad substrate spectrums and roles in drug resistance. Within our identified core functional microbiome genes involved in multidrug efflux systems are: *mdtA*, *mdtB*, *mdtC*, *mdtE*, *mdtK*, *mdtN*, *emrA*, *emrB*, *acrA*, *mexA*, *mexB*, *tcaB* and *mepA*. Genes encoding penicillin binding proteins, *mrcB*, *pbb2A*, *pbbB*, *pbbC*, were also detected in the core functional microbiome. Finally, genes that could help to provide colonisation resistance to resident microbes were also identified within the core, such as genes associated with adhesins, biofilms and hemolysis; *icaA*, *fimC*, *fimD*, *tlyC* respectively.

To further investigate differences within the core and accessory function of the canine skin microbiome, an enrichment analysis was conducted at pathway level. The hypergeometric test was conducted to test over-representation of pathways in the core genes of the canine skin microbiome (Fig. 8a). Most of these pathways are ones that are essential for bacterial survival, such as pathways for DNA replication, aminoacyl-rRNA biosynthesis, carbon metabolism and biosynthesis of cofactors. A pathway for pantothenate (vitamin B5) and CoA (coenzyme A) biosynthesis was identified as enriched within the core. CoA is essential in various metabolic reactions, including in the synthesis of phospholipids which are a major class of lipids represented in the skin. Additionally,

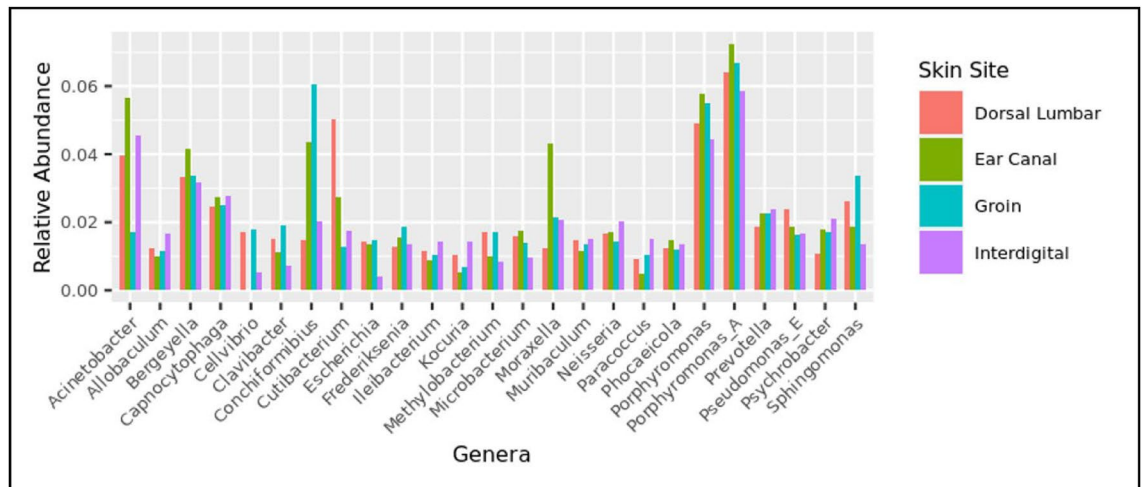


Figure 6. Average relative abundance, across skin sites, of the top 24 genera present within the core canine skin microbiome.

<i>Capnocytophaga canimorsus</i>
<i>Porphyromonas gulae</i>
<i>Capnocytophaga canis</i>
<i>Paracoccus marcusii</i>
<i>Sphingomonas aerolata</i>
<i>Bergeyella zoohelcum</i>
<i>Capnocytophaga cynodegmi</i>
<i>Curtobacterium flaccumfaciens A</i>
<i>Cutibacterium acnes</i>
<i>Porphyromonas A cangingivalis</i>
<i>Porphyromonas A canoris</i>
<i>Clavibacter michiganensis K</i>
<i>Muribaculum sp002492595</i>
<i>Psychrobacter sp001652315</i>
<i>Rathayibacter sp002930885</i>

Table 1. List of species of interest within the canine core microbiome that are represented by two or more strains.

the average relative abundance of the pathways enriched in the core was assessed (Fig. 8b). This revealed the biosynthesis of secondary metabolites pathway as the most highly abundant. Secondary metabolites can include bioactive compounds and antimicrobials which may mediate environmental responses or interactions between members of the microbiome.

Discussion

This study utilised a shotgun metagenomic approach to characterise the healthy canine skin microbiome of 72 dogs and establish a core microbiome at the taxonomic and functional level. In addition, this study assessed four distinct skin sites and four breeds to determine factors influencing variation within the healthy canine skin microbiome.

Previous studies into the canine skin microbiome have generally utilised 16S sequencing approaches where the hypervariable region of the 16S gene studied can bias the reported microbial composition^{1,8–19}. There is only one study published to date utilising shotgun sequencing for a small number of canine skin samples (n = 11)¹⁷. The specificity of databases, such as RefSeq, for canine skin samples has been documented as a pitfall to utilising shotgun sequencing approaches¹⁷. In this study, we employed a canine specific database (MetaGeneCanine™, Diversigen, US) to enhance our taxonomic assignment and increase species and strain resolution. Overall, we experienced relatively low mapping rates (mean 12.5% mapped reads) however, this is still a substantial uplift on the mapping reads we have observed when using RefSeq to assign taxonomy to this dataset (mean 0.65% mapped reads, data not shown). The low mapping rate suggests there is still novelty within the canine skin microbiome that is not yet captured by the databases, including reads from plants, fungi and viruses, and there is a risk that taxa of interest in other species (specifically human) are focused on due to their representation in the databases

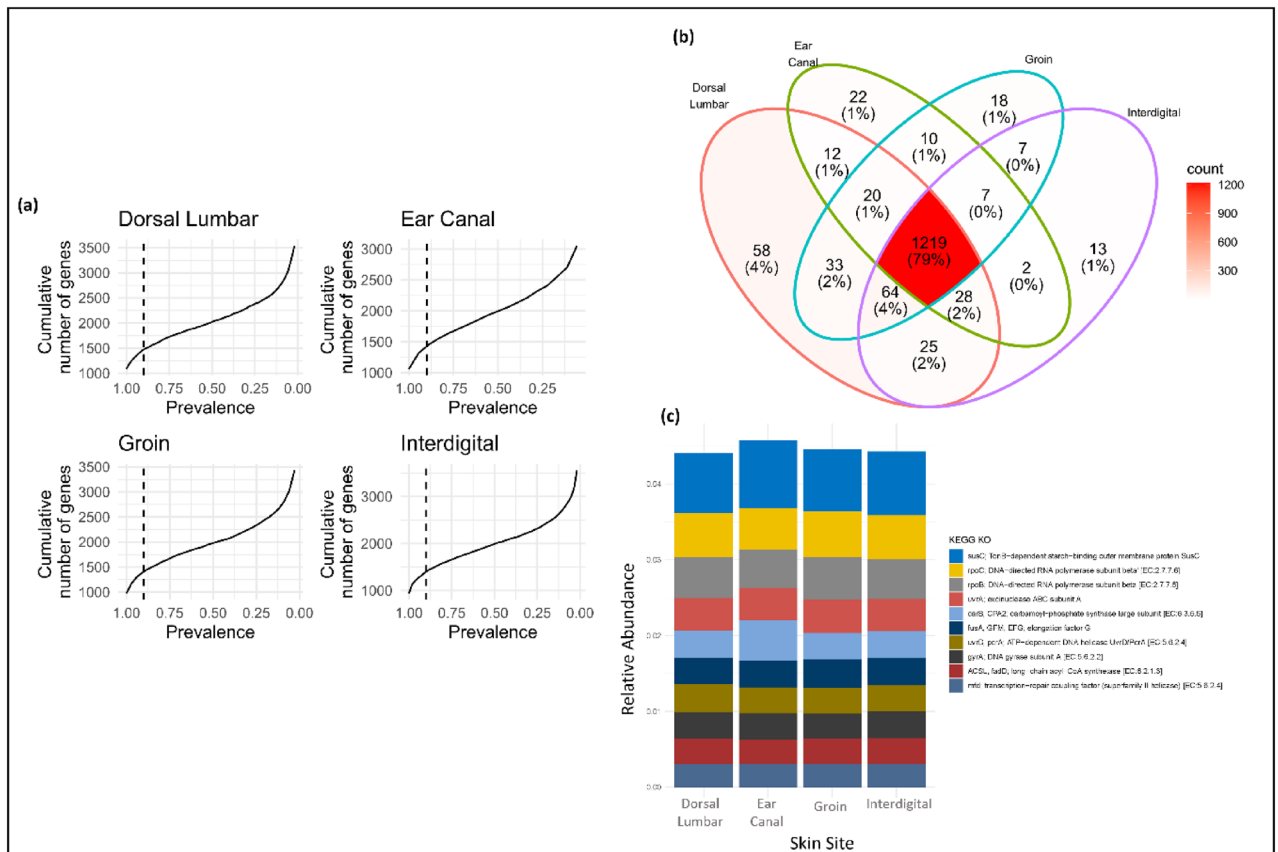


Figure 7. (a) Prevalence of skin microbiome gene families across sites (clockwise from top left; dorsal lumbar, ear canal, interdigital, groin). The dashed line represents a threshold of 90% prevalence, (b) Venn diagram showing the presence of 1538 gene families across skin sites and the overlap of 1219 core gene families with greater than 90% prevalence across all sites, (c) relative abundance of the top 20 gene families within the core microbiome, note the remaining gene families present within the core microbiome have been excluded from this figure.

and canine specific taxa are under studied. Further work investigating metagenome assembled genomes (MAGs) from this dataset could be valuable in advancing our understanding of the canine skin microbiome, specifically at the more granular taxonomic levels (species and strain).

The findings from our study are consistent with other reports of the healthy canine skin microbiome, with Proteobacteria, Actinobacteriota and Bacteroidota described as among the most abundant phyla. Firmicutes have also been described as highly abundant and again this was consistent in our study. We have identified a set of 230 taxa that are highly prevalent ($\geq 80\%$) across samples and skin sites. A previous study in dogs using 16S sequencing has aimed to establish a core microbiota across individual and skin site, using an 85% prevalence threshold¹¹. The authors described a total of 39 different families that make up the core microbiota and considerable overlap with the families detected in the core microbiome in this study were discovered. Of the 39 families detected by Cusco and colleagues¹¹, 20 families were consistent between the studies namely, *Sphingomonadaceae*, *Lachnospiraceae*, *Propionibacteriaceae* and *Porphyromonadaceae*, among others.

Unlike previous studies we have been able to define core taxa at a species level. We have identified that many of the most commonly occurring species within the core skin microbiome (defined as species where more than one strain belonging to that species were detected within the core) are also commonly isolated from the canine oral cavity, including *C. canimorsus*, *P. gulae*, *C. canis*, *B. zoohelcum*, *C. cynodegmi*, *P. cangingivalis*, *C. flaccumfaciens* and *P. canoris*^{21,22}. The inclusion of a high number of species commonly occurring in the oral cavity of dogs on the skin microbiome is expected due to the cleaning and licking behaviours of dogs.

Within the canine core microbiome defined within this study, we have identified species belonging to genera previously reported to be present on the canine skin microbiome, such as *Pyschrobacter* spp., *Sphingomonas* spp. and *Cutibacterium* spp., and *Rathayibacter* spp. which have been previously isolated from groin samples from humans²³. Finally, we have detected strains belonging to the species *P. marsusii* within the canine core microbiome. This species has been associated with healthy skin in humans and enriched after cleansing²⁴. Additionally, it is thought to have antimicrobial activity with some strains containing extrachromosomal elements and gene clusters potentially involved in the production of bacteriocins and bioactive polyketides²⁵. This could point towards a role of the skin microbiome in colonisation resistance against opportunistic pathogens.

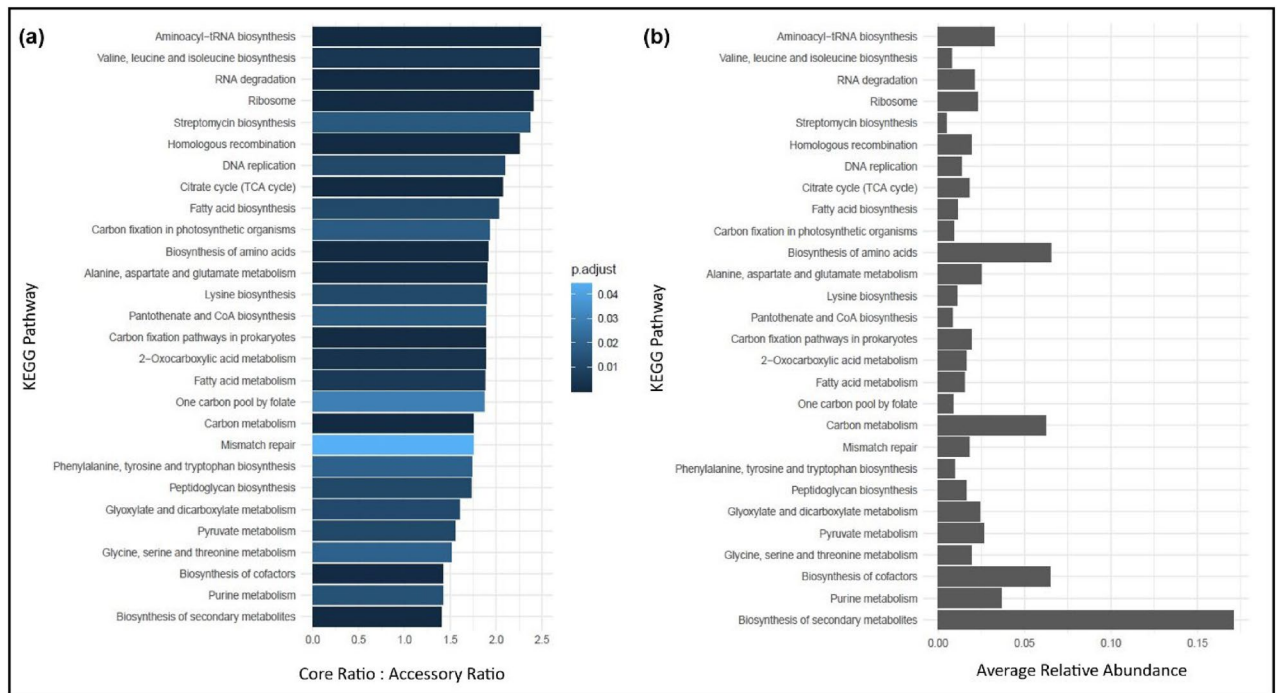


Figure 8. Pathways enriched in the functional core canine skin microbiome, (a) enrichment analysis of pathways with hypergeometric test overlaid, pathways with a core ratio: accessory ratio greater than 1 are enriched in the core functional microbiome, (b) average relative abundance of the pathways enriched within the core functional microbiome.

Shotgun sequencing technology has allowed us to elucidate the core function of the canine skin microbiome. We established core function by assessing overall prevalence of gene families across breeds and sites and have shown that, although many of the core functions are involved in bacterial metabolism and survival, many functions alluding to the role of the skin microbiome in colonisation resistance and protection from invading pathogens have been identified. The gene cluster involved in the synthesis, transport and cleavage of a well-known bacteriocin, Pep5 was discovered. Bacteriocins are antimicrobial peptides produced by bacteria and their inhibitory activity has the potential to give the producing bacterial strain a competitive advantage over other colonisers or invading pathogens. Pep5 is a lantibiotic produced by *Staphylococcus epidermidis*, a common human skin microbiome commensal and has shown antimicrobial activity to methicillin resistant *Staphylococcus aureus* (MRSA) strains²⁶. An increased abundance of *Staphylococcus* species have been detected in the skin microbiome of dogs with atopic dermatitis^{8,10} and *Staphylococcus pseudintermedius* is commonly associated. Lauková and colleagues²⁷ have reported the susceptibility of *S. pseudintermedius* isolated from the skin lesions of dogs to another known bacteriocin (Gallidermin) produced by *Staphylococcus gallinarum*. Therefore, it is possible that the production of Pep5 by members of the canine skin microbiome could be providing resistance and controlling colonisation of *S. pseudintermedius* in healthy dogs. We also discovered genes encoding outer membrane receptors for ferrienterochelin and colicins. These receptors are involved in the active transport of iron which is essential for replication and growth. Ferrienterochelin contains an enterobactin which is known to be deployed by commensal and pathogenic species belonging to Enterobacteriaceae during colonisation²⁸. Although the genes involved in the production of enterobactin and colicins (another bacteriocin) were not detected within the core function of the microbiome, it is not unlikely that the presence of receptor proteins are indicative of its role in colonisation, protection from oxidative stress and production of biofilms, all of which would be useful traits of members of the skin microbiome. Additionally, we found that the pathway for the biosynthesis of secondary metabolites was highly abundant and enriched in the core functional microbiome. This pathway is responsible for the production of secondary metabolites, such as antimicrobials, which may further indicate the role of the core functional microbiome in colonisation resistance.

In this study we also identified genes associated with adhesion, biofilm production and virulence, further suggesting the role of the canine skin microbiome in colonisation resistance and protection from invading pathogens. Genes belonging to the *ica* locus were identified which have been implicated in virulence and mediating intracellular adhesion in some *S. epidermidis* strains²⁹. Although these may be beneficial attributes, they may also be implicated in opportunistic invasion of skin commensals. Finally, we have identified genes involved in multidrug resistance, such as *mexB* and *acrB*³⁰, which may indicate another role of the core functional skin microbiome in protection against pathogens and in colonisation resistance. The presence of intrinsic resistance systems in members of the skin microbiome could allow them a competitive advantage over other transient organisms and allow them to persist during antimicrobial treatment, meaning dysbiosis which may be associated with skin diseases such as atopic dermatitis, occur less frequently.

Within this study, we have also shown that breed and skin site are involved in driving variation within the skin microbiome. Particularly, we observed the microbial profile of all sites (except for the ear canal) were different when compared to each other when measured using Bray–Curtis dissimilarity. This is in keeping with previous studies into the canine skin microbiome where differences in the microbial profile of skin sites have been observed, especially between different types of skin sites, such as haired, mucosal or mucocutaneous junctions^{8,12,18}. Cusco and colleagues¹² conducted network analysis and demonstrated interactions that were exclusive to skin site. The differences in the microbial composition at different skin sites could be due to physiological characteristics at each site. The dorsal lumbar potentially has higher sebum production and a greater number of sebaceous glands due to more dense hair at this site than other sites. We were expecting the ear canal to be the most different site due to the slightly more anaerobic conditions that can be experienced and the presence of wax however, this was not observed when beta diversity was measured. A dog's paws will be in more regular contact with the environment than other areas, specifically the dorsal lumbar, and may attribute to the changes in the microbial composition between the interdigital site and others. Additionally, we observed a difference in the species richness between ear canal and interdigital sites but no other differences in alpha diversity were observed between sites. Apostolopoulos and colleagues¹⁸ described significantly higher species richness in axilla, interdigital and groin sites in comparison to the ear canal in non-allergic dogs. A possible explanation for this due to the chamber like construction of the ear canal and potential bactericidal activity of cerumen was motioned.

The microbial profile of Beagles and Norfolk Terriers appeared most pronounced using Bray–Curtis dissimilarity. The coat type and length of Norfolk Terriers is different to Beagles, with Norfolk Terriers having a longer coat which could influence the differences in the skin microbiome. Additionally, small breed dogs (such as Norfolk Terriers) are known to have periodontitis more commonly, where increased levels of *P. gingivitis* can be detected in the oral cavity. This could influence the composition of transferred microbes to the skin through licking. Our results are consistent with results reported elsewhere regarding the influence of breed on the variation of the skin microbiome. Cusco and colleagues¹² reported that breed explained 9% of the variation in the skin microbiome in their study where breeds with different hair length were studied, French Bulldog, German Shepherd, and West Highland White Terrier. Although these are different breeds to those within the present cohort, this is further evidence that breed, and hair type, can be a driver for skin microbiome differences. It is important to note that diet was not controlled during this study and therefore care should be taken when differences between breeds are interpreted.

Although this study sampled a reasonably large cohort of dogs (72) they were all housed at the Waltham Petcare Science Institute where they are part of a colony cohort. The dogs within this study were exposed to the same housing conditions and environmental influences which may impact the diversity and variation within their skin microbiomes and may not be representative of dogs living in the general pet population. Although this is a limitation, these factors mean that the effect of environmental contamination on the skin may be reduced. Additionally, we collected, processed and stored skin swab samples in a controlled, consistent manner using a commercially available preservation solution. It is known that collection method may impact the microbial profile observed so this should be considered when comparing the results to other studies using owned pet dogs.

Conclusion

In this study we have demonstrated that a core group of taxa and genes are present within the skin microbiome of healthy dogs. We have shown the importance of utilising shotgun sequencing coupled with a well annotated species-specific database to understand the function of the skin microbiome and to increase granularity at species and strain taxonomic levels. Establishing a clear picture of health is vital to further our understanding of the canine skin microbiome in disease, such as atopic dermatitis, where species and strain specific bacteria are likely to be playing an important role. We have established that functionally the skin microbiome may play a role in colonisation resistance and protection from invading pathogens. We have also uncovered the novelty of the canine skin microbiome and shown that further investigation is required to increase the suitability of databases for shotgun sequencing of canine skin samples.

Materials and methods

Cohort description

The cohort comprised 75 healthy adult dogs housed at the Waltham Petcare Science Institute (Leicestershire, UK). The dogs belonged to four breeds: Labrador Retriever (n = 40), Beagle (n = 13), Petit Basset Griffon Vendéen (n = 7) and Norfolk Terrier (n = 15). At the time of sampling (April–August 2020), the cohort had a mean age of 3.8 years (range 0.9–9.3 years) and a mean bodyweight of 27.92 kg (Labrador Retrievers, range 20.62–36.20 kg), 14.43 kg (Beagle, range 11.07–17.66 kg), 14.16 kg (Petit Basset Griffon Vendéen, range 12.24–17.03 kg) and 6.01 kg (Norfolk Terrier, range 3.55–8.28 kg). The cohort contained 38 male and 37 female dogs of which 51 were neutered and 24 were entire. The metadata associated with the cohort is provided in supplementary material Table S6. Dogs were housed in pairs in a pen, not restricted by breed, that provided continuous inside and outside access; in addition, dogs from adjacent pens were socialised in group paddocks during the day. Normally, dogs would also have off-lead exercise and socialisation with other dogs. However, on the day of sample collection, dogs were confined to their pens with access to the paddocks if concreted, until sampling had been completed.

The dogs had no concurrent illnesses that may impact the skin, had not been treated with any antimicrobials in the 3 months prior to sampling and had not been washed with any detergent or shampoo, nor had any topical treatments in the previous 24 h to sampling. All dogs were fed commercially available complete dry diets throughout the study, appropriate for their life stage (puppy, adult) and/or weight, and diet remained consistent throughout the sampling phase. This study was approved by the Waltham Petcare Science Institute Animal

Welfare and Ethical Review Board (AWERB). All methods were performed in accordance with the relevant guidelines and regulations and is reported in accordance with the ARRIVE guidelines.

Sample collection

Skin microbiome samples were collected from four different sites from all dogs, totalling 300 samples. The skin sites selected for sampling were: right ear canal, interdigital region of the left fore paw (between each digit), dorsal lumbar and right groin, named A, B, C and D respectively (Fig. 1a). The hair at each site was parted by the sample collector to ensure access to the skin surface, and areas were not shaved prior to sample collection. Dogs were taken to a procedure room on their home unit for sample collection. For each skin site, two sterile flocked swabs with 80 mm breakpoint (Norgen Biotek Corp.) were used. Swabs were soaked in sterile wetting solution (Tris buffer (pH 8.0), 2 mM EDTA and Triton X-100, Norgen Biotek Corp.) then applied, with rotation, to the desired skin area for a period of at least one minute. Duplicate swabs for each sample site were stored in stabilisation solution (Swab Collection and DNA Preservation System, Norgen Biotek Corp.) and stored at 4 °C until DNA extraction. To minimise cross contamination between sample sites and individual animals, a new pair of nitrile gloves were worn by the sampling person for each sampling site.

DNA extraction

DNA extraction was conducted at Alkek Center for Metagenomics and Microbiome Research (CMMR), Baylor College of Medicine. Genomic DNA from skin swabs were extracted using the Saliva DNA Isolation Kit (Norgen Biotech Corp.) according to the manufacturers Supplementary Protocol for the Isolation of DNA from Norgen's Swab Collection and DNA Preservation System using Norgen's Saliva DNA Isolation Kit.

Shotgun sequencing

Library preparation and shotgun sequencing was conducted at Alkek Center for Metagenomics and Microbiome Research (CMMR), Baylor College of Medicine. Six samples were removed as they did not reach the desired thresholds for sequencing. These included four samples from the same dog (DogNo. 2). A total of 294 collected samples from 74 dogs were successfully sequenced to a depth of 5 gb/sample using an Illumina NovaSeq S4 sequencer. A negative control and positive control (ATCC MSA1003) were also included to check pipeline sensitivity and specificity.

Bioinformatics

Sequencing data was processed at Diversigen (New Brighton, MN, USA). Firstly, host (canine) sequences were removed using Bowtie³¹ before DNA sequences were aligned to Diversigen's curated database containing all representative genomes in RefSeq³² for bacteria with additional manually curated strains (DivDB-Canine). Strains were defined as clusters of genomes sharing average nucleotide identity > 99%. Every input sequence (read) was compared to every reference sequence in DivDB-Canine using fully-gapped alignment with BURST and an identity threshold of 97%. Ties were broken by minimizing the overall number of unique Operational Taxonomic Units (OTUs). For taxonomy assignment, each sequencing read was assigned to the lowest common ancestor that was consistent across at least 80% of all reference sequences tied for best hit. Kyoto Encyclopedia of Genes and Genomes Orthology³³ groups (KEGG K0s) were observed directly via alignment to a gene database derived from Div-DB-Canine. All sequencing reads were aligned to all reference gene sequences at an identity threshold of 97% using fully-gapped alignment with BURST³⁴. Ambiguously mapped reads were excluded from the resulting functional feature table. The mean read depth per sample was 28 million reads (min 9710, max 122 million). Post host removal, the mean read depth per sample was 7.68 million reads. Of these reads, a large proportion could not be assigned to taxonomy; reads mapping ranged from 2642 reads (0.76%) to 6.6 million reads (68.97%), mean 9.1 million reads (12.50%). Previously, we used RefSeq to assign taxonomy to this dataset and we observed a range of 0.07–2.00% of reads mapping (mean 0.63%). For gene families, we observed a mean of 405,311 reads mapping post host removal (5.55%), with a range of 1104 reads (0.18%) to 3.2 million reads (33.08%). We applied a conservative threshold of 500,000 reads to be mapped to taxa for samples to be taken forward in the study. This was based on previous published observations of sequencing depth required for shallow shotgun sequencing.³⁵ This reduced the number of samples by 55%, from 294 samples from 74 different dogs to 162 samples, across skin sites and breeds, from 72 different dogs (Table S1).

Statistical analysis

Downstream analyses were performed using R version 4.1.3³⁶ and the raw count tables (taxonomic Table S7 and gene families Table S8), using only the samples that have reached the 500,000 mapped reads threshold ($n = 162$). Prior to analyses, a noise removal step was conducted. Any count that was lower than 0.01% of total counts was inputted with 0, any taxa that had 0 across all samples was subsequently removed. Additionally, taxa that were only present in one sample were removed. Prior to alpha diversity calculations, samples were rarefied to the read depth of the lowest sample (502,635 reads). Alpha diversity analyses to compare the diversity of communities within samples were assessed using two metrics; Shannon Diversity to assess evenness and Observed Species to assess richness. Shannon diversity and species richness of each sample were, separately, fit to a linear mixed effects model with the relevant grouping (skin site or breed) as the fixed effect and individual dog as the random effect. Contrasts were made between the groups, and the family-wise 95% confidence intervals and p values were obtained. Beta diversity analyses to compare the diversity of communities between samples was assessed using Bray–Curtis dissimilarity on relative abundances and visualised using non-metric multidimensional scaling (NMDS). PERMANOVA (permutational multivariate analysis of variance) was used to assess statistical differences between skin sites and confounding factors (breed and sex). Pairwise PERMANOVA was implemented

with the pairwiseAdonis package³⁷ and p values adjusted using the Bonferroni method. Dispersion tests were conducted on the same pairwise comparisons using the vegdist function from the vegan package³⁸ and p values adjusted using the Bonferroni method. Prevalence of a feature (taxon or gene) was estimated per skin site defined as the proportion of samples where the feature was present. The number of features that was observed above a certain prevalence threshold (cumulative number of features) was then plotted. Core was defined as the overlap of features across the four sites; all other features were categorised as accessory. For comparisons between core and accessory taxa, abundances at the phylum level were estimated relative to the counts in the core or accessory sets, respectively. KEGG pathway enrichment analysis was conducted using MicrobiomeProfiler³⁹. All detected genes were used as universe background genes. Adjustment of p values was performed using the Benjamini & Hochberg method.

Data availability

Data is available upon reasonable request to the corresponding author: Michaella Whittle, Email address: micky.whittle@effem.com.

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Author contributions

M.J.W.: study design, methodology, project administration, statistical analysis, visualisation, data interpretation, writing—original draft. J.C-F.: statistical analysis, visualisation, data interpretation, writing—review and editing. G.C.A.A.: technical support, supervision, writing—review and editing. P.W.: study conceptualisation, funding acquisition, supervision, writing—review and editing.

Competing interests

M.J.W., J.C-F., G.C.A.A. and P.W. are employees of Mars Petcare, a manufacturer of pet food and provider of veterinary services. Funding was provided by the Waltham Petcare Science Institute, Mars Petcare. Mars Petcare has filed a patent relating to this research.

Additional information

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