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Bacterial associations with periodontal disease in Yorkshire terriers

Corrin Wallis[1*], Alison Colyer[1] and Lucy J. Holcombe[1]

Abstract

Background Periodontal disease, an inflammatory condition initiated by the build-up of plaque on the tooth surface, is a common problem seen within veterinary practices. There are an increasing number of studies which indicate distinctive microbial profiles associated with healthy gingiva and periodontal disease. Most have been based on analysis of samples from populations of mixed breed dogs collected at a specific point in time. A study investigating the development of periodontal disease in Yorkshire terriers provided an opportunity to investigate the concurrent changes in the oral microbiota in this specific breed.

Results Analysis of 42 subgingival plaque samples from 22 dogs (1 to 4 samples per dog) by 454 pyrosequencing of the V1-V3 region of the 16S rRNA gene resulted in 796,091 filtered sequence reads which were assigned to 286 operational taxonomic units (excluding those deemed noise). Statistical analysis showed that health and mild gingivitis were associated with higher relative abundance of taxa belonging to the phylum Proteobacteria (e.g., *Moraxella* and Pasteurellaceae). In moderate gingivitis there was increased representation of taxa belonging to the phylum Firmicutes (e.g., Peptostreptococcaceae, Lachnospiraceae, Erysipelotrichaceae and *Frigovirgula*) and Bacteroidetes (*Porphyromonas canoris*). Periodontitis was also associated with an increased representation of some taxa belonging to the phylum Firmicutes (e.g., Peptosteptococcaceae), Spirochaetea (e.g., *Treponema)* and Synergistetes (e.g., Synergistales).

Conclusions This study further advances our understanding of the bacterial changes associated with early periodontal disease. These can be leveraged to improve disease diagnosis, drive awareness and support recommendations for effective preventative and management strategies.

Keywords Dog, Canine, Gingivitis, Periodontal disease, Microbiota

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Background

Periodontal disease is one of the most common disorders in dogs but can be challenging to diagnose in first opinion practice [1–6]. The disease is initiated by the formation of plaque biofilms on the tooth surface, resulting in host-mediated inflammation [7–9]. Initially the inflammation is limited to the gingiva (gingivitis) but, without effective treatment, this can progress into the tissues that support the tooth (periodontium) resulting in pocket formation, gingival recession and alveolar bone loss (periodontitis). Periodontitis can lead to the development of oronasal fistula, ocular problems, perio-endo lesions, pathologic jaw fractures or spontaneous tooth exfoliation [10, 11]. The onset of several systemic conditions has also been associated with periodontal disease [12–15].

The microbial communities in dental plaque are complex. Early culture-based studies of the canine oral microbiota were limited with most confined to investigating bacterial species known to be associated with oral health or late-stage periodontal disease in humans [16–18]. However, subsequent cloning and sequencing studies showed that 80% of bacteria in canine plaque were novel with only 16.4% previously identified in the human oral cavity [19]. More recently, high-throughput sequencing has enabled larger-scale studies and identification of more of the bacterial species within microbial communities. This has led to further research which indicates canine oral microbial profiles differ according to whether the dog has healthy gingiva or periodontal disease [20–22]. These studies also confirmed the early findings that, despite some similarities, the plaque microbiota of dogs substantially differs to that of humans.

Most canine oral microbiota studies have been based on analysis of samples from populations of mixed breed dogs collected at a specific point in time. A recent study of miniature schnauzers explored shifts in bacterial community composition during the development of canine periodontal disease. The findings were consistent with previous suggestions that periodontal disease progression is associated with an increase in taxa with a previously low abundance concomitant with a reduction in taxa with a previously high abundance, rather than the appearance of previously undetected bacterial species [21]. These insights indicate that specific bacteria do not initiate the inflammatory response, but it is the result of dysbiosis, where the proportions of more virulent species increase at sites of inflammation and tissue destruction [23]. The objective of this study was to investigate changes in the microbiota composition of subgingival plaque with gingivitis and periodontitis in Yorkshire terriers, an extra-small breed of dog that has been shown to have a higher prevalence of periodontal disease compared to larger breeds possibly due to tooth overcrowding resulting in a greater accumulation of plaque [24, 25]. The hypothesis was that differences in the microbial profile with the extent of gingivitis and proportion of periodontitis teeth in the mouth would be evident. The elucidation of oral microbiota associations with periodontal disease will enable the development of diagnostic and prognostic tests which can be used to drive awareness and support recommendations for effective home-care regimes to help prevent or slow disease progression.

Results

Clinical status

Assessment of the periodontal health status indicated that all 22 dogs had gingivitis and early periodontitis (Supplementary Table 1). The average gingivitis score across the four timepoints was 1.5 +/- 0.23 (range 0.9 to 2.1). The average number of periodontitis teeth across the four timepoints was 15 +/- 5 (range 5 to 28). All teeth were early periodontitis (< 25% attachment loss) with exception of two incisors (attachment loss ≥ 4 mm) and three canines (attachment loss ≥ 5 mm).

Sequence quality

A total of 42 subgingival plaque samples from 22 Yorkshire terriers (1 to 4 samples per dog) were analysed by 454 pyrosequencing of the V1-V3 region of the 16S rRNA gene. This resulted in the generation of 999,761 sequence reads that passed the sequence providers initial quality filter. Following amplicon noise filtering 796,091 sequence reads were retained. The number of sequence reads per sample ranged from 8,525 to 16,314. Clustering of the sequence reads at ≥ 98% identity, resulted in the identification of 4,056 operational taxonomic units (OTU), reduced to 286 following removal of the OTUs deemed noise. The removed OTUs accounted for 2.3% of sequence reads.

Bacterial community composition

Taxonomic assignment of the 286 OTUs from the Yorkshire terrier samples resulted in 58.3% mapping with ≥ 98% identity to taxa previously identified in the oral cavity of dogs or cats [19, 26]. A further 31.2% mapped to other sequences within the Silva database with ≥ 98% identity. The remaining 10.5% of sequences shared between 86.7% and 97.7% identity to sequences within the Silva database.

The majority of OTUs identified were assigned to 7 phyla: Firmicutes (31.8%), Proteobacteria (17.8%), Bacteroidetes (17.1%), Spirochaetae (11.2%), Actinobacteria (9.1%), Fusobacteria (4.5%) and TM7 (3.1%). There were a further 7 phyla that each represented less than 1.5% of the OTU assignments: SR1, Chlorobi, Synergistetes, Chloroflexi, BD1-5, Tenericutes, Elusimicrobia. The 20 most abundant OTUs in subgingival plaque from Yorkshire terriers represented 39.2% of the sequence reads

Table 1 The 20 most abundant bacterial species observed in subgingival plaque of Yorkshire Terriers. COT/FOT depict previously identified canine/feline oral taxa [19, 24]

Species	Proportion of total sequence reads
Porphyromonas cangingivalis	5.29%
Parvimonas sp. COT-035	4.31%
Moraxella sp. COT-017	3.82%
Aquaspirillum sp. FOT-079	3.15%
Actinobacteria COT-406	2.39%
Leptotrichia sp. COT-345	1.88%
Granulicatella sp. COT-095	1.54%
Actinomyces COT-404	1.46%
Treponema sp.	1.39%
Bergeyella sp.	1.38%
Fusobacterium sp.	1.35%
Euzebya sp.	1.34%
Corynebacterium mustelae	1.30%
Fusobacterium sp.	1.30%
Abiotrophia sp.	1.30%
Fusobacterium sp.	1.26%
Neisseria sp.	1.24%
Treponema sp. COT-359	1.20%
Campylobacter sp. FOT-100/COT-011	1.19%
Actinomyces sp. FOT-320/COT-083	1.11%

(Table 1). The most abundant bacterial species was *Porphyromonas cangingivalis*.

Bacterial diversity

There was no statistically significant relationship between bacterial diversity and periodontal health status of the Yorkshire terriers. On average there was an increase in Shannon diversity of 0.03 (-0.45, 0.52) when comparing 0–75% periodontitis teeth in the mouth ($p = 0.90$) (Fig. 1a). With respect to gingivitis, there was an average increase of 0.24 (-0.23, 0.71) in Shannon diversity with an increase in average gingivitis score of 1 ($p = 0.31$) (Fig. 1b).

Bacterial associations with gingivitis and periodontitis

An exploratory non-metric multidimensional scaling (nMDS) showed no discrete clustering of plaque microbiota by proportion of periodontitis teeth in the mouth (Fig. 2a) or mean gingivitis mouth score (Fig. 2b). However, analysis of the individual OTUs using Generalised Linear Models (GLM) identified several that were significantly associated with the proportion of periodontitis teeth in the mouth and mean gingivitis mouth score. Of the 286 individual OTUs, 7 showed a significant association with gingivitis when comparing a mean gingivitis mouth score of 2 to a mean gingivitis mouth score of 1. Of these, 6 OTUs had a higher relative abundance when the statistically estimated mean gingivitis mouth score was 2 (Table 2; Fig. 3). Five belonged to the phylum Firmicutes (Peptostreptococcaceae COT-307/FOT-060, Peptostreptococcaceae COT-077, Lachnospiraceae COT-099, Erysipelotrichaceae COT-381 and *Frigovirgula* sp. COT-032) and one to the phylum Bacteroidetes (*Porphyromonas canoris*). There was 1 OTU, Pasteurellaceae COT-080 a member of the phylum Proteobacteria, with a higher relative abundance when the statistically estimated mean gingivitis mouth score was 1.

Exploration of the bacterial association with periodontitis in Yorkshire terriers identified 10 OTUs that significantly differed in their relative abundance when comparing 0–75% periodontitis teeth in the mouth (Table 3; Fig. 4). Of these, three had greater than 80% of samples with zero abundance so were not considered further. Six of the remaining OTUs were significantly associated with periodontitis; two from the phylum Firmicutes (Peptosteptococcaceae COT-129 and Peptosteptococcaceae COT-021), three from the phylum Spirochaetae (2 novel species of *Treponema* and *Treponema* sp. COT-355) and one from the phylum Synergistetes (Synergistales bacterium COT-244). One OTU from the phylum Proteobacteria (*Moraxella* sp. FOT-087) was significantly associated with periodontal health.

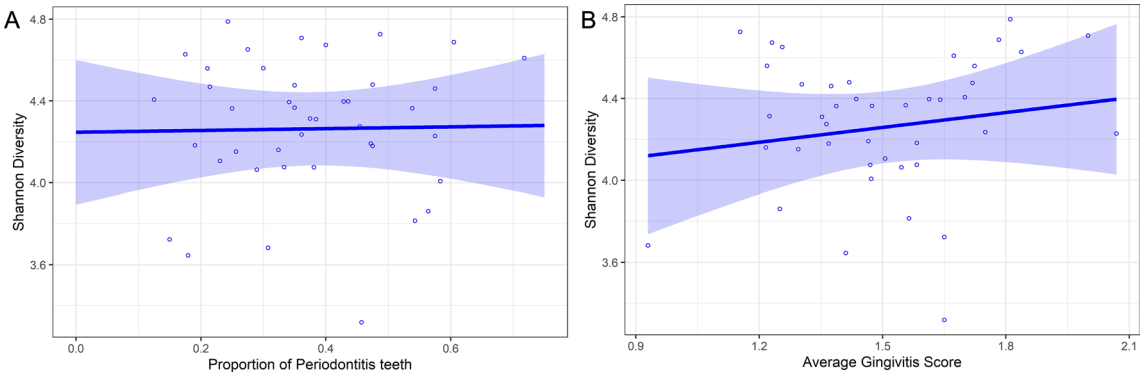

Fig. 1 Changes in Shannon diversity index with (**a**) increasing proportion of periodontitis teeth in the mouth and (**b**) mean gingivitis mouth score. Open circles represent the data, blue line the estimated relationship and blue shaded area 95% confidence region

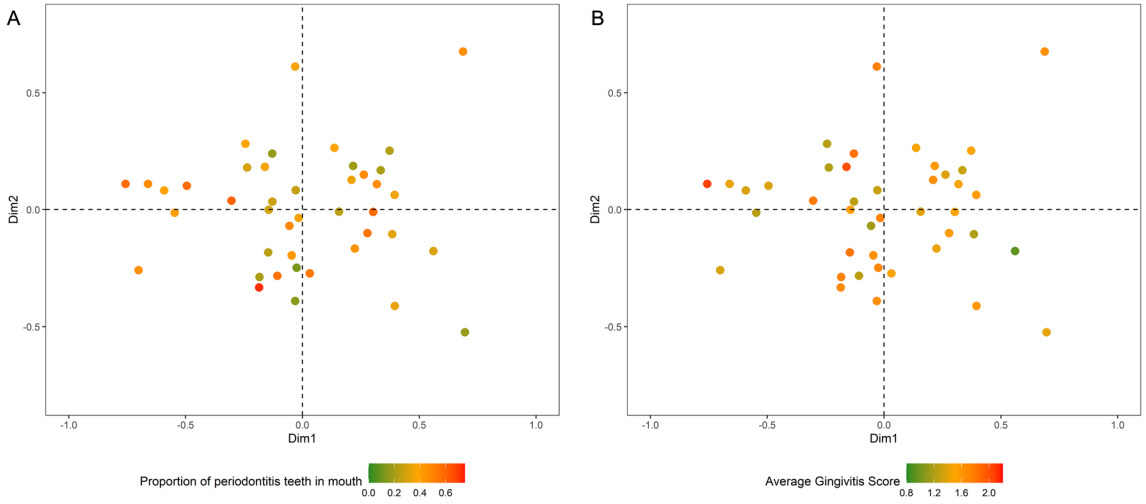

Fig. 2 Non-metric multidimensional scaling dimensions 1 (Dim1) and 2 (Dim2) coloured by (**a**) proportion of periodontitis teeth in the mouth and, (**b**) mean gingivitis mouth score

Table 2 Estimated odds ratio (OR) with 95% confidence limits (CL) and the associated *p*-values adjusted for multiplicity by Benjamani-Hochberg [36] of the OTUs significantly associated with gingivitis when comparing a mean mouth gingivitis score of 2 to a mean mouth gingivitis score of 1

Phylum	Species	OR	Lower 95% CL	Upper 95% CL	*p*-value
Firmicutes	Peptostreptococcaceae COT-307/FOT-060	15.0	2.9	77.0	0.048
Firmicutes	Peptostreptococcaceae COT-077	343.2	47.2	2500.0	< 0.001
Firmicutes	Lachnospiraceae COT-099	184.1	22.5	1508.3	< 0.001
Firmicutes	Erysipelotrichaceae COT-381	63.0	6.8	584.1	0.013
Firmicutes	*Frigovirgula* sp. COT-032	101.6	21.3	484.5	< 0.001
Bacteroidetes	*Porphyromonas canoris*	12.2	3.9	38.0	0.001
Proteobacteria	Pasteurellaceae COT-080	0.08	0.02	0.28	0.006

Discussion

This study of Yorkshire terriers provided a unique opportunity to investigate the bacterial associations with periodontal disease in a highly predisposed breed of dog. Analysis of subgingival plaque samples by 454 pyrosequencing indicated that the percentage of sequences mapping to the bacterial 16S rRNA gene databases, and the taxa identified, are representative of previous studies of the canine plaque microbiota [20–22, 27]. The study identified taxa significantly associated with average gingivitis score and the proportion of periodontitis teeth in the mouth. The increased relative abundance of taxa belonging to the phyla Proteobacteria in periodontal health and the shifts to increased relative abundance of the phyla Firmicutes and Spirochaetes in moderate gingivitis and periodontitis agrees with findings from previous cross-sectional studies [20, 22, 27, 28]. These studies of dogs with healthy gingiva and periodontal disease have reported that the phylum Proteobacteria, particularly species belonging to the genera *Moraxella*, *Neisseria* and family Pasteurellaceae, and also members of the phylum Bacteroidetes such as *Bergeyella*, *Capnocytophaga* and *Porphyromonas* were significantly more abundant in dogs with healthy gingiva [20, 22, 27, 28]. In parallel the studies also reported increased relative abundance with periodontal disease of taxa belonging to the phyla Firmicutes, such as Peptostreptococcaceae, Lachnospiraceae and Clostridiales, and Spirochaetes, especially the genus *Treponema*.

The findings of the current study also support those from a one-year longitudinal study of miniature schnauzers which showed that periodontitis and gingivitis were characterised by an increase in abundance of species belonging to the phylum Firmicutes, particularly bacterial species belonging to the family Peptostreptococcaceae [21]. Periodontal health was also associated with a greater proportion of species belonging to the phyla Bacteroidetes (particularly *Bergeyella zoohelcum*) and Proteobacteria (particularly *Moraxella* sp. COT-017 and members of the Pasteurellaceae family). Although, there were many similarities between the Yorkshire terrier and miniature schnauzer studies, Yorkshire terrier microbiota showed some distinct differences, mainly in the association of bacterial species belonging to the genus *Treponema* with periodontitis. The increased abundance of *Treponema* in Yorkshire terriers might be explained by the fact they had a greater number of teeth that progressed to periodontitis (47% of teeth assessed) compared to the miniature

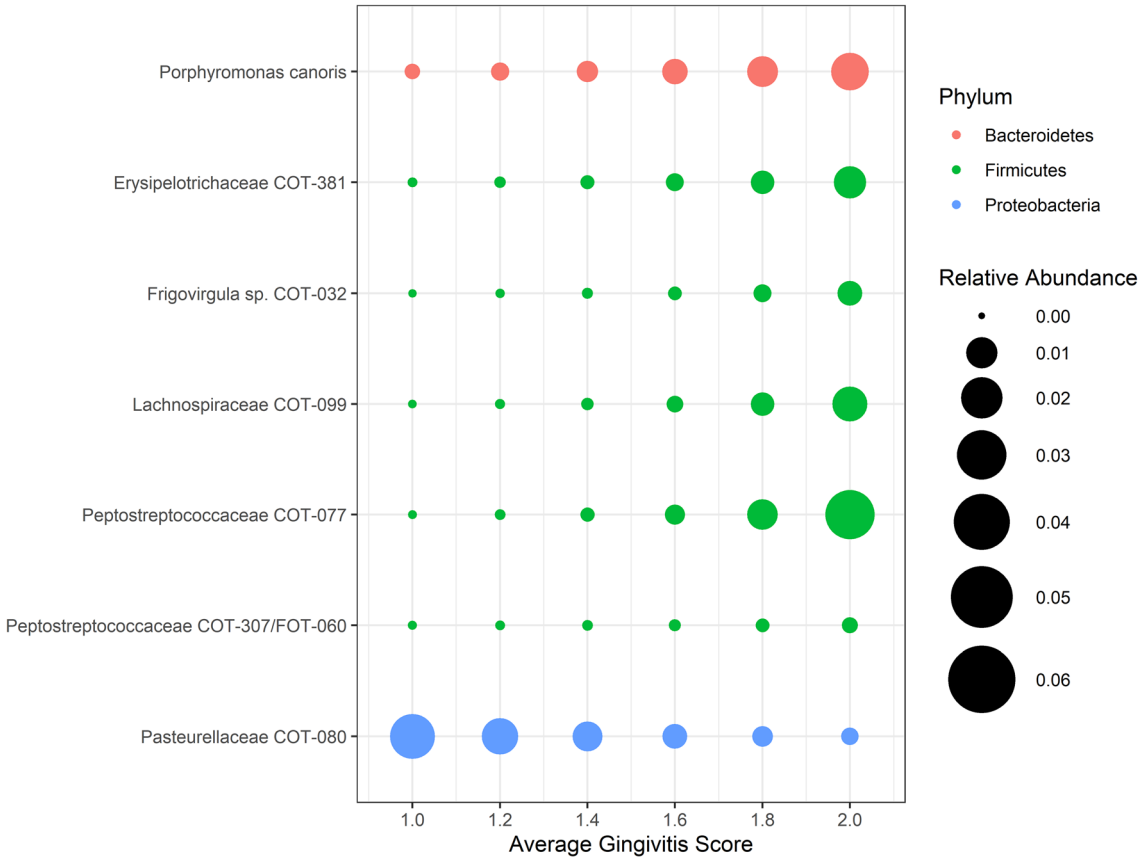

Fig. 3 Bacterial species that showed significant associations with mean gingivitis mouth score. The size of the circles represents the estimated proportion, and the colour represents the phylum

schnauzers (27%). The presence of *Treponema* indicates that smaller breeds such as Yorkshire terriers have more "disease-like" bacterial profiles at a younger age (less than 1 year old) than breeds such as the miniature schnauzer. Recently it has been proposed that *Treponema denticola* can be used as a prognostic biomarker for the diagnosis of canine periodontitis [29]. Studies in other species have also indicated that *T. denticola* plays a key role in periodontal disease with its small size, motility and array of virulence factors enabling it to invade gingival epithelial and fibroblast cells resulting in tissue destruction [30]. However, further research is needed to elucidate *T. denticola* mechanisms of pathogenesis in relation to periodontal disease and the significance of its role in dogs.

The current study showed a small increase in bacterial diversity with periodontal disease progression although this was not statistically significant. The literature pertaining to changes in species richness and diversity is controversial with some supporting the findings of this study [21, 22, 28] and others indicating a significant increase in diversity in dogs with periodontal disease versus those with healthy gingiva [20]. The Yorkshire terriers in this study were only exhibiting early stages of the disease (gingivitis and < 25% attachment loss, PD2) and, if the dogs were allowed to continue to develop into the later stages of disease, the changes in bacterial diversity may have been greater.

One of the main limitations of this study was that a composite (whole mouth) plaque sample from all the teeth was collected representing bacterial species from sites with gingivitis and periodontitis. These two conditions often occur together making it difficult to distinguish which is associated with the corresponding bacterial changes observed in periodontal disease. Despite this these conditions are not always correlated i.e. you can have severe gingivitis without periodontitis and vice versa. Therefore, the statistical model undertaken, which investigated cumulative proportion of periodontitis teeth in the mouth or mean gingivitis scores separately as continuous fixed effects, is appropriate and clearly showed the bacterial associations with increasing gingivitis scores differed to those associated with increasing proportion of periodontitis teeth. Another limitation is that the use of molecular methods such as 16S amplicon sequencing detects bacterial DNA that could be from both live and dead bacteria. To determine the viability, and consequently virulence, other methods that only

Table 3 Estimated odds ratio (OR) with 95% confidence limits (CL) and the associated *p*-values adjusted for multiplicity by Benjamani-Hochberg [36] of the OTUs significantly associated with periodontitis when comparing 0–75% periodontitis teeth in the mouth

Phylum	Species	OR	Lower 95% CL	Upper 95% CL	*p*-value
Spirochaetae	Novel *Treponema* 1	194.02	119.62	314.76	< 0.001
Spirochaetae	*Treponema* sp. COT-355	12.73	8.86	18.29	< 0.001
Synergistetes	Synergistales bacterium COT-244	3.47	1.99	6.04	< 0.001
Firmicutes	Peptostreptococcaceae bacterium COT-129	3.61	2.58	5.06	< 0.001
Firmicutes	Peptostreptococcaceae bacterium COT-021	1.47	1.28	1.69	< 0.001
Spirochaetae	Novel *Treponema* 2	3.48	2.03	5.98	< 0.001
Proteobacteria	*Moraxella* sp. FOT-087	0.18	0.16	0.20	< 0.001

detect living bacteria (e.g. bacterial culture) would need to be used.

Studies designed to investigate the development of periodontal disease in breeds of dog with differing susceptibilities to periodontal disease have provided a unique opportunity to gain preliminary insights as to whether dog breed or clinical status of the periodontium had the greatest impact on the bacterial community composition of canine subgingival plaque [24, 31]. The Yorkshire terrier dogs investigated in this study had the highest prevalence of periodontitis teeth (47% of teeth assessed progressed to periodontitis over a 10-month period) compared to miniature schnauzers (26.8% of teeth assessed progressed to periodontitis over approximately 14 months). These studies are not directly comparable due to differences in sampling approaches (single teeth versus whole mouth collections), DNA extraction methods (Nucleospin 96 Tissue kit, Macherey-Nagel versus Masterpure™ Gram positive DNA purification kit, Epicentre) and the proportions of periodontitis teeth. However, the consistency in bacterial changes observed across the studies support the theory that clinical status of the periodontium is the principal determinant of plaque microbiota composition. Another related theory is that these breeds may have differing rates of plaque deposition, which in turn influences the rate of change in periodontal health state. A recent study of the bacteriome of the oral cavity in healthy and periodontally diseased dogs showed that early colonisers were significantly more abundant in dogs with healthy gingiva and late colonisers were enriched in dogs with severe periodontitis [22]. Breed as well as individual differences in susceptibility and clinical manifestation of periodontitis could be due to immune function where in some instances the microbial biofilm is tolerated (homeostasis) and in others there is an aberrant response, leading to dysbiosis and inflammation-driven tissue destruction [32].

Conclusion

This study further advances our understanding of the bacterial associations with periodontal health and disease in dogs and supports the dysbiosis theory with bacterial species varying by orders of magnitude in their relative abundance with the advancement of disease. While further research is needed to understand which species drive the disease process as opposed to merely being better suited to the environment, these bacterial biomarkers of canine periodontal disease can be leveraged, through the development of screening tools. These tools can be used to improve periodontal disease awareness, and support conversations between dog owners and veterinarians about effective and tailored management strategies.

Materials and methods

Study cohort and sample collection

Bacterial plaque samples were collected in parallel with a study undertaken to determine the extent of gingivitis and periodontitis in Yorkshire terriers [24]. All dogs were housed at the Waltham Petcare Science Institute and received regular dental assessments under general anaesthesia during which the levels of gingivitis and periodontitis (clinical attachment loss based on probing depth, gingival recession and furcation exposure) were assessed [24]. Gingivitis was scored on a 4-point scale based on time to bleeding on probing where G0 was no inflammation or bleeding on probing and G3 was inflammation and immediate bleeding on probing. A tooth was classified as periodontitis if clinical attachment loss was ≥ 2 mm (≥ 2.5 mm on the canine teeth) or if furcation exposure was present [24]. Gingivitis and periodontitis were assessed on 4 aspects of every tooth in the mouth by people trained for consistency by a Diplomate of the European Veterinary Dental College as described previously [24]. Dogs were assessed every eight weeks (+/- 1 week) from 37 weeks up to a maximum of 61 weeks of age. Not all dogs were assessed at every time point as it was a pre-requisite of Waltham's Animal Welfare and Ethical Review Body that dogs were removed from study and given a whole mouth scale and polish when 12 or

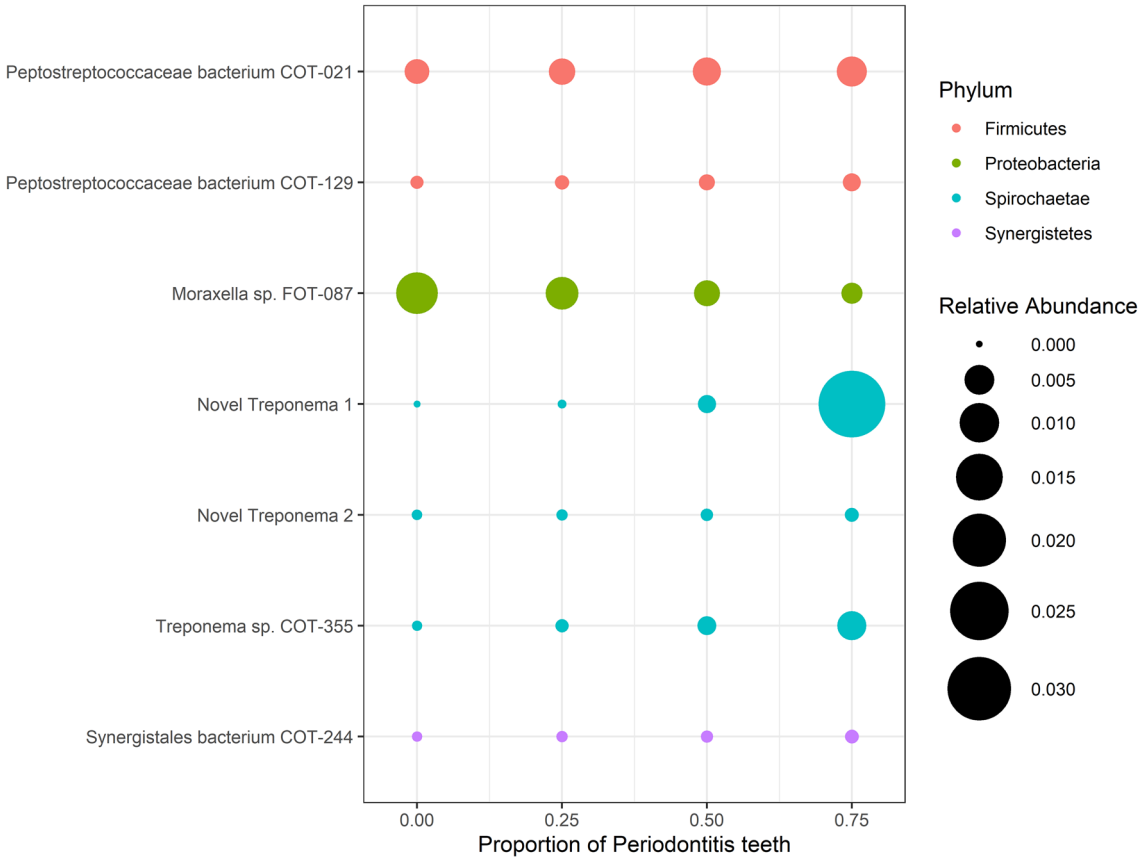

Fig. 4 Bacterial species that showed significant changes with proportion of periodontitis teeth. The size of the circles represents the estimated proportion, and the colour represents the phylum

more teeth developed the early stages of periodontitis. Further details on the criteria for scoring gingivitis and periodontitis, and the general anaesthesia protocol, can be found in the primary manuscript [24].

Subgingival plaque was collected from every tooth in the mouth whilst the level of gingivitis was being assessed. This resulted in one composite (whole mouth) plaque sample from all the teeth for each dog at each sampling occasion. A total of 22 Yorkshire terriers (5 litters) were included with sampling based on the duration they were on the clinical trial: 21 dogs were sampled at 37 weeks of age, 15 at 45 weeks, 5 at 53 weeks and 1 at 61 weeks resulting in 1 to 4 samples per dog. Subgingival plaque collection involved placing a sterile periodontal probe under the gingival margin and sweeping it around the entire circumference of the tooth along the base of the crown. The probe was placed into a 0.5 ml Eppendorf tube containing 300 µl TE buffer (10mM Tris-HCL, 1 mM disodium EDTA, pH8.0; Sigma-Aldrich) and agitated to remove the plaque. Samples were stored on dry ice for a maximum of 30 min prior to storage at -80ºC.

DNA extraction

DNA was extracted using the Masterpure™ Gram positive DNA purification kit (Epicentre, #MGP04100) according to the manufacturer's instructions but with an additional overnight lysis. After centrifugation of the plaque samples at 5000 x g for 10 min the cell pellet was resuspended in 150 µl of TE buffer and 1 µl Ready-Lyse™ Lysozyme Solution added. The lysis mix was incubated at 37 °C for 18 h. Following DNA extraction, the DNA pellet was suspended in TE buffer. The quantity and the purity of the DNA was determined using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies Inc).

Amplification and sequencing of 16S rDNA

The V1-V3 region of the 16S rRNA gene was amplified using universal primers and the Extensor Hi-Fidelity PCR Master Mix (# AB-0792, Thermo Scientific) according to the manufacturer's instructions. Forward primers were a mix of FLX_27FYM (5'-CGTATCGCCTCCCTCGCGCCATCAG**AGAGTTTGATYMTGGCTCAG-3'**) used at 9.5pmol/µl and FLX_27F_Bif (5'-CGTATCGCCTCCCTCGCGCCATCAGAGGGTTCGATTCTGGCTCAG-**3'**) used at 0.5pmol/µl [33]. The forward primers comprised, from the 5' end, the 454-sequencing adapter A (italicised

letters) and the 16S rRNA gene primer sequences (bold letters). Primer FLX_27F_Bif was included to ensure representation of the genus *Bifidobacter;* a lower concentration was chosen due to the low representation of this genus in previous studies of canine plaque. The reverse primer was used at 10 pmol/µl (5'-CTATGCGCCTTG CCAGCCCGCTCAGXXXXXXX**TYACCGCGGCTGCTGG-3'**) [34]. This comprised from the 5' end, 454 sequencing adapter B (italicised letters), 7 bp MID tag (denoted by X) and rRNA specific sequence 533R (bold text).

For each PCR reaction, 5 µl of template DNA was used and the PCR cycling conditions were as follows; 94˚C for 3 min, 10 cycles of 94˚C for 45 s (s), 55˚C for 30 s and 72 °C for 1 min, followed by a further 20 cycles of 94˚C for 45 s, 55˚C for 30 s and 72 °C for 90 s and a final extension of 72˚C for 5 min 30 s. Amplicon size and abundance was ascertained using agarose gel electrophoresis.

Library preparation, emulsion PCR and sequencing of the V1-V3 region of the 16S rDNA were conducted according to the GS FLX Titanium Series amplicon library protocol by Eurofins Genomics. Libraries were sequenced on a Roche Genome Sequencer FLX Titanium System™. Only the FLX Titanium B primer was utilised resulting in unidirectional sequences.

Sequencing data processing

The standard flowgram files were initially filtered by selecting reads with at least 360 flows and truncating long reads to 720 flows. Reads were filtered and denoised using the AmpliconNoise software (version V1.21) [35]. For the initial filtering step, reads were truncated when flow signals dropped below 0.7, indicative of poor quality. Subsequently, reads were denoised in three stages; (1) Pyronoise to remove noise from flowgrams resulting from 454 sequencing errors (PyronoiseM parameters -s 60, -c 0.01), (2) Seqnoise to remove errors resulting from PCR amplification (SeqNoiseM parameters -s 25, -c 0.08), (3) Perseus to detect and remove chimeras resulting from PCR recombination. The denoised sequences were then clustered at ≥98% identity using the script pick_otus.py within QIIME, which utilises the Uclust software program [36]. Uclust was run with modified parameters, with gap opening penalty set to 2.0 and gap extension penalty set to 1.0 and –A flag to ensure optimum alignment. The most abundant sequence in each OTU was selected as the representative.

The representative sequences were annotated using blastall 2.2.25 [37] and the Silva database (version 138). The Silva database contains full-length 16S rRNA sequences to previously identified canine oral taxa (COT) and feline oral taxa (FOT) [38]. These were deposited in GenBank and received accession numbers JN713151–JN713566 and KM461942–KM462187 [19, 26]. OTUs present in fewer than two samples or with an average relative abundance ≤ 0.05% were grouped together and deemed noise. This cut-off was defined based on false positive/negative species identification based on analysis of mock communities [20].

Statistical analysis

The Shannon diversity index was calculated for each sample using all OTUs prior to removal of OTUs classified as noise. Linear changes in Shannon diversity with proportion of periodontitis teeth in the mouth or mean gingivitis score (as continuous fixed effects) were investigated using linear mixed models, with dog nested in litter as the random effects.

nMDS was performed using a Bray Curtis distance matrix calculated from the non-rare OTU proportions (count of OTU out of the total number of sequences) to determine whether there was any association of the bacterial composition with mean gingivitis score or proportion of periodontitis teeth.

The individual OTUs (excluding those classified as noise) were analysed using GLM for proportions (the count for the OTU out of the total sequence depth for the sample), using a binomial distribution and logit link. Initially GLM were explored to account for correlations within a dog and litter, however the resulting models were found to have convergence and variance estimation problems. Thus, the final models dropped the random terms but included an overdispersion parameter to adjust the estimation for inflated variance at low proportions, (by using the "quasibinomial" family within the glm function to allow the variance parameter to not be fixed at 1) [39]. Two counts were added to each OTU count and four counts were added to the total sequence depth for each sample prior to analyses to enable better estimation with many zeros [40]. Cumulative proportion of periodontitis teeth in the mouth or mean gingivitis scores were investigated separately as continuous fixed effects. Mean gingivitis was calculated as the mean score for each tooth and then the mean of all teeth in the mouth. The odds ratio for the associated effects were estimated with 95% confidence intervals and the associated *p*-values adjusted for multiplicity by Benjamini-Hochberg to maintain a false discovery rate of 5% [41].

Statistical analyses were performed in R v3.2.4 statistical software [42] using the *vegan* library for the nMDS [43], *lme4* for exploring generalised linear mixed models [44], *multcomp* for calculating simultaneous comparisons and confidence intervals [45] and *ggplot2* for graphical representation [46].

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-025-04541-1.

Supplementary Material 1

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Author contributions
CW was involved in the conception and design of the study, acquisition, analysis and interpretation of the data, and drafting and revising the manuscript. AC input into the design of the study, performed the statistical analysis, helped with data interpretation and revised the manuscript. LH was involved in the conception and design of the study and revision of the manuscript. All authors read and approved the final manuscript.

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This work was funded by Mars Petcare who designed the study, collected, analysed and interpreted the data, and wrote the manuscript.

Data availability
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The study was approved by Waltham's Animal Welfare and Ethical Review Body and run under licensed authority in accordance with the UK Animals (Scientific Procedures) Act 1986.

Consent for publication
Not applicable.

Competing interests
CW, AC and LH were employees of Mars Petcare, a manufacturer of commercial petfood and provider of veterinary services, at the time of writing this manuscript. The data in this manuscript has been used to support a patent application.

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