Development of the Oral Microbiome in Kittens

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Abstract

The oral cavity harbors a complex microbiome, and next-generation sequencing studies indicate that the adult cat oral microbiome can be altered by food type and health status. To study development of the oral microbiome, plaque samples were collected from kittens within 24 hours of birth and throughout the preweaning period. Microbial diversity significantly increased with age. Although four phyla were predominant throughout, many significant differences in relative abundance were noted with age. These data indicate that the kitten oral microbiome rapidly changes during preweaning. Potential dietary interventions during this age may improve lifelong oral health in cats.

Glossary of Abbreviations
OTU: Operational Taxonomic Unit
PCA: Principal Component Analysis
PCoA: Principal Coordinate Analysis
PD: Phylogenetic Diversity
PERMANOVA: Permutational Multivariate Analysis of Variance

Introduction

The oral cavity harbors a complex ecosystem consisting of bacteria, protozoa, fungi, and viruses. Bacteria colonize oral surfaces through the establishment of plaque biofilm, which allows the bacterial community to form a stable structure by embedding in an extracellular matrix of polymers of host and bacterial origin. Commensal bacteria in the plaque biofilm play an important role in host defenses against pathogens by excluding their colonization in this biofilm.[1] However, continuous accumulation of the biofilm can lead to dysbiosis and predisposition to disease.

Until recently, oral microbial research was limited to culture-dependent techniques, which allowed for identification of only a fraction of the community present. With the advent of culture-independent techniques such as next-generation sequencing, a deeper analysis of the oral microbiome in health and disease is possible. Culture-independent analysis of the oral microbiome has greatly increased the ability to identify bacteria present in this complex environment. While culture-dependent techniques identified 280 bacteria in the human mouth, culture-independent techniques resulted in characterization of over 700 bacteria species in healthy humans.[2] Although there is individual variation in the oral microbiome and bacteria populations differ based on sample location (epithelium versus teeth), a major portion of the oral microbiome is shared by unrelated healthy adults.[3] Increasing the ability to identify bacteria present in the oral microbiome allows for a clearer understanding of changes due to age, diet, and health status. The acquisition and establishment of the oral microbiome in infants is of particular interest, as early bacteria colonizers may affect lifelong health.

Early Establishment of Oral Bacteria in humans

While it was believed that infants are sterile *in utero*, recent studies have shown that amniotic fluid contains bacteria with a composition most similar to the oral microbiome of the mother.[4] It has been hypothesized that this bacteria population may play a role in establishing immune tolerance for subsequent bacterial colonization following birth.[4] Birth mode (vaginal or caesarian) has been shown to impact the oral bacteria population of infants, with vaginally born infants having greater bacterial diversity.[5,6] Following birth, infants acquire oral bacteria from their environment through breathing, breast-feeding, and contact with others. The mother is a significant contributor of microorganism exposure during this time. Total aerobic bacteria in the saliva of mothers and newborn infants has been found to be significantly correlated.[7] Early colonizers of the infant oral microbiome are able to adhere to the epithelium and promote attachment by other bacteria, allowing for a rapid increase in diversity.[8] By 5 months of age, the oral microbiome of infants is distinct from their mother due to continued exposure to bacteria in the environment.[9] Diet has been shown to play a role in shaping the microbial community, as formula or breast-feeding affects the infant oral microbiome composition. Breast-fed infants have increased populations of *Lactobacilli* with antimicrobial properties compared to formula-fed infants.[10,11]

While the early colonization of the oral microbiome has been studied in human infants, it has not been examined in companion animals. However, this critical period could be important in establishing a healthy microbiome for lifelong health in pets.

Importance of the Oral Microbiome in Companion Animals

Research activity in the pet oral microbiome is less developed than in humans. Key differences exist in the oral cavity of pets compared to humans, so human research is not as easily transferable as compared to other areas of research.

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For example, salivary pH of companion animals is neutral to basic (7.3 to 7.8 in dogs and 7.5 in cats), while human salivary pH ranges from 6.2 to 7.4.[12,13] Unlike humans, pets lack salivary amylase, which initiates carbohydrate digestion.[13] Key differences in electrolytes and dentition result in an altered oral microbiome composition in pets compared to humans. For example, pets do not naturally have *Streptococcus mutans*, a key bacteria in the formation of dental caries, in their plaque. While 92% of adult humans have dental caries in their permanent dentition, the incidence is less than 5% in dogs and almost nonexistent in cats.[14]

Although dental caries are rare, companion animals are susceptible to periodontal disease. Gingivitis affects 95 to 100% of pets, while periodontitis affects 50 to 70% of the population.[15] Pathogenic microorganisms in the subgingival plaque degrade nitrogenous substrates to cytotoxic compounds (short-chain fatty acids, ammonia, sulfur compounds, indoles), inducing tissue inflammation and promoting apoptosis.[16] Upper and lower premolars are particularly affected by periodontal disease in cats,[17] and the incidence increases with age.[18] Prevalence of specific periodontic pathogens (*P. gulae, T. forsythia, C. rectus*), gingivitis, and periodontal disease significantly increases with age in dogs as well.[19] While inflammation from gingivitis can be reversed with professional dental cleaning and regular dental care, severe periodontitis results in permanent bone loss. In addition to periodontal disease, several studies have demonstrated a link in pets between poor oral hygiene and overall systemic health. Gingivitis and periodontal disease have been associated with increased systemic inflammation,[20] kidney disease,[21] endocarditis,[22] and parenchymal liver disease.[23]

While it has been demonstrated that oral bacteria affects both dental and systemic health, little research is available on the core oral microbiome of dogs and cats. The advent of next-generation sequencing has greatly increased our ability to study the populations and diversity associated with health and further identify putative pathogens in disease states. However, research in the area of oral health has lagged behind other areas.

Cat Oral Microbiome Studies

Very limited research has been published with regard to the oral microbiome in cats. Early studies were limited to culture-dependent methods and focused on identifying specific pathogens associated with infections from cat bites. Bacteria isolated from infections are similar in composition to the cultivatable oral bacteria in cats.[24] *Pasteurella multocida*, a common bacteria in the oral cavity of cats,[25] was isolated in 70% of infected bite wounds in humans.[26] Additionally, *P. multocida* was the most common facultative anaerobe isolated from infections in feline pyothorax[27] and in subcutaneous abcesses.[24] Love, et al., also demonstrated that *Bacteroides* species can be found in both the oral cavity of cats and in subcutaneous abcesses.[28] Norris and Love showed that three species of *Porphyromonas*, a common oral bacteria, could be isolated from subcutaneous abscesses in cats.[29] While these early studies do not directly address the common oral bacteria in healthy cats, they demonstrate that bacteria associated with infections from cat bites also can be found in samples taken from the oral cavity.

Love, et al., first characterized bacteria in the gingival margin of 14 healthy cats using anaerobic plating techniques.[30] *Actinomyces* was the most common facultative anaerobic taxa identified. *Bacteroides* and *Fusobacterium* comprised 77% of cultivatable obligate anaerobes. The most common species identified were *C. villosum* and *P. multocida*. These results were very similar to what had been cultivated in infections and abscesses associated with cat fights.

Next-generation sequencing has allowed deeper insights into the oral microbiome of healthy cats. Dewhirst, et al., established a 16S rRNA-based taxonomy for common feline oral bacteria.[31] Subgingival bacteria from cats with healthy gingiva or with periodontitis were collected, and the samples were pooled into two groups (healthy and periodontitis). Purified 16S rDNA were sequenced and assigned taxonomical groups through comparison against several 16S rRNA gene databases. A total of 171 feline oral taxa were identified and categorized into 11 phyla. Approximately 89% of the oral taxa were assigned to *Firmicutes, Proteobacteria, Bacteroidetes,* or *Spirochaetes*. Establishment of a gene reference set was an important step in better characterizing the oral microbiome of cats.

Supragingival plaque was collected from adult cats and analyzed via next-generation pyrosequencing in 2014.[32] Eleven cats living in households and receiving commercial diets were selected (six households; five of six housed two cats each). Bacteria identified were divided into 18 phyla, and 97.6% of bacteria were represented by seven phyla. Proteobacteria, Bacteroides, Firmicutes, and SR1 were the four most prevalent phyla. Bacteria diversity was similar to dog oral microbiome studies. Multivariate analysis indicated that cats living in the same household had a more similar oral microbiome. This study demonstrated that the oral microbiome of healthy cats is diverse and may be affected by the environment.

Two studies have compared the healthy feline oral microbiome to that of cats with periodontal disease. Mallonee, et al., aimed to study the relationship between healthy feline oral bacteria and periodontal disease.[33] Subgingival bacterial samples were collected from 32 cats (15 with periodontal disease, 11 with gingivitis, and six with no oral problems) and analyzed using culture-dependent techniques. The relationship of bacteria populations to gingival scores was evaluated. Higher gingival index scores (indicating increased inflammation) were associated with higher anaerobic Gram-negative rods. *Bacteroides* and *Peptostreptococcus* increased with periodontal disease, while *P. multocida* decreased. This early study concluded that oral bacteria populations

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shift during the progression of periodontal disease.

Next-generation sequencing of the V1-3 region was used to study subgingival plaque samples collected from 92 client-owned cats categorized as healthy (n = 20), gingivitis (n = 50), or mild periodontitis (n = 22).[34] Regardless of health status, over 90% of bacteria were classified in seven phyla, with Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria dominating. However, the proportion of phyla changed based on health state. When compared to healthy cats, Firmicutes doubled, while Bacteroidetes and Proteobacteria decreased in cats with periodontal disease. Five phyla increased with periodontal disease (Firmicutes, Spirochetes, Syngeristetes, Chloroflexi, TM7). Principal component analysis (PCA) indicated discrete clustering of healthy and mild periodontitis groups, while cats with gingivitis overlapped both groups. Although differences were noted in bacterial populations, the total number of operational taxonomic units (OTUs) detected and bacterial diversity were not significantly different among the groups.

Only one published study has evaluated the impact of diet type on the feline oral microbiome.[35] Supragingival plaque was collected from client-owned cats that were fed exclusively dry extruded kibble (n = 6) or wet food (n = 4), and the V1-V3 region was amplified and analyzed using next-generation sequencing. Regardless of diet, three phyla constituted 76% of the bacteria sequenced (Bacteroidetes, Firmicutes, Proteobacteria). Cats receiving the dry diet had significantly higher diversity as measured by Shannon index ($P < 0.05$). A differential abundance test was used to determine what caused the increase in diversity. Cats receiving the dry diet had a higher abundance of *Porphyromonas* spp. and *Treponema* spp., while cats consuming wet food had increased *C. kuhniae* ($p < 0.05$). The authors concluded that diet type affected the feline oral microbiome composition and diversity, but further research was needed to determine if one diet type was best for promoting health.

While limited research regarding the impact of health status and food form on the oral microbiome of cats exists, no research has been published on the development of the oral microbiome in kittens. The objective of our research was to assess changes in the oral microbiome of healthy kittens during the preweaning period. Oral bacteria at birth and changes in the microbiome due to age and diet type (milk or commercial food) were of particular interest.

Materials and Methods

Plaque sampling. Sixteen newborn kittens determined to be healthy and thriving at birth were selected for this study. Kittens were removed from the study if they became ill at any time during the preweaning period. The study was approved by the Nestlé Purina Institutional Animal Care and Use Committee.

Litters were housed and raised according to standard kitten rearing protocol at the Nestlé Purina PetCare Center. Kittens consumed milk exclusively for their first 2 weeks of age. Moistened extruded dry and wet commercial foods were slowly introduced to kittens starting at week three. The proportion of milk:solid food was transitioned over the preweaning period until 8 weeks of age when kittens were consuming solid food exclusively. All kittens received the same commercial foods during this study.

A total of five supragingival plaque samples were collected from kittens (within 24 hours of birth and at 2, 4, 6, and 8 weeks of age). To collect plaque, the mouth of the kitten was gently opened, and teeth and gingiva were gently swabbed for 10 to 15 seconds using a sterile cotton-tipped swab. If teeth had not yet erupted, gum ridges were swabbed. Plaque samples were stored at -800C within 15 minutes of collection until further analysis.

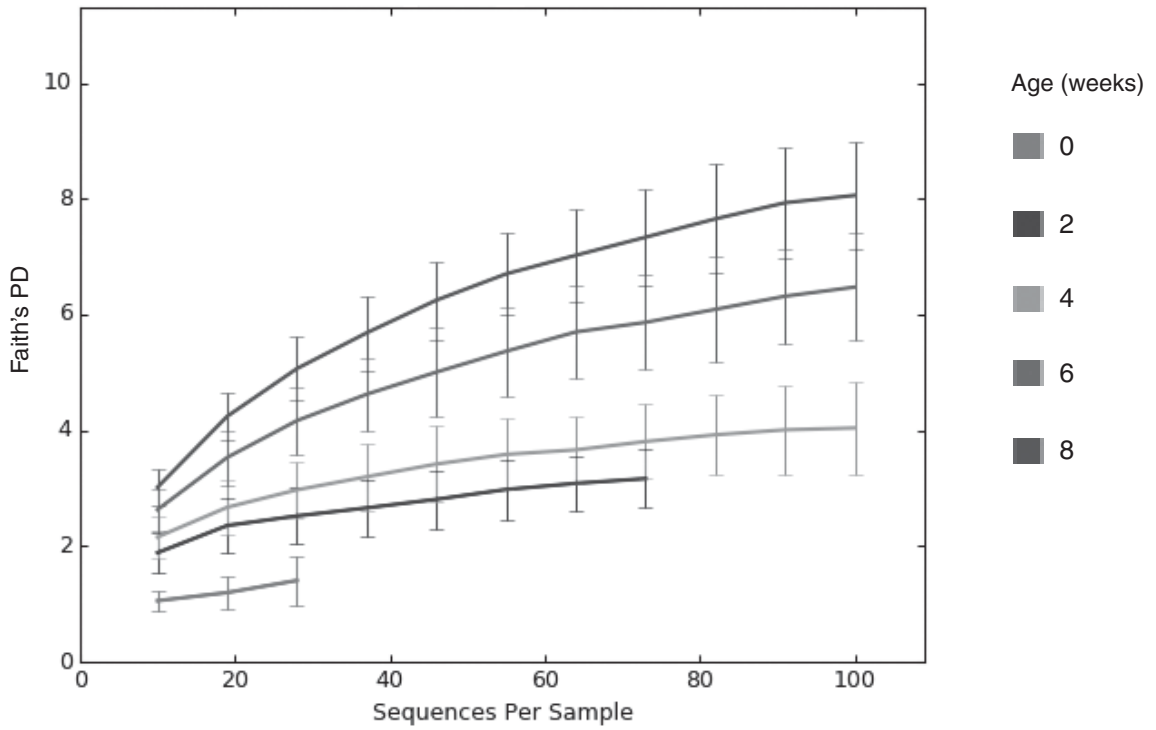
DNA Extraction. DNA extractions were performed using a modified protocol of the GenElute Bacterial Genomic DNA Kit, which allowed for the isolation of both Gram-positive and Gram-negative genomic DNA. Genomic DNA was quantified using the Quant-It PicoGreen dsDNA Assay kit on a microplate fluorescence reader (Bio-Tek Instruments Inc.). Samples were stored at -80^{0}C until sequence analysis.

16S rRNA gene sequencing. 454 pyrosequencing was performed on the regions of V1-3 of bacterial 16S rRNA gene. The sequencing data were processed as previously described with slight modifications.[36] Sequenced reads that were longer than 550 bases or shorter than 200 bases were removed. A total of 690,121 sequences was obtained from 92 samples, of which 16 samples were removed due to low sequence counts (less than 200).

Sequence processing. Sequencing noise characteristic to 454-pyrosequencing was removed by flowgram clustering as implemented in QIIME.[37,38] Chimeric sequences were detected and removed using a reference-based approach as implemented in UCHIME.[39] After that, a total of 277,667 high-quality sequences was obtained for the remaining 76 samples with the average of 3,653 sequences per sample. The average length for the sequences was 481 bases. OTUs were picked using a closed reference-based OTU picking method with UCLUST[39,40] with similarity threshold of 97%. The reference data file was obtained from the Silva database (release 123).[41] Taxonomical information was assigned to each OTU using the naïve Bayesian classifier at a minimum confidence internal of 0.8.[42]

Alpha and beta diversity indexes. The OTU table was first randomly subsampled at the level of 100 sequences per sample. The subsampled OTU table was used to calculate Faith's phylogenetic diversity. Additionally, a rarified OTU table was used to calculate a dissimilarity matrix based on weighted unifrac distance metric.[43] Principal coordinates analysis (PCoA) was performed on the distance metrics. Permutational multivariate analysis of variance (PERMANOVA)[44] was performed to test whether there was a significant difference among the groups on the beta diversity distance matrix using

Figure 1. Faith's phylogenetic diversity of kitten oral microbiome samples.[a]



[a] Curves for weeks 0 and 2 are short due to some samples having less than 100 sequence counts.

10,000 permutations. A phylogenic tree showing the similarity of the oral microbiome of the 16 cats also was created.

Statistical Analysis. Total sum scaling was used to account for uneven sequencing depths across samples. In addition, bacteria with less than 0.01% relative abundance in all of the conductions were removed.

A two-part zero-inflated Beta regression model with random effects (ZIBR)[45] was used to examine the main impact of time (two, four and eight weeks) on bacterial relative abundance. The ZIBR model includes two parts: 1) a logistic regression component that examines the presence/absence of a bacteria, and 2) a beta regression component that examines the non-zero relative abundance of a bacteria. Both components allow for the addition of a random effects parameter to account for the correlation between measures from the same subject. In the present study, this allowed us to account for the nonindependence of the data as each kitten was measured at the three points in time. Previous research has shown that this method has outperformed earlier methods such as linear mixed effects models.[45] A p-level less than 0.05 was considered significant.

In this paper, relative abundance of the newborn microbiome is reported. Comparisons are made between the kitten microbiome phyla at 2 weeks of age (exclusively milk diet), 4 weeks of age (mix of milk and solid diet), and 8 weeks of age (exclusively solid diet).

Results and Discussion

Kittens. Sixteen kittens (eight male, eight female; five litters with an average litter size of 3.2 kittens) completed the study. One litter was born via cesarean section, and one litter consisted of a single kitten. Average birth weight of kittens was 133 grams, and the average weight at 8 weeks of age was 920 grams. All kittens that completed the study maintained normal growth and remained healthy throughout the study.

Diversity. To investigate diversity of the oral microbiome community, alpha-diversity (within sample difference) and beta-diversity (between sample differences) were calculated. Faith's phylogenetic diversity (PD) on age is shown in Figure 1. Samples separated at each collection time, with diversity being lowest in samples collected within 24 hours of birth (age 0) and highest at 8 weeks of age. The number of sequences per sample also increased with age.

Principal coordinate analysis of the weighted UniFrac metrices was performed (Figure 2). The first principal coordinate (PC1) of the PCoA plot explained 54.7% of the variation in the dataset. The closer the points are to each other in the graph, the more similar the samples are to each other. Oral microbiome PCoA analysis shows clear clustering of plaque samples by age. Samples collected within 24 hours of birth cluster tightly together. Plaque samples at 6 and 8 weeks of age overlap, meaning they are the most similar. Beta diversity of the oral microbiome at different ages in the preweaning period differs significantly, suggesting a strong age influence on oral microbiome composition in kittens.

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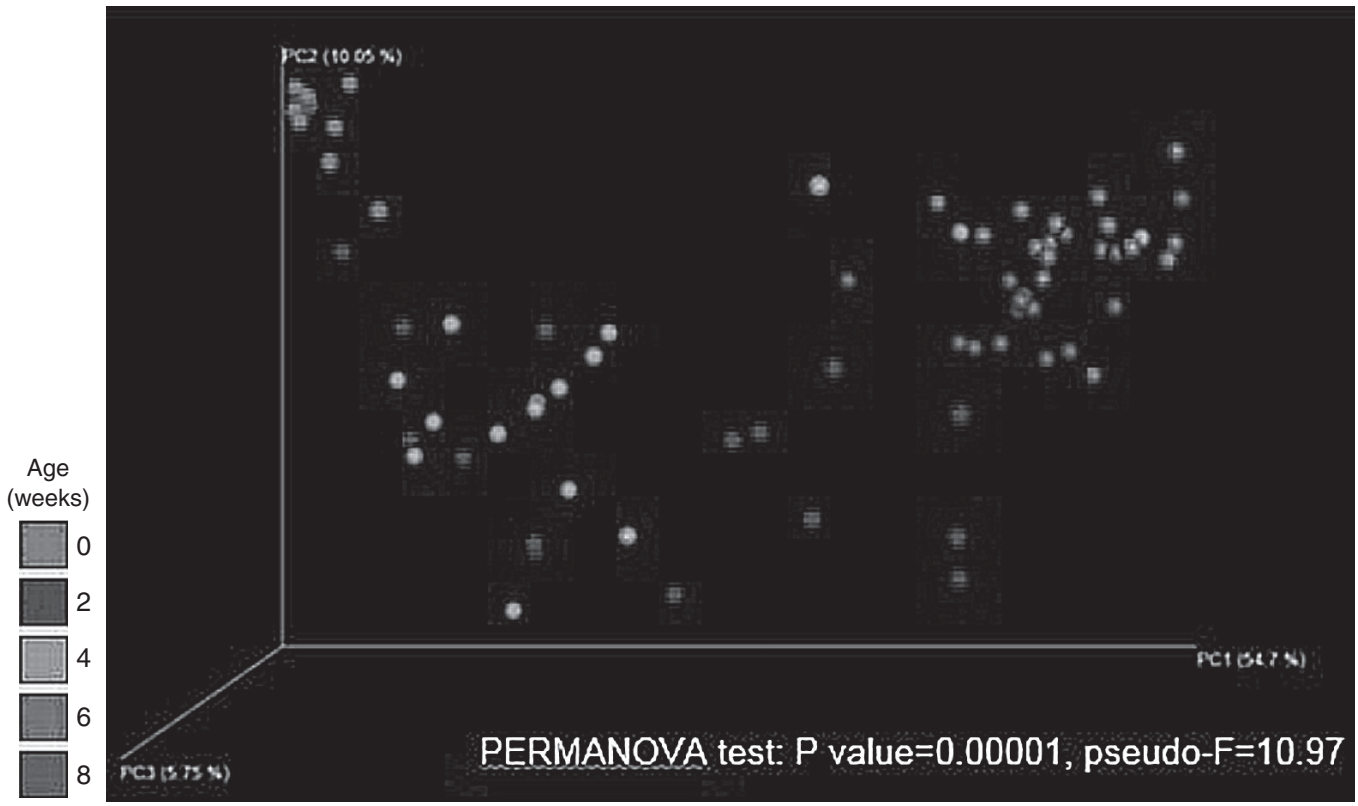
Figure 2. Principal coordinate analysis (PCoA) of weighted UniFrac metrices on kitten oral microbiome samples.



Sample size was as follows: 0 weeks = 11 samples, 2 weeks = 15 samples, 4-8 weeks = 16 samples.

Significant differences were noted in alpha and beta diversity measures. Differences in diversity could be due to age, development of teeth, and increase in exposure to environmental factors, such as diet. That the microbiome is most similar between samples when kittens were eating an increased amount of solid food:milk (week six) and solid food exclusively (week eight) indicates that commercial diets may play a role in stabilizing the developing oral microbiome of kittens.

Newborn phylogenetic analysis. To our knowledge, this is the first time that oral bacteria populations in newborn kittens have been studied. Within 24 hours of birth, the overall number of oral bacteria reads was significantly lower for kittens born via C-section compared to vaginal birth (48.3 versus 4614.7 reads, respectively). Due to low sequence counts, C-section kittens were not included in phylogenetic analysis at birth.

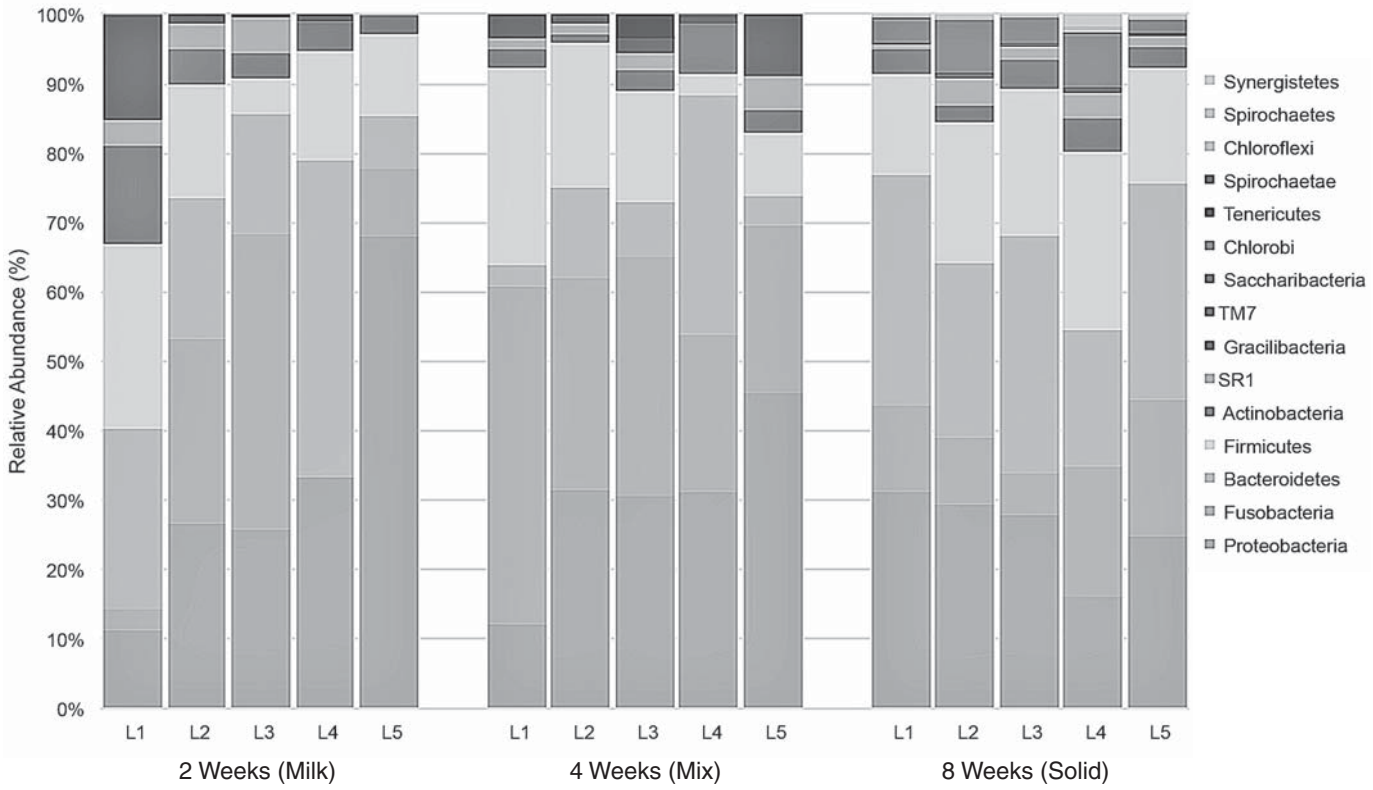
The majority of OTUs could not be classified at the phylum level at birth (77.4%; range from 63.8 to 92.5% by litter). Throughout the preweaning period, the percentage of unknown oral bacteria decreased with age (84.9%, 69.8%, and 26.0% unknown bacteria at 2, 4, and 8 weeks of age, respectively; $p < 0.05$). This decrease in unclassified OTUs during preweaning indicates that novel microbial taxa may play a key role in the early colonization of the cat oral microbiome and that they are different than those identified in adult cats.

Overall, the oral bacteria at birth that could be identified were clustered into seven different phyla. Proteobacteria (90.0%) and Firmicutes (9.3%) were the two predominant phyla with a relative abundance greater than 1.0%, representing over 99% of the categorized sequences. Other phyla present were Bacteroidetes, Actinobacteria, Fusobacteria, Chlorobi, and SR1.

The three predominant oral bacteria phyla reported in healthy adult cats are Proteobacteria, Firmicutes, and Bacteroidetes (Sturgeon, et al., 2014; Harris, et al., 2015), but Bacteroidetes was present in less than 0.5% of classified bacteria in newborn kittens in this study. These initial data indicate that Proteobacteria plays a larger role in the establishment of oral microbiome of newborn kittens. However, while differences in relative distribution of bacteria were noted between newborn kittens and studies investigating adult cats, the large proportion of unclassified oral bacteria at birth make it difficult to draw firm conclusions. Further research is needed to identify bacteria that could not be classified in this study and determine if birth mode may impact establishment of the oral microbiome in kittens as it does in human infants.

Phylogenetic analysis during growth. Predominant oral bacteria phyla of litters at 2, 4, and 8 weeks of age are presented in Figure 3. Overall, several differences were noted in bacteria phylum over time. The number of bacteria phyla

Figure 3. Relative abundance (%) of identified bacteria phylum by age.[a]



[a] Age of kitten (primary food source). L1-L5 specify litters of kittens. Litter 4 was a single kitten, and Litter 5 was delivered by C-section.

increased with age (10 phyla present at 2 and 4 weeks of age and 13 phyla present at 8 weeks of age), reflecting the increase in overall bacterial diversity previously noted. While variation occured among litters and between time points, five phyla were predominant in the oral microbiome (Proteobacteria, Fusobacteria, Bacteroidetes, Firmicutes, Actinobacteria).

Distribution of the major phyla was more varied among litters at 2 weeks of age, when milk was the sole source of nutrition. The predominant phyla at this age varied by litter. While Bacteroidetes was the major phylum present in Litters 1 and 4, Fusobacteria was greater in Litter 3. Litter 5, which was born via C-section, had a greater representation of Proteobacteria compared to the other litters. By 4 weeks of age, teeth had erupted, and kittens were receiving solid food in addition to milk. The oral microbiome was more similar at a phyla level among litters by this time period. With the exception of Litter 1, Proteobacteria was the dominant phyla present. At 8 weeks of age, all kittens were receiving the same commercial solid food. Phyla distribution was the most similar among litters at this time point, with Proteobacteria and Fusobacteria being most prevalent. These shifts in the predominant phyla during the preweaning period are indicative of the rapid transition of the oral microbiome during this period due to age, diet, and environmental exposure. Overall, the oral microbiome of kittens became more similar as the diet was standardized, indicating that the food source plays a major role in shaping the oral microbiome.

Overall, several significant differences were noted in the relative abundance of bacteria phyla with age (Table 1). Using ZIBR analysis, comparisons were made between the relative abundance of bacteria phyla as the kittens aged (2 weeks versus 4 weeks; 2 weeks versus 8 weeks; 4 weeks versus 8 weeks). Four bacteria phyla were increased ($p < 0.05$) at week four compared to week two (Proteobacteria, Tenericutes, Gracilibacteria, TM7). All phyla except candidate division SR1 and Tenericutes were significantly increased from week 2 to week 8. Nine phyla were significantly increased from week 4 to week 8 (Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, TM7, Chlorobi, Chloroflexi, Spirochaetes, Synergistes), while three phyla were decreased (Fusobacteria, Gracilibacteria, Tenericutes).

Numerous changes were noted in oral bacteria populations of kittens. Both the major and minor phyla were altered over time. Whether these changes are due solely to age, diet, or increased exposure to other environmental factors is still to be determined. However, the changes noted in this dataset indicate that the oral microbiome of kittens develops and changes rapidly over a relatively short amount of time.

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Table 1. Relative abundance of oral bacteria phyla in kittens at 2, 4, and 8 weeks of age.

	Age of Kitten (weeks)			P-Value		
Phylum	2	4	8	2 vs 4	2 vs 8	4 vs 8
Proteobacteria	4.53	9.87	20.31	0.0018	< 0.0001	< 0.0001
Fusobacteria	3.98	10.38	8.91	0.0722	0.0011	0.0033
Bacteroidetes	2.86	3.45	22.46	0.4694	< 0.0001	< 0.0001
Firmicutes	2.50	3.84	14.01	0.3337	< 0.0001	< 0.0001
Actinobacteria	0.57	1.03	2.59	0.1538	< 0.0001	0.0003
Candidate division SR1	0.46	0.55	1.56	0.3607	0.0011	0.0722
Gracilibacteria	0.24	0.88	0.20	0.0058	0.1847	0.0098
TM7	0.01	0.01	0.26	0.0150	0.0005	0.0014
Chlorobi	0.00	0.00	3.13	---	< 0.0001	< 0.0001
Tenericutes	0.00	0.24	0.03	0.0017	0.0104	0.2146
Chloroflexi	0.00	0.00	0.08	---	0.0104	0.0104
Spirochaetes	0.00	0.00	0.10	---	< 0.0001	< 0.0001
Synergistetes	0.00	0.00	0.33	---	< 0.0001	< 0.0001
Unknown	84.85	69.75	26.04	0.0024	< 0.0001	< 0.0001

Summary and Conclusions

The oral cavity harbors a complex microbiome that plays a role in health and disease of humans and companion animals. Research in infants indicates that oral microbiome establishment can be impacted by birth mode and diet. Very limited research has evaluated the oral microbiome in companion animals, and the majority of cat research has focused on bacteria associated with infections. Culture-independent methods have proven that the oral microbiome of adult cats is complex and can be altered by food and disease. Our work, for the first time, explored the development of the microbiome of kittens at birth and in the preweaning period. The majority of oral bacteria were unclassified taxa at birth, and there is an indication that birth mode may affect bacteria load in the mouth in the first 24 hours of life. The oral microbiome was altered in 2-week-old kittens born via C-section compared to those born vaginally. Diversity significantly increased as the kittens aged and their diet changed from milk to commercial food. Although four phyla were responsible for the majority of bacteria present in the mouth of kittens during preweaning, many significant differences were noted in the phylum with age. These data indicate that oral bacteria are rapidly changing over this short time period. This period of microbiome development may be more malleable than the oral microbiome of adult cats. Potential dietary interventions during preweaning may be able to shape the oral bacteria during this time to improve lifelong oral health in cats.

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