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Influence of wet and dry commercial diets on the oral microbiota of Yorkshire terriers

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Abstract

Background Periodontal disease is common in dogs and is initiated by the build-up of plaque on the tooth surface. There is evidence that the feeding of dry diets may help prevent the build-up of plaque and calculus compared to softer wet diets. The primary objective of this study was to determine whether diet format influences the microbial composition of dental plaque.

Results Subgingival (SG) and gingival margin (GM) plaque samples were collected from 28 Yorkshire terriers, housed within a research facility, between 37 and 53 weeks of age. Dogs were fed either wet commercial diets, dry commercial diets, or a simultaneous offering of the two. Illumina sequencing of the 16 S rRNA gene (variable regions 3 and 4) of 43 SG and 43 GM plaque samples resulted in the generation of 6,725,682 paired end reads. Exploratory factor analysis, a statistical method for dimensionality reduction of multivariate data, was used to identify groups of covarying bacterial species. Subsequent mixed effects modelling revealed significant differences in the scores of two of these groupings indicating systematic differences in prevalences of their component taxa. One grouping revealed that for GM plaque samples, the profile of bacterial species most descriptive of the wet diet was biased towards those associated with periodontal disease whereas for the dry diet it was biased towards those associated with healthy gingiva. The dogs fed a mixture of wet and dry diets had bacterial profiles in between the wet and dry diets, i.e. a mix of both health and disease associated taxa. The other bacterial grouping indicated that, in dogs fed a wet diet only, GM plaque was significantly associated with bacteria that preferred aerobic conditions whereas SG plaque was associated with taxa that favoured anaerobic conditions.

Conclusions Although dry diets shifted the bacterial community towards a healthier profile compared to wet diets there was no evidence of improved periodontal health. Additional methods to maintain dental hygiene should therefore be promoted to ensure effective management of periodontal disease in dogs.

Keywords Canine, Dog, Microbiome, Oral, Periodontal disease, Bacterial plaque

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Background

Periodontal disease is common in dogs, with prevalence estimates of 44–100% [1–6]. Despite the high prevalence, less than 20% of dogs are diagnosed with the condition in first opinion veterinary practices [7–10]. This is likely due to the majority of diagnoses being based on conscious visual assessments and therefore potentially underestimating the extent of the disease [11, 12]. More detailed assessments of the periodontium (gingiva, cementum, periodontal ligament, and alveolar bone) under general anesthesia are required to ascertain the full extent of disease [13].

The initial stage of periodontal disease is apparent as red and inflamed gingiva and, without effective treatment, this can progress to periodontitis. Periodontitis is where the inflammation extends deeper into the periodontium causing irreversible damage. This can lead to periodontal abscesses and is the principal cause of tooth loss in dogs [14–17]. Several studies also suggest there is an association between periodontitis and systemic disease [18–21].

Biofilm development on the tooth surface is generally believed to initiate the disease process [17, 22, 23]. The first stage of biofilm formation is the adherence of salivary glycoproteins to the tooth surface. Primary bacterial colonisers then attach to this salivary pellicle, followed by secondary and tertiary colonisers ultimately resulting in mature plaque [17, 24–26]. The bacterial communities within these biofilms are complex and their composition and structure constantly adapting to their environment [27]. Despite this, distinct bacterial populations have been shown to be associated with canine periodontal health and disease [28–30]. Differences in the community composition of canine subgingival (SG) and gingival margin plaque (GM) have also been reported, which are likely partly due to the depletion of oxygen as bacteria infiltrate below the gingiva and into the sulcular epithelium [31].

Reduction of biofilm formation is the most effective means of maintaining healthy gingiva [15, 32, 33]. This can be achieved with an effective homecare regime combined with regular health checks, and veterinary treatment if required. A variety of homecare regimes have been shown to help prevent the build-up of plaque on the tooth surface and these include tooth brushing, dental chews, dental diets and oral solutions or gels[1] [12]. Some of these products work via mechanical abrasion resulting in the cleaning of the tooth and oral surfaces whereas others contain active ingredients. Recently, it has been shown that feeding dental chews to dogs can shift the bacterial composition of dental plaque towards a profile associated with periodontal health [34, 35]. There is also some evidence indicating that dry diets help to prevent the build-up of plaque and calculus and, reduce the levels of gingivitis compared to softer wet diets [36, 37]. However, other studies have found no correlations between dietary consistency and the levels of plaque, calculus or periodontal health status [6, 36, 38, 39].

An opportunity arose to study a population of Yorkshire terriers being acquired by the Waltham Petcare Science Institute. The primary research focus was to determine the incidence of gingivitis and periodontitis within this breed of dog [40]. In parallel, the study provided an opportunity to collect SG and GM plaque samples for a preliminary exploration of the impact of dietary composition (wet commercial diet, dry commercial diet or a simultaneous offering of the two) on microbial diversity and composition. The hypothesis was dogs fed a commercial dry diet, as opposed to commercial wet diets, would have plaque bacterial profiles more similar to those observed in dogs with healthy gingiva.

Results

Sequence quality and taxonomic assignment

Sequencing of the 43 SG and 43 GM samples using the Illumina MiSeq platform resulted in the generation of 7,141,323 paired end reads. Following removal of sequences deemed noise, 6,725,682 paired-end reads remained (94.2%) with an average number of reads per sample of 83,039 (+/- 43,277).

Clustering of sequence reads at ≥ 98% identity resulted in the generation of 16,419 operational taxonomic units (OTUs). After filtering for those lower than 0.05%, or that were not present in two or more samples, 172 OTUs remained. Of these, 165 (95.9%) had a sequence identity of ≥ 98% to sequences within the Silva database and the remaining 7 OTUs (4.1%) had identities ranging from 96.06 to 97.79%. A total of 118 OTUs (68.6%) were mapped to sequences previously isolated from plaque samples from dogs and cats. With respect to the other OTUs, 28 (16.3%) could be assigned taxonomy at the species level, 19 (11.1%) to genus, 5 (2.9%) to family, 1 (0.6%) to class and 1 (0.6%) to order.

In total, 12 phyla, 22 classes, 36 orders, 58 families, 86 genera and 163 species were identified within the canine SG and GM plaque samples. The most abundant phyla (sum of sequence reads across all samples) were Firmicutes (34.9%), Proteobacteria (19.0%), Bacteroidetes (18.0%), Actinobacteria (10.2%) and Fusobacteria (6.1%). The remaining 7 phyla comprised Patescibacteria (4.3%), Spirochaetes (3.3%), Epsilonbacteraeota (2.3%), Synergistetes (0.9%), SR1 (0.5%), Chlorobi (0.4%) and Tenericutes (0.2%).

Twenty-six species were present at a relative abundance > 1.0% and these together accounted for 58.2% of total sequence reads (Table 1). Taxa with COT and FOT

[1] http://www.vohc.org/VOHCAcceptedProductsTable_Dogs.pdf.

Table 1 Most abundant bacterial species within canine plaque (subgingival and gingival margin combined). COT and FOT identifiers indicate canine and feline oral taxon identified in previous studies of dogs and cats

Species	Number of sequences	Percentage of sequences
Porphyromonas cangingivalis	402,233	5.98%
Moraxella sp. FOT-350	375,202	5.58%
Actinobacteria bacterium COT-406	261,956	3.89%
Peptococcus sp. FOT-012/COT-044	237,916	3.54%
Parvimonas sp. COT-035/FOT-132	220,394	3.28%
Bergeyella zoohelcum	200,389	2.98%
Proteocatella sp. FOT-127/*Frigovirgula sp.* COT-007	185,228	2.75%
Peptostreptococcaceae bacterium COT-047/FOT-015	169,090	2.51%
Unclassified *Neisseria*	159,495	2.37%
Fusobacterium canifelinum/Fusobacterium nucleatum subsp. Polymorphum/*Fusobacterium nucleatum subsp.* Nucleatum	153,718	2.29%
Fusobacterium sp. FOT-120	145,827	2.17%
Granulicatella sp. COT-095	130,540	1.94%
Unclassified Saccharimonadaceae	113,662	1.69%
Peptostreptococcaceae bacterium COT-003	109,100	1.62%
TM7 phylum sp. COT-363	108,590	1.61%
Frederiksenia canicola	107,289	1.60%
Actinomyces canis	97,391	1.45%
Filifactor villosus/Filifactor FOT-044	95,715	1.42%
Peptostreptococcaceae bacterium COT-005/004/FOT-036	92,952	1.38%
Campylobacter rectus/ Campylobacter sp. FOT-100/COT-011	92,073	1.37%
Porphyromonas canis	88,228	1.31%
Peptostreptococcaceae FOT-068/COT-168	79,207	1.18%
Moraxella sp. FOT-087/COT-018	76,971	1.14%
Aquaspirillum sp. FOT-079	71,073	1.06%
Unclassified *Actinomyces*	69,283	1.03%
Peptostreptococcus sp. COT-033/FOT-053	68,969	1.03%

identifiers indicate canine and feline oral taxon identified in previous studies of dogs and cats [41, 42]. The species with the highest relative abundance in canine plaque was *Porphyromonas cangingivalis* representing 6% of the total sequence reads.

Changes in microbiota across sample type and diet

Multivariate analysis using Non-metric multidimensional scaling (nMDS) of all 86 samples (43 SG & 43 GM) revealed no clear separation of experimental groups, with all data ellipses overlapping (Fig. 1). However, there was some visual separation between GM and SG sample types, suggesting the two locations may have differing bacterial communities.

The Shannon diversity index did not significantly differ between either SG and GM plaque, dogs fed commercial dry, commercial wet, or a mixture of the two diets, or the interaction of these factors (all pairwise comparisons $p > 0.05$; Fig. 2).

Visual inspection of the phylum level data indicated some differences in relative abundance of the most prevalent phyla by sample type and diet group (Fig. 3). For example, dogs fed the dry diet had a lower relative abundance of Firmicutes in both GM and SG plaque. In SG plaque, this was associated with a higher relative abundance of Proteobacteria whereas, in GM plaque it was linked with a higher relative abundance of Proteobacteria and Bacteroidetes. However, a series of general linear model-based univariate analyses examining phylum level abundances did not find any of these differences, between the most prevalent phyla, to be statistically significant. Nonetheless, 14 significant phylum level differences were identified, involving a total of eight different phyla. Notably, all these differences related to sample type (GM vs. SG), and none to either diet or the interaction (Table 2). The most notable finding was the significantly higher relative abundance of Spirochaetes in SG plaque when compared to GM plaque across all 3 diet groups ($p = 0.033$ dry, $p = 0.021$ mixed, $p = 0.01$ wet).

Exploratory Factor Analysis, a multivariate statistical method, was used to determine whether any underlying structure existed within the OTUs identified in GM and SG plaque samples [43]. Essentially, the method groups OTUs with related patterns of response– i.e., uses the inter-correlations that exist between OTUs and reduces them into groups known as factors. Subsequent analysis is then applied to evaluate potential differences between these groups of covarying microbiota. A 'parallel analysis' procedure for factor selection identified a total of eight factors (i.e., taxonomic groupings). Subsequent pairwise comparisons, following linear mixed effects modelling of factor scores, indicated a significant difference in scores associated with Factor Group 3 between wet and dry commercial diets, in GM plaque ($p = 0.019$; Fig. 4). Also, for Factor Group 7, between GM and SG plaque samples, in dogs fed a wet diet ($p = 0.045$; Fig. 5).

The OTUs that were most characteristic of Factor Group 3 (i.e., that 'loaded' most heavily onto this factor with loadings > 0.5 or <-0.5), and were associated with changes in microbiota composition between wet and dry commercial diets in GM plaque, are shown in Table 3. GM plaque from dogs fed the wet diet was associated with OTUs with positive loadings, and most were members of the phylum Firmicutes (e.g., species belonging to the genus *Schwartzia* and *Selomonas* and the family Peptostreptococcaceae) and Spirochaetes (e.g., species belonging to the genus *Treponema*). The latter is consistent with the findings from the phylum level differences

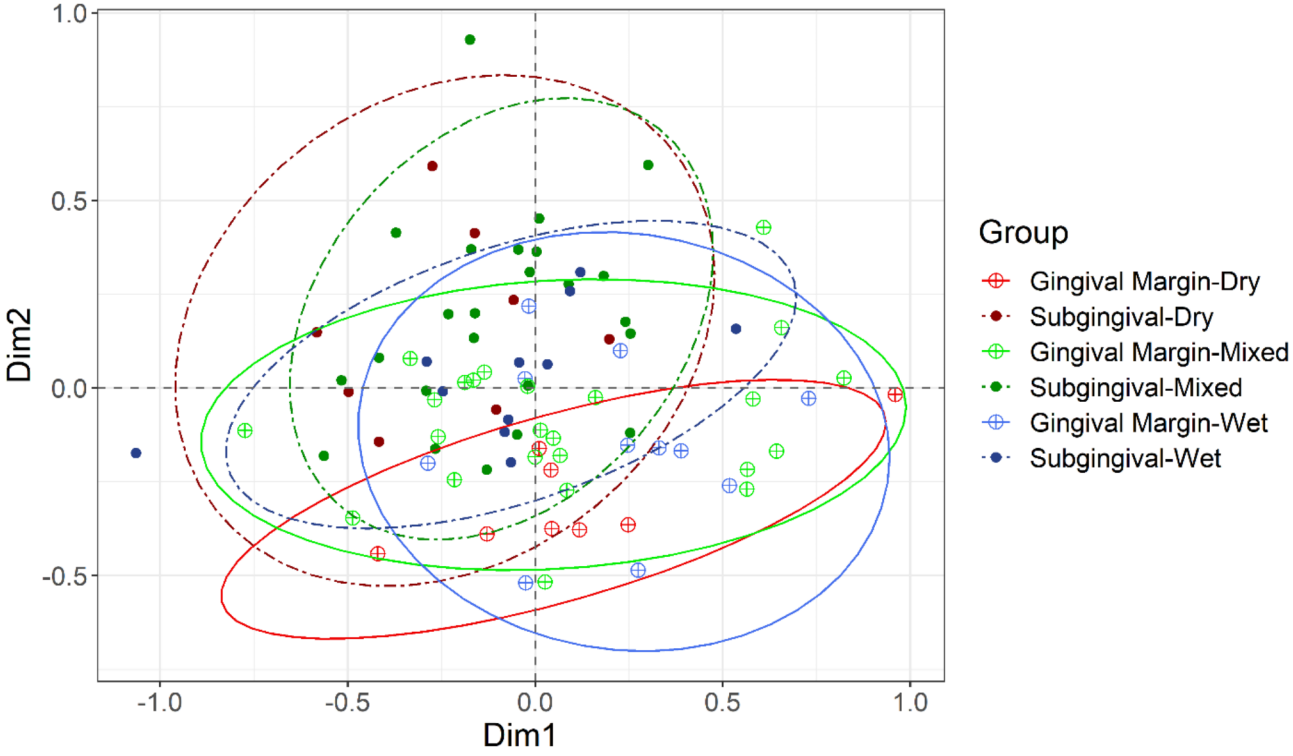

Fig. 1 Non-metric multidimensional scaling dimensions 1 (Dim1) and 2 (Dim2) labelled by sample type (gingival margin plaque (crosshair) and subgingival plaque (filled circle)) and diet (wet (blue), dry (red) or mixture of the two (green)). Ellipses represent 95% bivariate data quantiles for each experimental group

between GM and SG plaque. In contrast, GM plaque from dogs fed the dry diet was associated with OTUs with negative loadings and belonged to the phylum Bacteroidetes (e.g., species belonging to the genus *Capnocytophaga*) and Proteobacteria (e.g., species from the genus *Pasteurella*). The dogs fed a mixture of wet and dry diets had a mixture of OTUs with positive and negative loadings.

The OTUs that were most characteristic of Factor Group 7 (i.e., those with loadings of >0.3 or <-0.3) and are associated with changes in microbiota composition between GM and SG plaque from dogs fed the same wet diet are shown in Table 4. GM plaque samples were associated with OTUs with positive loadings and most belonged to the phylum Proteobacteria (e.g., species belonging to the genus *Aquaspirillum*, *Helomonas* and *Conchiformibius*) and Saccharibacteria (formerly known as TM7). SG plaque samples were associated with OTUs with negative loadings which generally belonged to the phylum Firmicutes (e.g., species belonging to the family Peptostreptococcaceae and the genus *Parvimonas*, *Proteocatella*, *Frigovirgula* and *Peptostreptococcus)*, Fusobacteria (e.g., species belonging to the genus *Leptotrichia* and *Fusobacterium*) and Proteobacteria (e.g., species belonging to the genus *Eikenella* and *Cardibacterium*). These taxa showed no obvious patterns in terms of their previous associations with periodontal health and disease. However, there were patterns in the oxygen requirements and Gram stain status for each of the most descriptive OTUs, as ascertained by performing literature and Internet searches on the taxonomic name assigned to each sequence. This indicated that OTUs with positive loadings were predominantly a mixture of Gram-negative aerobes and facultative anaerobes whereas those with negative loadings values were anaerobes or facultative anaerobes. Therefore, for dogs fed a wet diet only, GM plaque was significantly associated with bacteria that prefer aerobic conditions whereas SG plaque was associated with taxa that favour anaerobic conditions.

Periodontal health status

The clinical health status was similar across diet groups, with the average gingivitis score ranging from 1.40 to 1.52, and the proportion of periodontitis teeth ranging from 12.13 to 14.29 (Table 5). This suggests that the consistency of the diet did not differentially alter the periodontal health status of this population of Yorkshire terriers over the first year of their life.

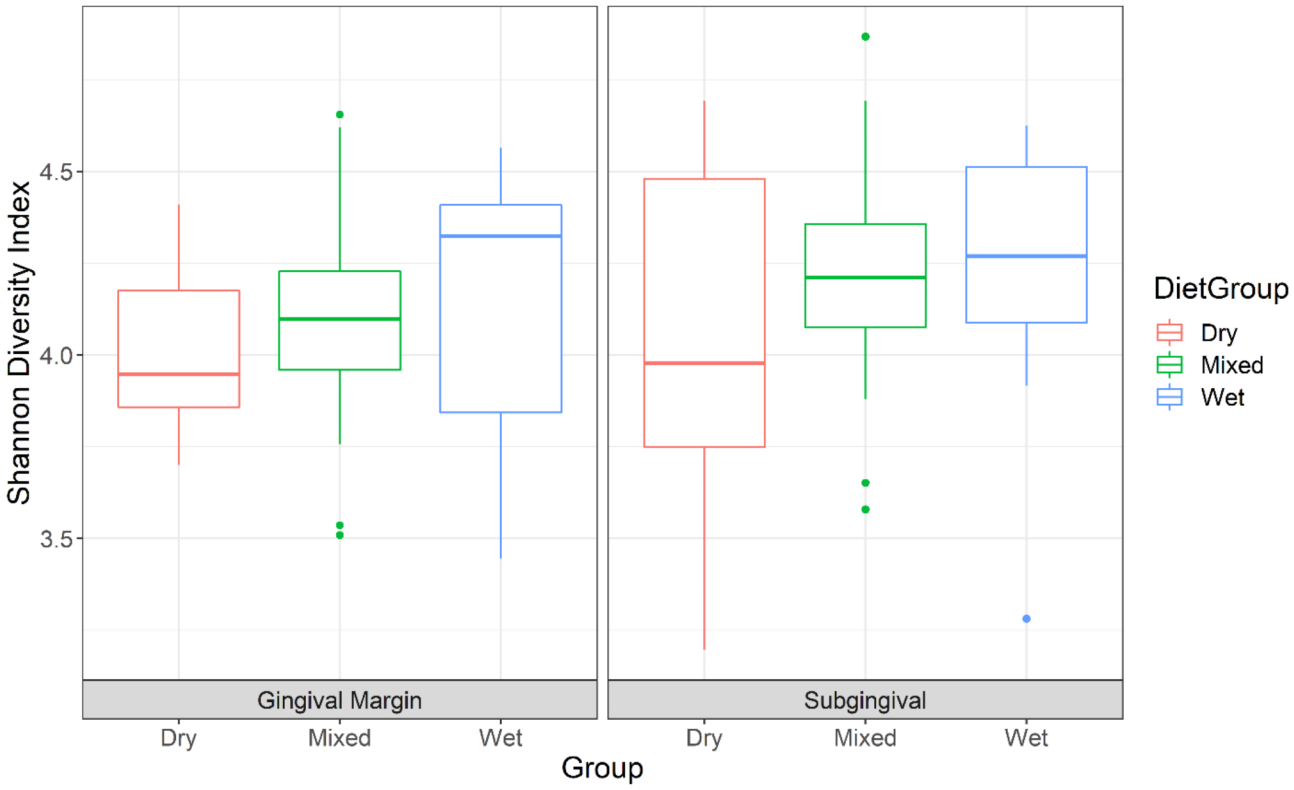

Fig. 2 Boxplots of Shannon diversity for gingival margin and subgingival plaque samples when dogs were fed a dry commercial diet (red), wet commercial diet (blue) or a mixture of the two (green). Individual dots represent outliers

Discussion

This study highlights the potential impact of commercial main meal diets on the bacterial composition of canine dental plaque. The phylum and species level bacterial compositions broadly agreed with other published studies of the plaque microbiota of dogs [28, 31, 44, 45]. Both GM and SG plaque samples from Yorkshire terriers fed a commercial dry diet had a lower relative abundance of Firmicutes when compared to those fed a commercial wet diet. This dry diet was also associated with a higher relative abundance of Proteobacteria (SG & GM) and Bacteroidetes (GM only). A number of bacterial species belonging to the phylum Firmicutes have been associated with early periodontal disease in dogs, whereas many of the bacterial species from the phyla Bacteroidetes and Proteobacteria have been associated with periodontal health [28, 29, 45]. These visually observed differences in relative phylum abundances between diets were not found to be significant. However, in the case of GM plaque, factor analysis provided statistical support for the visual observations of a diet-related difference. The OTUs in GM plaque most associated with the wet diet (positive loadings) were taxa previously associated with periodontal disease, whereas the OTUs most linked with the dry diet (negative loadings) were taxa previously associated with healthy gingiva [28]. This concurs with a recent study of 12 adult dogs which also reported that dogs fed dry diets generally had a higher abundance of taxa associated with oral health, and a lower abundance of taxa associated with poor oral health [46]. These colony dogs when fed commercial wet food (canned) for 6 weeks compared to those fed a commercial dry diet (extruded) had a significantly enriched relative abundances of Firmicutes and Synergistetes in subgingival plaque [46]. Taxa in supragingival plaque also significantly differed between diets with Chloroflexi, Fusobacteria and Synergistetes enriched in dogs fed dry food. Dogs fed wet food also had a higher microbial alpha-diversity [46]. A number of OTUs have also been shown to be differentially abundant in supragingival plaque collected from cats fed dry extruded kibble, when compared to those fed wet canned and/or fresh meat [47]. The most enriched genera in cats fed dry diets were *Actinobacillus*, *Acholeplasma*, *Treponema* and *Porphyromonas* whereas Proteobacteria from the Neisseriaceae family predominated in cats fed wet diets. Analysis of supragingival plaque samples from cats also indicated a significant effect of diet on species richness and evenness, where cats fed dry diets had a higher diversity of OTUs [47]. This contrasts with the Yorkshire terriers in this study where diet, and sample types, did not significantly impact bacterial diversity. It is not clear if the effects observed on the plaque microbiota

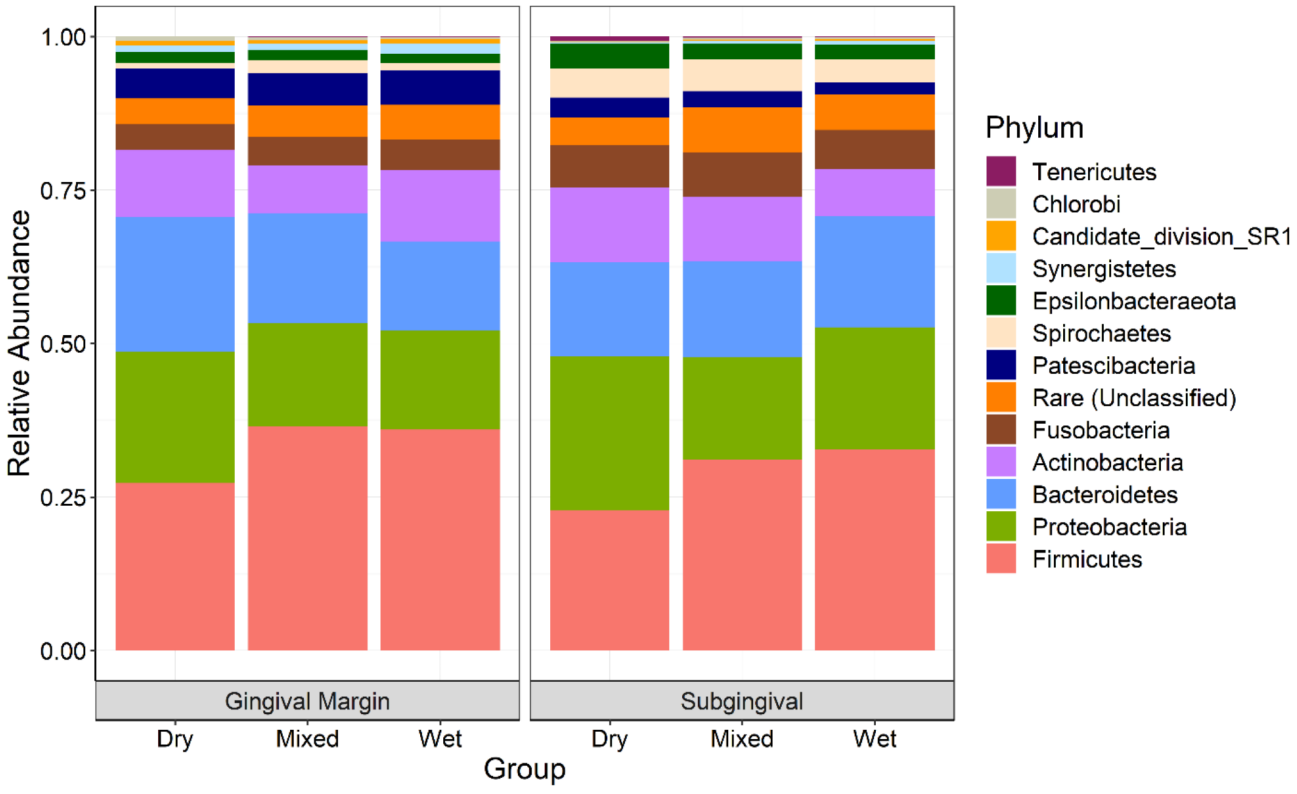

Fig. 3 Stacked bar chart showing the phylum level composition of gingival margin and subgingival plaque when dogs are fed wet commercial diets (wet), dry commercial diets (dry), and a mixture of the two (mixed)

of Yorkshire terriers are due to differences in dietary texture or the composition of the diets.

It has been hypothesized that the physical consistency and texture of foods affects the oral health of dogs and cats [36, 48]. Early studies suggested that soft food diets were associated with increased frequency and severity of periodontal disease compared to harder foods [36, 48]. However, the data are inconclusive and comparisons between studies difficult, often due to the small numbers of animals, the varying clinical evaluation methods used, and differences in data reporting [36]. A study of 63 dogs, with owner-reported diet and eating patterns, showed that dogs fed dry food had less gingivitis but there was no correlation between the feeding of soft diets and attachment loss [49]. An epidemiological study of 1350 client-owned dogs, again with owner reported feeding history, did not consistently demonstrate differences in the levels of gingival inflammation and periodontal bone loss when comparing dogs fed dry food only with those fed non-dry food only [38]. A study of 12 adult dogs fed either a commercial dry (extruded) or commercial wet (canned) food for 6 weeks did not significantly differ in their gingivitis scores [46]. In contrast, an epidemiological study of 17,184 dogs visiting Polish veterinary practices found that dogs fed only commercial pet foods, when at least part was a dry diet, had significantly better oral health than those fed home-prepared diets [37, 50]. In this study of Yorkshire terriers aged 37 to 53 weeks, the feeding of commercial wet, dry, and mixed diets had little effect on their overall periodontal health status (gingivitis and proportion of teeth with periodontitis), despite the fact Yorkshire terriers are prone to developing periodontal disease from a young age [40]. However, if more dogs had been included in the study and diets fed for a longer time a greater difference in their periodontal health status may have emerged.

The scientific literature on the effect of diet on a variety of oral health parameters is inconclusive. Several studies have reported that the feeding of soft diets is associated with increased plaque and calculus accumulation whereas others have reported no such effect [36, 46, 48]. Canned food has been reported to perform similarly to dry food in preventing the accumulation of plaque and calculus [38, 39]. A population survey of 63 dogs reported that dry food had little effect on plaque but reduced the levels of calculus [49]. A larger scale health survey of 2649 dogs reported less dental calculus where the diet was predominantly dry food compared to those whose diet composed mainly home-cooked, canned or leftovers [51]. Although the impact of wet and dry diets on plaque and calculus accumulation is not reported here, these data from Yorkshire terriers support the

Table 2 Generalised Linear Mixed effects models (GLM) - univariate analyses examining phylum level abundances between subgingival (SG) and gingival margin (GM) plaque when dogs fed dry commercial and wet commercial diets or a mixture of the two. Values in brackets represent 95% confidence intervals

Phylum	Mean relative abundance GM plaque			Mean relative abundance SG plaque			*P*-value (GM v SG)		
	Dry	Mixed	Wet	Dry	Mixed	Wet	Dry	Mixed	Wet
Actinobacteria	0.084 (0.051, 0.136)	0.068 (0.051, 0.091)	0.096 (0.063, 0.144)	0.131 (0.081, 0.205)	0.088 (0.066, 0.117)	0.069 (0.045, 0.105)	0.138	0.166	0.250
Bacteroidetes	0.188 (0.112, 0.297)	0.157 (0116, 0.208)	0.151 (0.096, 0.229)	0.152 (0.089, 0.246)	0.146 (0.108, 0.194)	0.167 (0.107, 0.251)	0.399	0.646	0.702
Candidate division SR1	0.005 (0.002, 0.016)	0.003 (0.002, 0.006)	0.005 (0.002, 0.013)	0.001 (0.0003, 0.003)	0.002 (0.0009, 0.003)	0.002 (0.0009, 0.006)	0.013	0.057	0.174
Chlorobi	0.003 (0.001, 0.01)	0.002 (0.001, 0.004)	0.002 (0.0008, 0.006)	0.0007 (0.0002, 0.002)	0.002 (0.0009, 0.003)	0.002 (0.0007, 0.005)	0.033	0.617	0.702
Epsilonbacteraeota	0.012 (0.0059, 0.024)	0.014 (0.009, 0.020)	0.011 (0.006, 0.020)	0.028 (0.014, 0.055)	0.024 (0.016, 0.035)	0.022 (0.012, 0.039)	0.033	0.021	0.071
Firmicutes	0.282 (0.191, 0.396)	0.331 (0.269, 0.399)	0.338 (0.249, 0.44)	0.249 (0.166, 0.357)	0.318 (0.258, 0.384)	0.319 (0.233, 0.419)	0.542	0.698	0.702
Fusobacteria	0.034 (0.015, 0.076)	0.033 (0.021, 0.053)	0.041 (0.02, 0.081)	0.052 (0.023, 0.113)	0.06 (0.038, 0.094)	0.054 (0.027, 0.10)	0.364	0.021	0.621
Patescibacteria	0.04 (0.021, 0.077)	0.037 (0.025, 0.054)	0.047 (0.027, 0.082)	0.022 (0.011, 0.043)	0.022 (0.015, 0.033)	0.018 (0.01, 0.032)	0.124	0.021	0.007
Proteobacteria	0.153 (0.072, 0.298)	0.163 (0.107, 0.241)	0.14 (0.073, 0.251)	0.179 (0.085, 0.339)	0.141 (0.091, 0.21)	0.169 (0.090, 0.295)	0.635	0.542	0.646
Spirochaetes	0.009 (0.004, 0.022)	0.016 (0.009, 0.026)	0.008 (0.004, 0.017)	0.03 (0.012, 0.071)	0.037 (0.022, 0.061)	0.03 (0.014, 0.063)	0.033	0.021	0.010
Synergistetes	0.006 (0.001, 0.029)	0.005 (0.002, 0.011)	0.007 (0.002, 0.024)	0.0014 (0.0003, 0.007)	0.003 (0.001, 0.006)	0.001 (0.0004, 0.005)	0.033	0.166	0.012
Tenericutes	0.0002 (0.00004, 0.0006)	0.0004 (0.0002, 0.001)	0.0003 (0.00009, 0.001)	0.002 (0.0005, 0.007)	0.001 (0.0004, 0.002)	0.0007 (0.0002, 0.002)	0.0003	0.046	0.169

hypothesis that dry diets result in lower levels of colonization of the tooth surface by bacterial species dominant in mature plaque biofilms and periodontal disease [44].

Exploratory factor analysis followed by further univariate analyses on resulting factor scores, identified significant factor differences relating to sample type. In dogs fed the commercial wet diet, the OTUs with the highest positive loadings within a grouping were more prevalent in GM plaque samples; these tended to be Gram-negative bacterial species that prefer aerobic conditions. In contrast, the SG plaque samples were correlated with OTUs with negative loadings, and these tended to be bacterial species that favour anaerobic conditions. This supports the most notable phylum level difference identified between sample types which was the increased relative abundance of spirochaetes in SG compared to GM plaque. This finding is consistent with a previous study of the SG and GM plaque microbiota of dogs [31]. Spirochaetes thrives in anaerobic conditions and several species in this phylum, such as *Treponema* sp., have been associated with periodontitis [52]. Previous studies have also shown SG plaque samples to have a significantly lower proportion of aerobic taxa and significantly higher proportion of anaerobic taxa than GM samples [31, 46]. The absence of a corresponding relationship between OTUs and sample type when dogs were fed a dry diet is consistent with the diet preventing the build-up of mature plaque biofilms both above and below the gum line. However, it must be noted that no significant interaction effect was evident, and this relationship may not have been found simply due to insufficient sample size.

The main considerations for this study were the relatively small number of dogs and the fact the study was not specifically designed to investigate the impact of diet consistency on the oral microbiota. The relatively small number of dogs per diet group means the study may not have been sufficiently powered, and therefore the significant differences in bacterial composition observed are likely biased towards those with the largest variation between diet groups and sample types. Likewise, the small number of dogs could be why differences in overall periodontal health status were not detected. Also, more insights may have been found if the duration of the study had been longer. Whilst client-owned dog studies are potentially more representative of the general population, they rely on self-reported data which is known to be unreliable with often other types of food being fed and not recorded. Although diet groups could not be balanced between wet and dry diet on this study because of the primary research goal for the population the highly controlled feeding environment enabled reliable data to be generated. The plaque samples were collected from

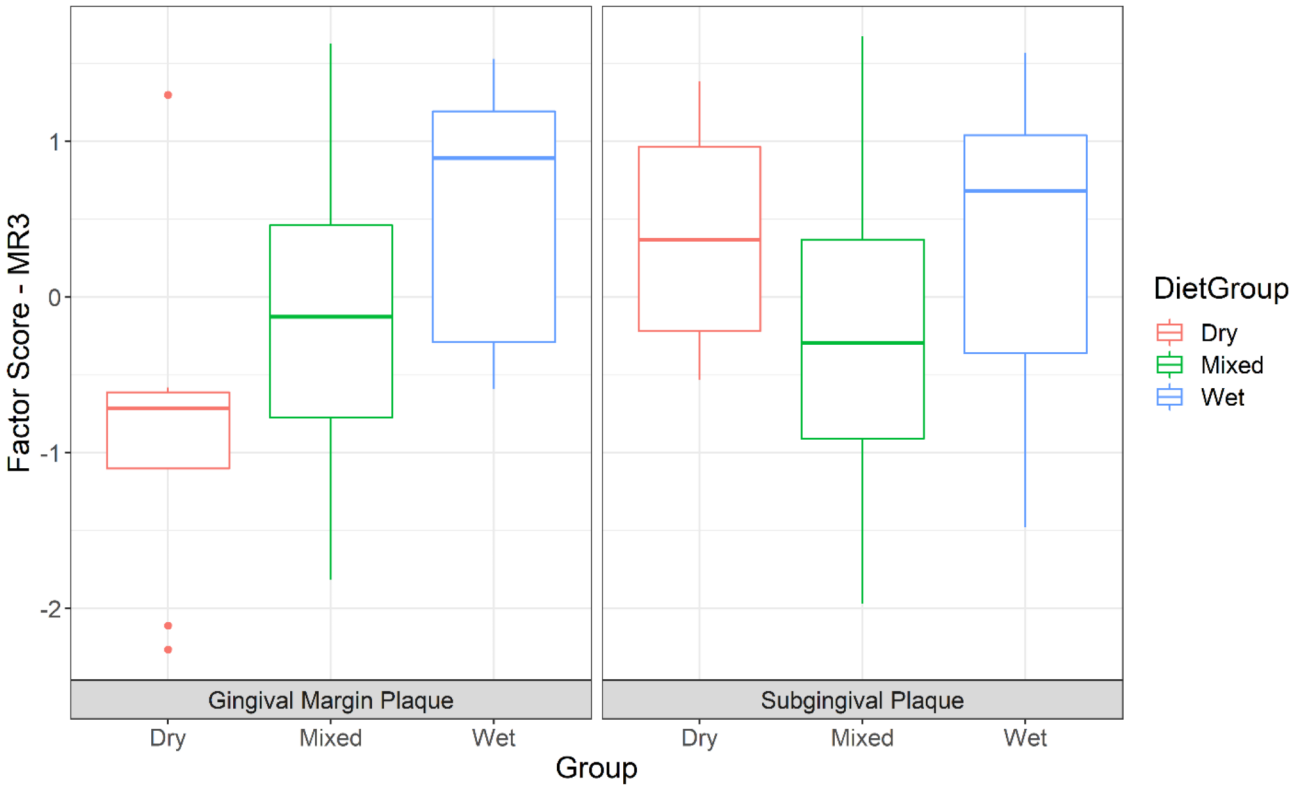

Fig. 4 Boxplots of the scores for Factor Group 3 for gingival margin and subgingival plaque for each diet group: Commercial dry (red), commercial wet (blue), mixture of commercial wet and dry (green). Individual dots represent outliers

dogs 37 to 53 weeks of age but there is limited data on whether the puppy microbiota is comparable to that of adult dogs. A study of 20 small breed dogs (9 breeds) aged 6–8 months indicated that the oral microbiota is similar to that of adult dogs although there were some differences suggesting the oral microbiota was still in the process of maturation [53]. Despite these limitations, the current study generated several insights which have been aided by the use of a single breed of dog maintained within a managed research environment where diet could be strictly controlled. Further research is necessary to determine the impact of dietary composition and texture on the canine oral microbiota before it can be ascertained whether the type of main meal diets fed can improve the periodontal health of dogs.

Conclusions

In this study of Yorkshire terriers during the first year of life, evidence was found to suggest that feeding a commercial dry diet may reduce colonization of the tooth surface by the types of bacterial species found in mature plaque biofilms and associated with periodontal disease. This, however, was not matched with clear evidence of the dry diet preventing the development of periodontal disease in this predisposed breed. Additional methods, such as tooth brushing, to maintain dental hygiene are therefore required and these should be promoted to ensure effective management of periodontal disease in dogs.

Materials and methods

Study cohort

The 28 Yorkshire terriers (7 litters) investigated in this study were a subset of a larger study to determine the incidence of gingivitis and periodontitis in Yorkshire terriers [40]. This was an opportunistic study as the Waltham Petcare Science Institute were acquiring a population of Yorkshire terriers at their research facility. The dogs were acquired from UK breeders at around 8 weeks of age. All puppies included in the study had a genetic DNA test (Wisdom Panel™, Mars Petcare) which confirmed they were representative of the global Yorkshire terrier pet population. Puppies were sequentially enrolled onto the study at 37 weeks of age and remained on the study until a maximum of 53 weeks of age (+/- 1 week). There were 15 entire females and 13 neutered males. The dogs' body weight at 37 weeks of age ranged from 3.92 kg to 16.28 kg (average 11.69 +/- 1.97 kg). The dogs were housed in environmentally enriched kennels which included indoor and outdoor access. All dogs were provided with a comprehensive socialisation and training programme which was adjusted to their individual needs.

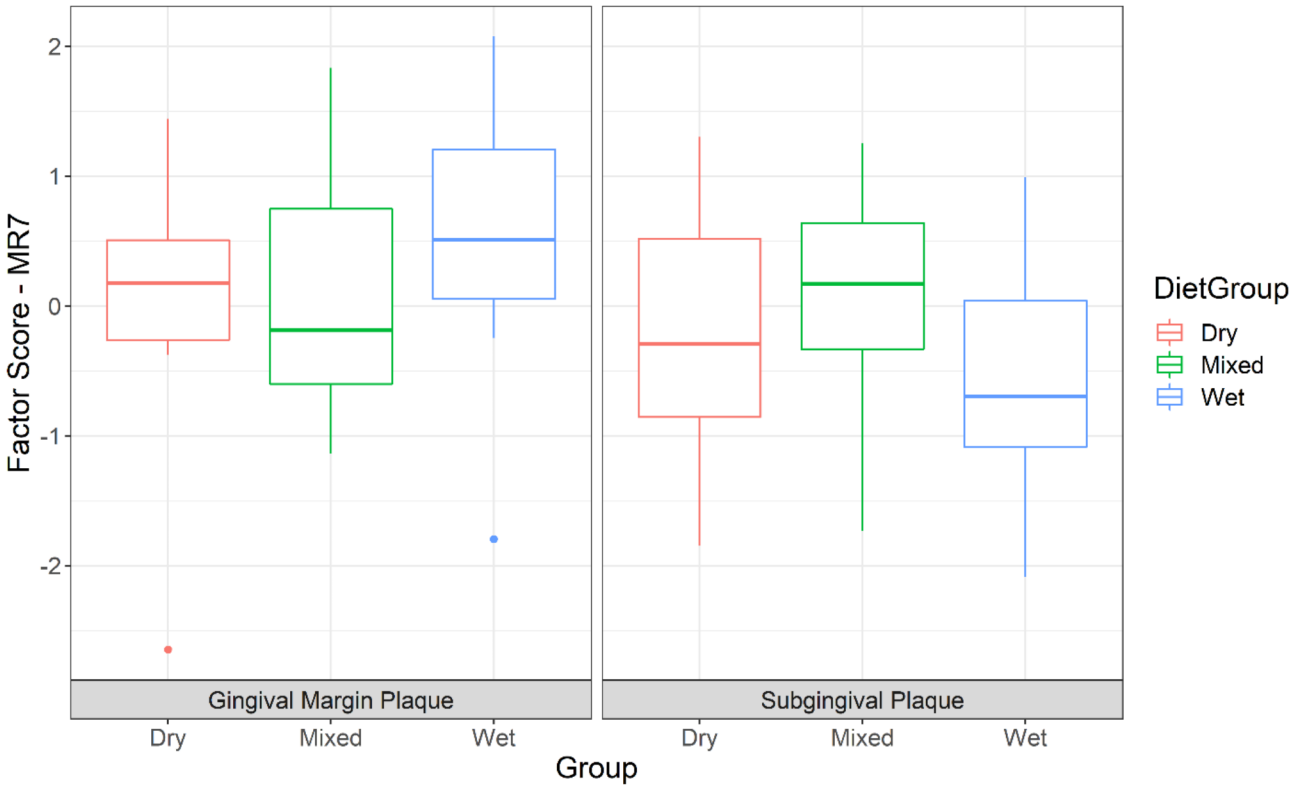

Fig. 5 Boxplots of the scores for Factor Group 7 for gingival margin and subgingival plaque for each diet type: Commercial dry (red), commercial wet (blue), mixture of commercial wet and dry (green). Individual dots represent outliers

The dogs were fed a commercial dry diet (Royal Canin® Yorkshire terrier 29 Junior) from weaning up to 14 weeks of age. At 14 weeks, 5 dogs remained on this diet, 7 were weaned onto a commercial wet diet (Cesar® puppy with chicken & rice with a carrot topping), and the remaining 16 dogs were fed a simultaneous offering of the dry and wet diets. Diets were nutritionally analysed (Eurofins) and the predicted metabolizable energy content calculated according to the equation described by the National Research Council [54]. The energy intake of the dogs was determined as described previously [55]. The Yorkshire terriers were shared across several research studies being undertaken by the Waltham Petcare Science Institute. Diet and diet groups were depicted by an unrelated feeding study (unpublished data). Dogs did not receive any oral care products such as dental diets, chews, gels, wipes or oral solutions.

This study was approved by the Waltham Animal Welfare and Ethical Review Body and run under licensed authority in accordance with the UK Animals (Scientific Procedures) Act 1986. The suitability of dogs for the study was determined by a veterinarian based on a physical examination and a review of the dogs' veterinary history. No dogs were excluded from taking part in the study.

Clinical measures

As part of the parent study, the dogs had their oral health determined at 37 weeks of age and then re-assessed at eight-week (+/- 1 week) intervals up to a maximum of 53 weeks of age [40]. The clinical assessments were performed under general anaesthesia during which the levels of gingivitis and periodontitis were assessed around the whole gingival margin of every tooth. Gingivitis was measured using time to bleeding on probing (scale 0–4; 0 being healthy gingiva and 4 severe gingivitis), and periodontitis was based on extent of clinical attachment loss (probing depth, gingival recession and furcation exposure) [40]. Full details on the clinical scoring methods and general anaesthesia protocol can be found in the published primary study of periodontal disease in Yorkshire terriers [40]. Dogs were examined by a veterinarian prior to each general anaesthesia and were removed from the study once 12 or more teeth developed the early signs of periodontitis [40]. This meant that 28 dogs were assessed at 37 weeks of age but only 13 and 2 dogs were assessed at 45 and 53 weeks respectively. Throughout the study routine veterinary care was permitted. This sometimes included the administration of antibiotics and anti-inflammatory drugs. Records of all veterinary treatments were maintained for each dog. No dogs received

Table 3 Most influential operational taxonomic units in factor group 3 and their periodontal health associations as determined from scientific publications

OTU #	Loading	Phylum	Class	Order	Family	Genus	Species	Health association
8534	0.527	Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	*Treponema*	*Treponema* sp. COT-207	Disease
11,699	0.531	Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	*Treponema*	*Treponema* sp. COT-247	Disease
10,845	0.534	Firmicutes	Negativicutes	Selenomonadales	Veillonellaceae	*Selenomonas/Schwartzia*	*Schwartzia* sp.FOT-014/ COT-063	Disease
9061	0.559	Firmicutes	Negativicutes	Selenomonadales	Veillonellaceae	*Selenomonas*	*Selenomonas* sp. COT-167	Unknown
5093	0.611	Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	*Treponema*	*Treponema* sp. COT-249	Disease
329	0.612	Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	*Treponema*	Unclassified *Treponema*	Disease
10,686	0.616	Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	*Treponema*	*Treponema* sp. COT-201	Disease
7737	0.628	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Unclassified Desulfovibrionales	Unclassified Desulfovibrionales	Desulfovibrionales bacterium COT-009	Disease
4245	0.635	Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	*Treponema*	*Treponema* sp. COT-350	Disease
8371	0.646	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	*Parvimonas*	Peptostreptococcaceae bacterium COT-030/ FOT-028	Disease
1959	-0.673	Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	*Capnocytophaga*	*Capnocytophaga cynodegmi/ Capnocytophaga canimorsus*	Health
4616	-0.635	Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	*Capnocytophaga*	*Capnocytophaga canimorsus/ Capnocytophaga* sp. COT-295/ FOT-311	Health
5402	-0.626	Proteobacteria	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	*Pasteurella*	*Pasteurella dagmatis/ Pasteurella stomatis/ Pasteurella mulocida/ Pasteurella* sp. FOT-354	Health
7264	-0.62	Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	*Capnocytophaga*	*Capnocytophaga canimorsus*	Health

antibiotic or anti-inflammatory drugs in the 4 weeks preceding their clinical assessment.

Sample collection

At each timepoint two plaque samples, one SG and one GM, were collected from each dog. Samples were collected from every tooth in the mouth when dogs were under general anaesthesia for assessment of periodontal health. This resulted in a total of 43 SG and 43 GM samples (1–3 SG and 1–3 GM samples per dog (Fig. 6). The reduction in the number of samples collected at later timepoints was due to prerequisites of the Waltham

Table 4 Most influential operational taxonomic units in factor group 7. The oxygen requirements were ascertained by performing literature and internet searches on the taxonomic name assigned to each sequence

OTU #	Loading	Phylum	Class	Order	Family	Genus	Species	Gram stain	Oxygen requirements
5151	0.429	Proteobacteria	Gammaproteobacteria/ Betaproteobacteria	Betaproteobacteriales/ Neisseriales	Aquaspirillaceae/ Neisseriaceae	*Aquaspirillum*	*Aquaspirillum* sp. FOT-082	G-	Aerobe
799	0.324	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	*Halomonas*	*Halomonas phoceae*	G-	Aerobe
11,517	0.316	Patescibacteria/ TM7	Saccharimonadia	Saccharimonadales	Saccharimonadaceae	Candidatus *Saccharimonas*		G+	Aerobe
15,463	0.315	Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	*Treponema*	*Treponema* sp. FOT-142/ COT-200	G-	Anaerobe/ Microaerophilic
78	0.308	Patescibacteria/ TM7	Saccharimonadia	Saccharimonadales	Saccharimonadaceae	Candidatus *Saccharimonas*		G+	Aerobe
11,245	0.306	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	*Alloprevotella*	*Prevotella* sp. COT-282	G-	Anaerobe
12,361	0.304	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Neisseriaceae	*Conchiformibius*	*Conchiformibius* sp. COT-289	G-	Aerobe
14,329	-0.307	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	*Eikenella*	*Eikenella* sp. COT-049	G-	Facultative anaerobe
6805	-0.311	Firmicutes	Clostridia	Clostridiales	Family_XI	*Parvimonas*	*Parvimonas* sp. COT-035/ FOT-132	G+	Anaerobe
7086	-0.328	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	*Proteocatella*/ *Frigovirgula*	*Proteocatella* sp. FOT-127/*Frigovirgula* sp. COT-007	G+	Anaerobe
6181	-0.334	Proteobacteria	Gammaproteobacteria	Cardiobacteriales	Cardiobacteriaceae	*Cardiobacterium*	*Cardiobacterium* sp. COT-176	G-	Facultative anaerobe
13,271	-0.341	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Unclassified Peptostreptococcaceae	Peptostreptococcaceae bacteriumCOT-047/ FOT-015	G+	Anaerobe
8646	-0.346	Fusobacteria	Fusobacteriia	Fusobacteriales	Leptotrichiaceae	*Leptotrichia*	*Leptotrichia* sp. COT-345	G-	Anaerobe/ Facultative anaerobe
6529	-0.366	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	*Peptostreptococcus*	*Peptostreptococcus* sp. COT-033/FOT-053/*anaerobius*	G+	Anaerobe
12,700	-0.416	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	*Fusobacterium*	*Fusobacterium canifelinum/nucleatum* subsp. Polymorphum/ Nucleatum	G-	Anaerobe

Animal Welfare and Ethical Review Body. This required dogs to be removed from the study, and given a scale and polish, when 12 or more teeth developed the early stages of periodontitis.

GM plaque was collected prior to scoring gingivitis. Collection involved sweeping a sterile periodontal probe across the crown of the tooth just above the gingival margin. The probe was placed into a 0.5 ml Eppendorf tube containing 300 µl TE buffer (10mM Tris-HCL, 1 mM

Table 5 Summary of Yorkshire Terriers mean gingivitis score (+/- standard deviation) and proportion of periodontitis teeth (+/- standard deviation) by diet group

Diet Group	Mean gingivitis score (scale 0–4)	Proportion periodontitis teeth
Dry	1.52 +/- 0.15	12.13 +/- 5.41
Mixed	1.46 +/- 0.17	14.29 +/- 6.43
Wet	1.40 +/- 0.25	12.55 +/- 8.20

disodium EDTA, pH8.0; Sigma-Aldrich) and agitated to remove the plaque. SG plaque samples were collected whilst scoring gingivitis. This involved placing a sterile periodontal probe under the gingival margin and sweeping it along the base of the crown of the tooth. Again, the probe was placed into a 0.5 ml Eppendorf tube containing 300 µl TE buffer and agitated to remove the plaque. The SG and GM 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). The manufacturer's instructions were followed 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 overnight. Following DNA extraction, the DNA pellet was resuspended in TE buffer.

Sequencing of the 16 S rRNA gene

The 16 S rRNA gene, variable regions 3 & 4, was amplified using the Extensor Hi-Fidelity PCR Enzyme Mix (AB-0792, Thermo, UK) and universal bacterial primers 319F and 806R. Each primer contains a linker sequence, index sequence and heterogeneity spacer [56]. The PCR mixture contained 25 µl Phusion® High-Fidelity PCR Master Mix with HF Buffer (MO531, New England Biolabs, UK), 5 µl of each primer (1µM), 10 µl template DNA, 3.5 µl nuclease free water and 1.5 µl DMSO (3%), prepared in a 96-well format. The PCR cycling conditions consisted of an initial denaturation step at 98 °C (30s), followed by 30 cycles of 98 °C (15s), 58 °C (15s) and 72 °C (15s) and a final elongation at 72 °C (60s).

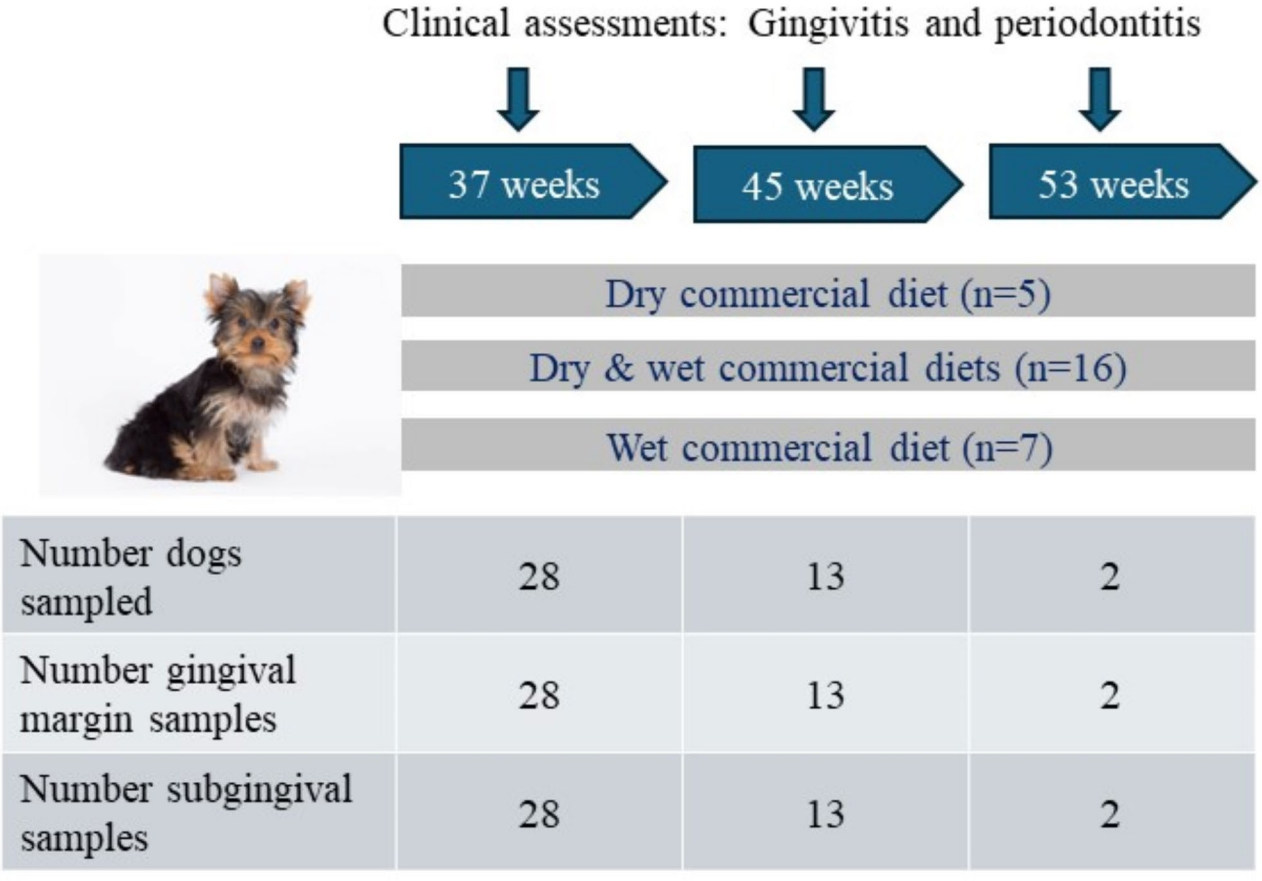

Fig. 6 Schematic of the study design together with a summary of the number of dogs sampled and gingival margin and subgingival plaque samples collected at each timepoint

Library preparation and sequencing was carried out by Eurofins Genomics, Germany. In brief, the 16S amplicons were quantified using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, UK) and then pooled in equimolar amounts. The 16S rRNA amplicon libraries were sequenced on a MiSeq (Illumina) using v3 chemistry and bi-directional 300 bp mode.

Processing of sequence data

Forward and reverse reads were assembled into contiguous sequences spanning the entire V3-V4 regions using *FLASH* assembler [57]. Linker sequences were removed using *TagCleaner* [58] and sequences de-multiplexed in *QIIME* (version 1.9) using split_libraries_fastq.py [59]. Chimeric sequences were removed using userarch6 [60]. Sequences were clustered at ≥98% identity using *uclust* [61] to generate OTUs. The most abundant sequence in each OTU were selected as the representative. The representative sequences were annotated using blastall 2.2.25 [62] and the Silva databases (version 138). The Silva database contains full-length 16 S rRNA sequences to previously identified canine oral taxa (COT) and feline oral taxa (FOT) [63]. These were deposited in GenBank and received accession numbers JN713151–JN713566 and KM461942–KM462187 [41, 42]. If the alignment matched the top BLAST hit with ≥98% sequence identity and ≥98% sequence coverage then a species level was assigned but if these criteria were not met the next appropriate level of taxonomic assignment was allocated: ≥94% genus; ≥92% family; ≥90% order, ≥85% phylum. OTUs present in fewer than two samples or with an average relative abundance ≤0.05% were deemed "noise". This cut-off was based on statistical analysis of mock communities to determine optimum false positive and negative rates [28].

Statistical analysis

Statistical analyses were performed using R statistical software (versions 4.0.0 & 4.1.3) [64] using packages *vegan* [65], *lme4* [66], *psych* [67], *multcomp* [68], and *ggplot2* [69]. The primary measures for oral health status of each dog were mean gingivitis score (average of four measurements for each tooth and then average of all teeth in the mouth), proportion of healthy teeth in the mouth and the proportion of teeth with periodontitis in the mouth. The primary microbiota measure was OTUs. Data from all 86 samples (43 SG and 43 GM) were included in the statistical analyses. Due to so few dogs providing the full complement of, or even multiple, samples (see end of Clinical measures sub-section), there were insufficient data to robustly evaluate time-related effects, and timepoints were assessed together. Repeat observations from the same dogs were accounted for within the statistical models where feasible and appropriate (e.g., through the inclusion of random effect terms for individual animal in LMEM formulae).

A multivariate analysis, using nMDS, was performed using a Bray-Curtis distance matrix calculated from the OTU proportions (count of OTU out of the total number of sequences), to determine whether overall microbial composition was visually separable by diet (wet, dry, or mixed) or sample type (GM or SG). Ellipses representing the 95% bivariate data quantiles for dimensions 1 and 2 were calculated, assuming a multivariate t-distribution [70].

A series of univariate analyses were also undertaken, to assess between group variation in alpha-diversity (Shannon index), abundance of individual phyla, and prevalence of 'Factor Groups' of species with related response patterns, as identified through factor analysis.

The Shannon diversity index was calculated for each sample, using all OTUs prior to removal of those deemed noise. A linear mixed effects model was fitted, with Shannon diversity index as the response variable, total sequence counts as a covariate, and diet group, sample type and their interaction as fixed effects. Individual dog was entered as the sole random effect (intercept-only). Normality and homogeneity of model residuals were assessed through visual inspection and verified using Shapiro [71] and Bartlett's tests [72], respectively.

The relative abundances of each of the 12 phyla identified in the study were calculated for each sample (count of phylum out of the total number of sequences). Generalised linear mixed effects models were then fitted, using a binomial distribution with a logit link function, to the proportions (prior to analyses. To aid model convergence when an OTU has many zero counts, 2 counts were added to each OTU count and 4 to the total sequence count, analogous to adding 2 successes and 2 failures [73]) for each of the respective outcome variables. Each model included fixed effects of diet group, sample location, and their interaction, plus random effects of both dog and observation; the observation level random effect being introduced to mitigate overdispersion [74]. Pairwise comparisons were then conducted, using the *R*-function *glht*, contained in the *multcomp* package. These compared the relative abundance of each phylum between diet groups (wet, dry, or mixed) for each sample type (GM or SG), between sample types for each diet group, and for the interaction between these factors (i.e., whether the difference between diets varied between sample types). A *p*-value correction was applied to adjust for multiple comparisons across the 13 models, according to the false discovery method of Benjamini and Hochberg [75], but no adjustment was made across pairwise comparisons within each model. A false discovery rate of 5% was applied.

To investigate potential differences in clusters of covarying OTUs an exploratory factor analysis was performed, followed by linear mixed effects modelling to evaluate differences in 'Factor Groups between diets and sample types. Factor analysis is a statistical method used to describe variability among observed, correlated variables in a lower number of unobserved 'latent' variables, called factors (i.e., dimension reduction) [67]. In the present case each identified factor should relate to a grouping of related OTUs in the dataset. Factor scores, representing the prevalence of each grouping, were subsequently compared between experimental groups. First, log transformed relative abundances (+2 to the count and +4 to the total) were explored for the optimal number of factors, using a parallel analysis procedure implemented through the *R*-package *psych* [76, 77]; factor eigenvalues were compared versus resampled data on a scree plot, and a total of eight factors were selected. Factor analysis was then conducted, with eight factors, using ordinary least squares regression (minimum residual method), and without factor rotation, to obtain factor scores (by experimental condition), and factor loadings (by OTU). Then linear mixed effect models were fitted, to assess variability of each Factor Group between experimental conditions. Here, respective factor scores were the response variable, with diet group (wet, dry, or mixed), sample type (GM or SG) and their interaction as fixed effects, and individual dog as the random effect. As previously, *p*-values were then determined for the pairwise differences between diet groups within each sample type, sample types within each diet group, and the two-way interactions. Again, these were adjusted to compensate for alpha-inflation across models, using the Benjamini-Hochberg (false discovery rate, FDR) method [75]. Periodontal health associations of the OTUs most associated with diet group were determined from the published literature [28]. Oxygen requirements and Gram stain status for each of the most descriptive OTUs were ascertained by performing literature and Internet searches on the taxonomic name assigned to each sequence.

Abbreviations

SG	Subgingival
GM	Gingival Margin
OUT	Operational Taxonomic Unit
TE	Tris-EDTA
nMDS	Non-metric Multidimensional Scaling
FDR	False Discovery Rate

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Author contributions

CW, LH were involved in the conception and design of the study. CW, ZE performed the analysis of the data and prepared the figures. CW, LH, ZE and GA interpreted the data and wrote the main manuscript. All authors reviewed the final manuscript.

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This study was funded by the Waltham Petcare Science Institute who were involved in study design, collection, analysis and interpretation of data, writing the manuscript, and in the decision to submit the article for publication.

Data availability

The high throughput sequencing datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request. The 16 S rRNA canine oral taxa and feline oral taxa sequences used to annotate the data were deposited in GenBank and received accession numbers JN713151-JN713566, KF030193-KF030235, JN713151-JN713566 and KF030193-KF030235.

Declarations

Ethics approval and consent to participate

This study was approved by the Waltham 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, ZE, GA, LH were employees of Mars Petcare, a manufacturer of commercial pet food, at the time of writing this manuscript. The data in this manuscript has been used to support a patent application.

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