THE EFFECT OF DIETARY TEXTURE ON THE COMPOSITION OF THE MURINE DENTAL MICROBIOME

By

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EFFECT OF DIETARY TEXTURE ON THE COMPOSITION OF THE DENTAL MICROBIOME

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DEDICITION

I would like to dedicate this work to Dr. Ryan Jennings. Thank you for introducing me to research and for encouraging me to follow a path that was unfamiliar. You have truly shown me the value of pursuing an idea not for the sake of finding, but for exploring. This is science, and it’s pretty cool.
ABSTRACT

LEA MARYAM SEDGHI
THE EFFECT OF DIETARY TEXTURE ON THE DENTAL MICROBIOME
Under the direction of LAURA SILO-SUH, Ph.D. and Dr. Craig Byron, Ph.D.

Dental plaque exists as a highly complex biofilm community that is known to lead to various states of dental disease. The pathogenicity of the dental biofilm is directly related to the species residing within the biofilm. While some oral biofilm communities are associated with states of oral health, others are interchangeable with states of dental disease and oral pathology. The microbial community population of the oral cavity is heavily influenced by host diet composition. Diet composition contributes to the establishment of differing microbial communities in terms of relative species abundance and diversity. Some diets are associated with oral bacteria related to a healthy oral environment while others show association with microbes that are related to states of dental disease.

Many studies have focused on the significance of dietary carbohydrate content on dental disease. However, the effect of dietary texture on dental disease has been comparatively overlooked. This study utilizes 16S rRNA analysis to better understand the influence of diet texture on oral microbial populations among the dental enamel surface in terms of microbial diversity and abundance. Four diet groups—1) Base (Control), 2) Base+Fiber, 3) Base+Sugar, and 4) Base+Fiber+Sugar—are used to
determine if dietary texture as well as carbohydrate content affects changes within the oral microbiome to potentially influence dental disease. This study ultimately seeks to offer insight into whether the loss of dietary texture in the Western diet has acted as a stimulus towards increased incidence of dental disease over time.

We found that dietary fiber, and not sugar, had a significant effect on dental microbiome alpha and beta diversity at the genus level. Additionally, beta diversity was significantly affected at the phylum level. Consistent with this finding fiber, and not sugar, also explained significant changes in microbial taxonomic abundance at the genus level. These changes were microbe-specific for each dietary group. A general finding was that fiber had a stronger influence on microbial diversity and abundance than did sugar. The only significant effects sugar demonstrated in this experiment were in its interaction with dietary fiber. Further analysis using metagenomic shotgun sequencing should be utilized to better understand the metabolic impact of the changes observed in this preliminary study.
INTRODUCTION TO THE STUDY

Microbiology: A Study of Communities

The study of microbiology has evolved to account for the polymicrobial relationships and interactions that exist within microbial communities, including those within the body that influence human health (1). Culture-dependent methods persisted as the gold standard for studying microbial communities and pathogens in the fields of microbial ecology and medical microbiology for over a century. Following the great plate anomaly in the 1960’s, culture-independent methods were developed to better account for microbial diversity within ecosystems that was unable to be reproduced using culture-dependent methods (Table 1) (2). It is now well established that culture-dependent methods neither account for the total diversity of a microbial community or describe the ecology of microbial communities (1, 3-7). The culture-dependent method involves the selective isolation of microorganisms from microbial communities by inoculating media. This method permits the detection of specific microorganisms within a sample as well as facilitates ease in enumerating live cells (8). However, cultivating microbes in vitro is limited by various factors, including but not limited to nonsufficient media formulations and the need to replicate strict anoxic conditions required for many anaerobic oral species. In addition to these limitations, the non-cultivability of many microbes is attributed to the lack of necessary microbial interactions that traditional culture methods
are unable to replicate (9, 10). Culture-dependent methods fail to wholly represent many or all of the members that exist within a microbial community, presenting problems for understanding microbial community dynamics and ecology.

Table 1: A Brief History of Microbial Ecology

<table>
<thead>
<tr>
<th>Year</th>
<th>Event</th>
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<tbody>
<tr>
<td>1674</td>
<td>Antonie van Leeuwenhoek observes and describes single-celled organisms (“animalcules”) with an early compound microscope while examining samples of dental plaque scrapings. van Leeuwenhoek is revered as the father of microbiology for his discoveries.</td>
</tr>
<tr>
<td>1860-1880</td>
<td>Two hundred years after van Leeuwenhoek’s discovery, the Golden Era of microbiology begins, with discoveries contributed by Louis Pasteur, Robert Koch, and Ferdinand Cohn. Isolating bacteria in pure culture was essential to the establishment of Koch’s postulates, which served as an outline to determine the etiology of disease.</td>
</tr>
<tr>
<td>1888-1890</td>
<td>Beijerink and Winogradsky utilize cultivation methods and enrichment for specific bacteria to understand the mechanisms of chemolithotrophy.</td>
</tr>
<tr>
<td>1900-</td>
<td>Culture-dependent methods and microscopic examination remain the gold standard of observation and discovery for microbiologists (especially environmental microbiologists) throughout the mid-late 20th century. A hunger for knowledge regarding understanding microbe-microbe interactions and microbe-environment interactions emerges during this time.</td>
</tr>
<tr>
<td>1960-</td>
<td>The “great plate anomaly” occurs, as traditional enrichment culture techniques did not produce results congruent with microscopic observations of microbial numbers observed in environmental samples. It was realized that 99% of bacteria could not be isolated via traditional culture enrichment methods.</td>
</tr>
<tr>
<td>1980-</td>
<td>In 1980, Carl Woese employs 16S rRNA sequence analysis to identify microbial phylogeny directly from samples. Researchers begin using rRNA sequences to identify bacteria in mixed communities in the absence of traditional cultivation. The cloning of 16S rRNA-encoding genes becomes the gold standard for the characterization of microbial community composition.</td>
</tr>
<tr>
<td>1985</td>
<td>Alternative culture-independent methods are created such as <em>in situ</em> hybridization and community fingerprinting (e.g. - terminal restriction fragment length polymorphisms analyses and denaturing gradient gel electrophoresis).</td>
</tr>
<tr>
<td>1998-</td>
<td>Metagenomics emerges as a tool for community analysis that is not dependent on the 16S rRNA-encoding gene, but instead involves direct cloning of DNAs extracted from environmental samples, suggested by Olsen in 1980. Metagenomics exists as functional and sequence-based analysis of total environmental DNA. Metagenomics continues to be used to explore the genetic capacity and activities of microbes in a wide range of environments.</td>
</tr>
</tbody>
</table>
Noticeable effect in advancing the study of microbial populations has been attained in the field of microbial ecology, in which newer culture-independent experimental approaches have provided detailed assessments of both the composition and functions of complex microbial communities (11). Such culture-independent methods, including but not limited to 16S rRNA gene community profiling, genomics, transcriptomics, proteomics, metabolomics, high throughput “next generation sequencing,” oligonucleotide and DNA microarrays, high sensitivity mass spectrometry, and nuclear magnetic resonance analysis have been developed to study living systems in the absence of cultivating samples, and to generate massive amounts of data even in a single experiment (2, 11, 12). *In vivo* studies relating to microbial communities (including 16S rRNA survey and metagenomics) better capture the population dynamics and metabolic activities that exist within these communities than do culture-dependent studies.

Studies that rely on *in vivo* as opposed to *in vitro* methods provide representative information regarding microbial communities in their native state, presumably without conflating effects caused by an unnatural setting of culture-dependent experiments. These methods entail the study of the microbiota (the microbial species present) as well as the microbiome (the genes and gene products of those species) (13). The 16S ribosomal RNA gene is a phylogenetic marker that has become the mainstay for measuring the taxonomic diversity that exists within communities of Bacteria and Archaea, and is used in the identification of specific organisms residing within microbial communities (1). Studies of system microbiomes are performed to better understand microbial community dynamics and the relationship of microbial communities to their
environments, including both abiotic and biotic microbial habitats, which harbor unique microbial communities (2, 14, 15).

Metagenomics is used to study the interactions occurring within microbial communities through methods that utilize microbial community genome sequencing and molecular analysis (14). The “metagenome” is a term that describes the collective genomes of microbes (typically of many different taxonomic varieties) residing within a specific environment (2, 12). Culture-independent methods relating to metagenomics have provided new knowledge in terms of understanding microbial communities; these methods demonstrate the ability to resolve greater species richness within these communities than typically reported using culture-dependent methods (16, 17).

The Human Microbiome

The human microbiome represents one of the many host-associated biotic environments available that harbors unique microbial communities. Study of the human microbiome is new only in terms of the system to which it applies, as similar studies have occurred among microbial ecologists studying macro-scale ecosystems (non-host associated) for decades. The term “human microbiome” was coined by Joshua Lederberg, and defined as the “ecological community of commensal, symbiotic, and pathogenic microbes that live on and within our bodies and are important determinants of health and disease” (18). In-depth analysis of the human microbiome is being achieved through the Human Microbiome Project (HMP), a continuing study designed to understand the microbial composition of the human genetic and metabolic elements, as well as how these communities impact human physiology and pathology (15).
Study of the human microbiome demonstrates the vast number of commensal and mutualistic relationships that exist among multi-cellular organisms and microbes across size scales. Microbial communities within the body are vital constituents of proper growth and development, as well as overall health and wellbeing. For example, proper immune system function and protection against pathogenic organisms is dependent upon the presence of, as well as the composition and diversity of, the microbiome (19). The microbiome also makes important contributions to human metabolism and physiology. The best-known example of this is the synthesis of the essential nutrient B12 by microbes, as the enzymes needed for B12 synthesis are absent in plants and animals (20). Additionally, the intestinal microbiome contributes significantly to the efficient utilization of dietary carbohydrates as well as impacts nitrogen balance by *de novo* synthesis of amino acids and urea cycling (20-22).

The detrimental outcome of the absence of commensal microbial communities is exaggerated in the development of gnotobiotic organisms (in which microbial species are absent). These gnotobiotic organisms are prone to chronic infection and tend to show an overall failure to thrive, as well as require 30% greater caloric intake to support their body weight (20-26). The microbes that constitute the microbiome even outnumber the amount of host eukaryotic cells. The human microbiome can be characterized as an indispensable aspect of the human body, as its presence and functions are comparable to that of an organ. The microbes on and within our bodies form a functional organ system that is fundamental to the proper development and continued well-being of our health and physiology. Together with our microbiomes, we form a holobiont, or a “superorganism” (27). The microbiome is essential to growth and development, protection from non-
commensal pathogens, maturation of the immune system, the development of the gastrointestinal (GI) system, and is able to provide the host with expanded metabolic capabilities (23, 24, 28, 29). The microbiome heavily influences human health and is reciprocally impacted by host health.

The Microbiome in Health and Disease

Medical microbiology historically has been largely defined by culture-dependent studies that sought to understand the relationship of specific microbial species to specified states of health and disease. The standard for proving the role of a specific organism in the cause of a disease was guided by Koch’s postulates, which state that a single organism will be present in all cases of a specified disease (30). Traditional definitions of disease and diagnostic methods have focused on establishing a single microbial species as the causative agent of disease. However, polymicrobial diseases or diseases involving microbial dysbiosis do not fulfill this traditional reasoning. Dysbiosis is defined as a change in microbial community composition or activity that elicits an aberrant host response to the microbiota, and this may be caused by either a single organism or many organisms (30, 31). The study of microbial communities has evolved from the traditional paradigm of diagnosis to account for the contribution of polymicrobial interactions to states of health and disease.

The composition of host-associated microbial communities largely impacts the wellbeing of the host. Variances in microbial community populations, including differences in relative species abundance and the presence or absence of certain microbes, is significant to defining host health and disease. Microbial community population dynamics show a substantial association to many disease states, including but
not limited to obesity, cardiovascular diseases, Crohn’s disease, various cancers, and inflammatory bowel diseases (19). Certain microbial community compositions are associated with a greater incidence of disease or are alternatively associated with states of health. Microbial communities are readily manipulated by various host factors, including but not limited to host genetics and diet.

Host genetics are an innate determinant that has an observable effect on microbial community diversity and population dynamics (32). The effect of host genetics on microbial populations has been observed by comparing the composition of microbial communities among individuals belonging to certain ethnic groups. Varying ethnic groups show differential outcomes in microbial community populations, with some ethnic groups having a greater relative abundance of certain microbial species than other ethnic groups (33). Individuals belonging to certain ethnic groups have demonstrated similar microbial community compositions that differ from individuals belonging to other ethnic groups. However, although differences in microbial communities have been observed among different ethnicities, microbial community composition is highly variable from one individual to another. This variability is due to the differences in genetic makeup between individuals as well as individualistic environmental factors, such as diet and behavior (34). Environmental factors are significant to the population dynamics and composition of the microbiome. These variances in microbial populations created by host environmental factors, such as diet and exercise, subsequently impact host health. The effect of diet on microbial populations is an attractive area of study due to its ability to be easily manipulated and its relevancy to diet-related pathology in modern human populations. Unlike host genetics, which are intrinsic and characteristic,
diet acts as a flexible variable that has the capacity to significant impact microbial community composition (35).

Impact of Diet on Microbial Communities

Consumption of particular foods modifies microbial community populations, microbial species diversity, and the relative abundance of species within microbial communities. Consumption of foods not only directly adds new microbial species to the GI tract, but also can modify the intestinal environment to promote the growth of certain microbial species over others. Fermented foods are an example of the impact of diet to host health; consumption of fermented foods directly adds microorganisms to the body as well as changes the environment of the GI tract, and ultimately impacts microbial community composition. The intestinal environment is altered by the enzymatic activity of the microbes as well as the food products that result from the process of fermentation. Nutritive properties of the foods change as a result of the enzymatic processes that ensue during fermentation, and fermentation additionally encourages the transformation of substrates into bioactive end products that may offer benefit to the host (36). Diet holds the ability to both directly add new microbes to the body as well as change the environment of the GI tract to favor the presence and growth certain microbial species and populations.

Variances in diet induce substantial changes in existing microbial population dynamics. The impact of diet on gut microbial community composition was observed via a comparative study of a high-fiber diet (rural African) to a diet high in fats and sugar (European diet). A large variability in the intestinal microbiota of the two diet types was observed, with the high-fiber diet promoting richer microbial species diversity that is
characteristic of a healthy microbial community (34). Anecdotally, in a study of the epidemiology of large bowel disease, a lack of non-infectious inflammatory bowel disease was noted among Africans consuming a high-fiber diet (37). The findings of these studies accentuate the influence of diet composition on microbial community populations within the microbiome.

Significance of Diet to Gastrointestinal Health

The effect of diet on both microbial community composition as well as overall health and disease has emerged as an increasingly important area of study. Special interest has been attributed to the effect of diet on (GI) microbial communities because of the GI tract’s significant influence on health and disease. This organ system is exposed to the external (outside of the body) environment more than any other organ in the body (other than the integument). It is traditionally reported that the epithelial gut barrier comprises ~300 m² of contact surface, although this measurement has recently been reduced (38). The gut epithelium maximizes nutrient absorption with intestinal villi that extend into the intestinal lumen to increase surface area. The villi and microvilli of the small intestine increase surface area 60-120 fold (38). This large exposure to the external environment makes the GI tract especially prone to invading pathogens. The small intestinal epithelium must allow the passage of water, electrolytes, and nutrients to be absorbed while also functioning as a component of the gut barrier. The epithelium, only a single-cell thick, must prevent the entry of harmful microbes, toxins, and antigens from passing into the bloodstream from the intestinal lumen (39, 40). Consequently, the GI tract exhibits both conflicting demands for maximal nutrient absorption and simultaneous host defense (41). The microbiome of the GI tract maintains all three domains of life:
Bacteria, Eukarya, and Archaea. More than any other ecosystem, the human gut houses the largest cell density of bacteria. The microbiota of the human intestine is comprised of trillions of microorganisms, most of which are defined as bacterial in origin and non-pathogenic (23, 28, 29, 42, 43).

Host diet choices shape the intestinal microbial community populations, creating an environment that proves more or less favorable for the viability of particular species. The presiding microbial community reciprocally impacts the nutrient absorption potential of the gut. For example, molecular analysis comparing the microbiota of lean and obese mice demonstrated that the obese mouse microbiome was rich with genes enabling breakdown of dietary polysaccharides, such as glucosidases, galactosidases, and amylases, as well as genes encoding proteins that transport and metabolize the products of polysaccharides (15, 23, 44).

The environment of the GI tract is readily manipulated due to daily variances in diet as well as shedding of the intestinal lumen. Cell apoptosis and the shedding of epithelial cells that occur at the villus tip, as well as the generation of new cells within the intestinal crypts, help to maintain tissue homeostasis. Near complete renewal of the functional villus epithelium by stem cells of the crypts occurs every two to six days (39, 45). Shedding of the intestinal epithelium is critical to preventing ingested pathogens from entering into the bloodstream. The intestinal epithelium prevents the transfer of harmful microorganisms, antigens, and toxins from the gut lumen into circulation by directly acting as a physical barrier as well as continually renewing to prevent the entry of pathogens that would otherwise breach the existing epithelial wall.
Diet rapidly changes the gut microbial community structure as well as produces differences in microbial gene expression. This demonstrates that microbial communities are altered in a rapid and diet-specific fashion. The short-term impact of diet variance on gut microbial communities was observed by comparing the contrasting impacts of an animal-based diet to that of a plant-based diet within short period of time (35). In this study, the animal-based diet increased the abundance of bile-tolerant organisms while it decreased the abundance of microbes possessing the ability to metabolize plant-derived polysaccharides. Here, diet impacted both existing microbial community composition and additionally impacted the transient colonization of foodborne microbes including bacteria, fungi, and viruses (35).

The ability of the gut microbiome to quickly interchange between herbivorous and carnivorous functional microbiotic profiles may reveal selective pressures that occurred throughout evolution to enhance human dietary flexibility (35, 46). Daily fluctuations in diet and the continual shedding of the intestinal lumen cause microbial communities of the GI tract to be readily prone to manipulation and adjustment. The GI tract exists as an attractive area of study as alterations in diet are able to instill significant changes to microbial community composition, and thus a secondary effect towards systemic health and disease.

The Oral Microbiome

The significance of the oral microbiome has been overshadowed in comparison to the attention on the GI tract (47). The oral microbiome is readily exposed to the external environment and experiences large day-to-day fluctuations within its local environment. Exposure to the external environment as well as the rapid fluctuations experienced among
the oral microbiome can be paralleled to the events and changes that occur within the GI tract. However, dental surfaces are unique from the GI tract in that they lack a natural shedding mechanism that aids in disrupting the formation of microbial biofilm communities. Dental biofilm, more commonly referred to as dental plaque, readily builds upon the surfaces of the oral cavity and subsequently results in states of dental disease if not addressed daily. Mechanical obstruction necessitated by the manual use of a toothbrush is performed daily to prevent the accumulation of dental biofilm (48). The oral cavity differs from all other human microbial habitats in that it maintains two types of surfaces for microbial colonization: the shedding surfaces of the mucosa and the non-shedding surfaces of the teeth and available dental prosthetics.

The availability of two different attachment strata within the oral cavity provides opportunities for colonization by a diverse range of microbial species (49). The teeth comprise the only inherent non-shedding surfaces in the human body; the stagnancy of these surfaces provides unique opportunities for extensive biofilm accumulation as well as a secure and fixed environment for microbial continuance (50). The surfaces of teeth include structures such as smooth surfaces, pits, fissures, proximal sites, and sometimes exposed root surfaces. These non-shedding surfaces allow large amounts of microbes to accumulate and evolve into a mature dental biofilm (51).

The composition of the oral biofilm is complex and highly heterogeneous, and is dependent on individual differences in oral environments, and is also dependent on the surface within the oral cavity on which it resides (52). The oral cavity consists of diverse structures and tissue surfaces, including the dental enamel surface, gingival sulcus, gingiva, tongue, cheeks, lips and palate. These sites within the oral cavity harbor unique
microbial communities, as they provide different habitats, growth conditions, and availability of nutrients. As a result, microbial communities differ depending on their intraoral location (19, 53). Although close in proximity, the microbiomes of various sites within the oral cavity differ significantly from one location to another. For example, the microbiome of saliva is more similar in composition to the microbiomes present on the dorsal and lateral surfaces of the tongue, while the soft tissue communities are more similar to one another than the microbiomes present on the dental surfaces and the dental surface and below the gingival margin.

Species residing within the oral cavity may be either site-specific at one or multiple sites, or subject specific. For example, many species, such as Streptococcus mitis and Granulicatella adjacens, are detected in multiple sites within the oral cavity. Species such as Rothia dentocariosa, Actinomyces spp., Streptococcus sanguinis, Streptococcus gordonii, and Abiotrophia defective alternatively primarily colonize dental surfaces, while Streptococcus salivarius resides mostly on the tongue (54). The oral microbiome should not be viewed as a singular entity and homogeneous in composition, but rather as a collection of diverse microbial communities (55).

The oral cavity exists as an environment that is widely dynamic in that it experiences daily mechanical influences including the movement of the mouth during mastication and speaking, as well as variances in salivary flow and secretion and diet that contribute to alterations in microbial community composition and resulting changes in the local pH. Saliva is critical in the preservation and maintenance of the health of oral tissues and structures. Saliva is an exocrine secretion consisting of 99% water, and contains a variety of electrolytes including sodium, potassium, calcium, chloride,
magnesium, bicarbonate, and phosphate, as well as proteins such as enzymes and immunoglobulins. Glucose and nitrogenous products such as urea and ammonia are also present in saliva. The constituents of saliva interact and contribute to the functions of saliva (56). One of the many functions of saliva is as a buffer system to protect the mouth; saliva neutralizes the acids produced by acidogenic microorganisms, and aids in preventing enamel demineralization.

Saliva holds a fundamental role in maintaining the integrity of dental enamel by modulating demineralization-remineralization cycles. Control of dental enamel hydroxyapatite de- and remineralization cycles is controlled primarily by concentrations of free calcium, phosphate, and fluoride in solution and the salivary pH. The high concentration of calcium and phosphate that is available in saliva aids in ionic exchanges that occur at the surface of the tooth. Remineralization of pre-carious lesions is made possible due to the availability of calcium and phosphate ions present in saliva (57-61). Several factors influence salivary flow and its composition, causing these to vary from one individual to another as well as within the same individual under different circumstances. Factors influencing salivary flow and composition include but are not limited to hydration, body weight, intrinsic size of salivary glands, increased salivation due to food or olfactory stimuli, as well as medications (58, 62-65).

In addition to endogenous nutrients such as saliva, exogenous nutrients are supplied in the form of host dietary-derived carbohydrates. Fermentable carbohydrates supplied from the host diet hold the largest influence on mouth ecology (66, 67). These fermentable carbohydrates are catabolized to acids that promote the growth of aciduric and acidogenic bacteria and inhibit the growth of commensal microbes. Sucrose is an
exogenous nutrient that can additionally be metabolized to extracellular glucan that promotes the formation of matrix in the dental biofilm. Extracellular matrices, largely comprised of exopolysaccharides, are characteristic of mature and stabilized biofilm communities. Approximately 40% of the dental biofilm is composed of polysaccharides that are largely comprised of glucans that are synthesized by microbial glycosyltransferases. Glucan-comprised exopolymers support the coherence of bacterial cells as well as tight adherence to the biofilm. The exopolysaccharide matrix provided by glucan synthesis provides physical resilience and strength to the biofilm community (68). Frequent exposure to low pH caused by carbohydrate catabolism disrupts biofilm homeostasis and leads to the proliferation of acidogenic and aciduric species (54). The impact of diet to dental disease is significant and will be addressed in detail following an overview of dental disease states and their underlying mechanisms.

The Oral Microbiome and Systemic Disease

The composition of the oral microbiome shows association to states of systemic health and disease. The association of dental disease to various medical conditions demonstrates the impact of oral health to systemic health. For example, it has been noted that increased risk of atherosclerosis, cardiovascular disease, premature birth, diabetes, respiratory disease, rheumatoid arthritis, inflammatory bowel disease, and stroke are often accompanied by poor oral health (32, 69-73). Periodontal disease shows an association to atherosclerosis, thus linking the composition of oral microbiota to an increased incidence of cardiovascular disease (74). Changes in the composition of the oral microbiome have also been noted to show association with certain types of cancer. For example, it is suggested that *Porphyromonas gingivalis* may play a significant role in
the development of various orodigestive cancers, with oral squamous cell carcinoma sharing the greatest association to *P. gingivalis*. An association between oral squamous cell carcinoma and high salivary counts of *Capnocytophaga gingivalis, Prevotella melaninogenica,* and *Streptococcus mitis* has also been noted (75). However, it remains unknown whether this correlation between poor oral health and systemic disease exists as causative or consequential in the pathological process.

The direct role of oral microbes in causing chronic disease is not demonstrated, yet a definite association with poor oral health to systemic disease exists. This association suggests that the same risk factors known to promote various systemic diseases (poor diet, lack of exercise, etc.) may also contribute to the manifestation of dental disease (76-78). For example, it is well established that severe periodontitis has an adverse effect on glycemic control in diabetes; severe periodontitis is also associated with an increased risk of type 2 diabetes onset. Periodontitis shares a dose-dependent relationship between the severity of periodontal disease and diabetic complication (72).

Bacteria originating from the oral cavity possess the ability to induce direct localized and systemic infection. Localized infections include the spread of bacteremia from infected dental structures, such as an abscess derived from infection of the periodontal pocket. These localized infections may spread to the tissues directly surrounding the dental structures or may spread to nearby bone and fascia (79). Three mechanisms exist for the association of oral infection to systemic disease. These include introduction of oral microbes into circulation via transient bacteremia caused by oral infection and invasive procedures, circulation of toxins created by oral microbes, and systemic inflammation induced by immunological response to oral microbes (80). Direct entry into the
bloodstream may occur due to trauma occurring within the oral cavity, invasive dental procedures, or prolonged infection with neglected treatment, such as with periodontal disease. Infection of the heart, brain, spleen, pancreas, liver, and bone by bacteria originating from the oral cavity has been recognized (9, 70, 71).

Microbial Dysbiosis and Dental Disease

The human microbiota contains more than 750 species of bacteria maintained within biofilms (81). Study of the oral microbiome has existed since the emergence of microscopy. In 1683, Leeuwenhoek noted the presence of the oral microbiome; Leeuwenhoek’s observation exists as both the first definitive report on microorganisms innate to humans and as the claim to the discovery of the oral flora (82). Despite longstanding knowledge of the oral microbiota and its presence, only half of the oral bacteria have been cultured in vitro. A possible explanation for this inability to cultivate oral microbial species is that many of these microorganisms exist within biofilm communities, and are thus bound in physical and metabolic interdependencies that precludes the use of traditional pure-culture methods (83).

The presumed etiologic agents of dental caries and periodontal disease were only recently defined in 1960 and 1980, respectively, through the use of in-vitro models (83). Although the agents of dental disease have been theoretically identified for dental caries and periodontal disease (Streptococcus mutans and Porphyromonas gingivalis) these etiologies are not explained in their entirety due to the polymicrobial factors that lead to dental disease states. The species-level identifications associated with culture-dependent methods do not fully represent the process of dental disease, and more information could be derived from functional characterization of the microbial community in vivo (82).
The dental biofilm exists as an extremely complex microbial community that is bound via physical and metabolic interdependencies. The oral biofilm builds in a sequential manner that is dependent upon ecological succession (84) and is characterized as a distinctive and polymicrobial consortium. Individual taxa are arranged and localized depending on their functional niche within the structure. For example, anaerobic bacteria are localized towards the interior whereas facultative or obligate aerobes are located at the periphery. Additionally, consumers and producers of complimenting metabolites are located near one another within the structure. Initial colonizers such as *Streptococcus* and *Actinomyces* spp. attach to the salivary pellicle that is present on the dental enamel surface.

The proportion of pioneer colonizers remains relatively constant between 18 hours and 4 days of initial colonization. It is within this “pre-organization phase” that the primary colonizers remain the predominant colonizers of the biofilm. The introduction of anaerobic bacteria such as *Porphyromonas*, *Fusobacterium*, *Prevotella*, *Veillonella*, and *Capnocyphaga* occurs over time, however. Ultimately these later colonizers largely comprise the overall population (85). The “microflora alteration phase” describes the shift in biofilm population dynamics from primary colonizers to anaerobic species that are characteristic of matured biofilm. Left undisturbed, the excessive biofilm will begin to accumulate on the dental surfaces and within the gum margin and induce pathological states (86). Dental disease results from a shift in the natural balance of the oral microbiota rather than as a result of exogenous infection by a pathogenic microbe.

Traditional modeling of the dental plaque architecture has assigned *Fusobacterium* as the genus that bridges early colonizers to later colonizers, as it has
attachment sites available to both. Recent biogeography studies of dental plaque reveal *Corynebacterium* as a foundational and necessary member to shaping the dental biofilm community. “Hedgehog” structures comprise dental plaque and are characterized by spiny and radially-oriented filaments. *Corynebacterium* extends central radial filaments that act as an attachment site for other microbial taxa. Along its length, a single *Corynebacterium* filament involves many distinct microenvironments. The hedgehog base is characterized by *Actinomyces* and *Corynebacterium* spp. The mid-filament annulus is occupied by *Fusobacterium*, *Leptotrichia*, and *Capnocytophaga*. The tip of the *Corynebacterium* filament is enclosed by a casing of *Streptococcus* and *Porphyromonas* spp, as well as *Haemophilus*, *Aggregatibacter*, and *Neisseriaceae* spp. (Figure 1).

The spatial stratification of specified taxa within the hedgehog structure is shaped by environmental and chemical gradients (87). Facultative aerobe *Streptococcal* spp. at the periphery of the structure utilize the oxidizing environment of the saliva to consume O$_2$ and create an anoxic environment (88). These outer Streptococcal species consume dietary-derived sugars and O$_2$ to produce lactate, acetate, and CO$_2$, which are subsequently utilized by other bacteria within the structure as metabolites (89, 90). For example, lactate is used by *Aggregatibacter* and CO$_2$ is used by *Capnocytophaga* for growth. It is important to understand dental biofilms as an interactive community rather than as a consortium of separate entities. The metabolic and physical interactions between two different microbes can substantially change both physiological and metabolic capabilities compared to those produced in isolation.
The inability to cultivate certain microbial species in vitro and the traditional reductionist approach of determining etiologic causes of dental disease has hindered the understanding of the processes leading to dental disease from a polymicrobial understanding. Assignment of specific microbes to specific disease states fails to account...
for the polymicrobial interactions leading to dysbiosis. It is because of this that an incomplete understanding of the etiology of dental disease has occurred. This is due, in part, to the inability to cultivate certain microbial species in vitro, as well as the inherent limitation of a single-species focused paradigm of traditional microbiology (91, 92). It is now accepted that dental disease results from a shift in the natural balance of the oral microbiota rather than as a result of exogenous infection by a pathogenic microbe. Microorganisms within the dental biofilm are bound by metabolic interdependencies and competition that are significant to the success of individual microbes as well as overall population dynamics and community success (86). Traditional etiologic agents of dental disease (S. mutans and P. gingivalis) are present in low numbers at healthy sites within the oral cavity, and thus are not invading pathogens but are rather normal constituents of a healthy biofilm community (93).

Rather than exogenous infection, dental disease can be attributed to processes of microbial dysbiosis that cause changes in population dynamics and relative species abundance. Dental disease can be prevented not only by inhibiting the proliferation of presumed pathogens, but also by modifying the environmental factors that drive the selection and enrichment of bacteria residing within the oral cavity (94). The inability to cultivate many oral microbes can be attributed to the lack of necessary microbial interactions that traditional culture methods are unable to provide (9, 56). To more fully understand dental disease, it is necessary to adopt a polymicrobial, if not systems biology, paradigm in the study of dental disease progression.

Differences in microbial community populations can be observed when comparing the microbiomes of healthy oral cavities to those demonstrating states of
dental disease. The use of microarray-based technologies, such as DNA-DNA hybridization techniques, has helped to identify the compositions of the oral microbiome most closely associated with either health or disease. These microbial community compositions, or “complexes,” denote the bacterial species that comprise the majority of the oral microbiome in various states of health or disease. Oral biofilms associated with states of oral health are comprised of bacteria belonging to the “yellow” and “purple” complexes. The yellow complex is largely composed of streptococcal species including *Streptococcus gordonii, Streptococcus intermedia*, *Streptococcus mitis, Streptococcus oralis*, and *Streptococcus sanguinis*. The purple complex is comprised of species such as *Actinomyces odontolyticus* and *Veillonella parvula*. The yellow and purple complex microbial community compositions are associated with healthy sites (75, 82).

The major genera with the largest representation to healthy oral microbiomes as follows: Firmicutes (genus *Streptococcus*, family Veillonellaceae, genus *Granulicatella*), Proteobacteria (genus *Neisseria, Haemophilus*), Actinobacteria (genus *Corynebacterium, Rothia, Actinomyces*), Bacteroidetes (genus *Prevotella, Capnocytophaga, Porphyromonas*), and Fusobacteria (genus *Fusobacterium*) (49, 55, 83, 95-97). Healthy oral cavities are additionally defined by having rich species diversity, in contrast to states of oral pathology in which species diversity is greatly reduced (98). Identification of species composition and molecular differences between states of health and disease may allow for the recognition and diagnoses of oral disease from a microbial standpoint, and may permit treatment at earlier and possibly reversible stages (49).

Commensal bacterial species help to maintain homeostasis among the oral microbiota via the production of ammonia from arginine and urea. *Streptococcus*
salivarius contributes significantly to alkali production within the mouth and expresses the urease gene when exposed to low pH conditions and an excess of dietary carbohydrates (99). Commensal bacterial species are additionally beneficial to the host due to their ability to compete with the colonization of pathogenic bacteria, as well as produce various bacteriocins that inhibit the proliferation of such species. In contrast, pathogenic bacteria possess the ability to infect both hard and soft tissue. Periodontitis and dental caries are characterized as the most common oral diseases. Dental caries are the single most common pathologies reported in childhood, and periodontal disease is demonstrated in 14% of adults aged 45-54, with this percentage increasing to 33% in older adults (100).

States of Dysbiosis: Periodontal Disease and Dental Caries

Periodontal diseases (gingivitis and periodontitis) are diseases of the oral soft tissues. Gingivitis is characterized as a reversible gingival condition that develops in the presence of excess dental plaque. If oral hygiene practices are ignored and plaque continues to accumulate, dental calculus forms as dental plaque becomes calcified by salivary calcium phosphate. The proportion of gram-negative and anaerobic species of the microbiota increases and causes endotoxin and lytic enzymes to pass into the gingivae. This induces inflammation, irritation, and swelling of the gums, as well as bleeding. It is the initial excess of dental biofilm that leads to the subsequent calcification of dental plaque, and ultimately periodontal disease. However, it is important to note that while the accumulation of biofilm triggers gingivitis, the presence of biofilm alone is not sufficient to evolve into periodontal disease (101). Periodontal disease ensues if gingival inflammation continues. Accumulation of dental biofilm
introduces host response, causing most tissue damage to be caused by an elevated immune response by the host and uncontrolled inflammation.

Periodontitis is characterized as a bacterial-induced chronic inflammation and pathology of the periodontium that ultimately affects the tissues that surround and support the tooth crowns, roots, and alveolar crypts (102, 103). The resulting local inflammation causes an increased flow of nutrient-rich gingival crevicular fluid (GCF) and bleeding, as well as the deprivation of oxygen among the infected site. GCF is a nutrient-rich serum-like exudate that bathes the gingival crevice. GFC contains host defense components such as antibodies and phagocytes, as well as various glycoproteins and proteins that serve as nutrients for bacteria residing within the gingival crevice. Many bacteria that colonize the gingival crevice are proteolytic in nature, and degrade host tissue to form CH$_4$, H$_2$S, H$_2$, and CO$_2$ (104, 105). The creation of an anaerobic environment results in the favored growth of obligate anaerobic and protein-dependent microbes among the gingival crevice, driving a shift from a normal to dysbiotic state (93).

The dysbiotic community induces the destruction of periodontal tissues via dysregulated host inflammatory response. This increased inflammatory response provides nutrients from tissue breakdown for the bacteria, thus driving disease progression, as well as inflammation-induced ulceration and leakage of blood (103, 106). Crosstalk among the host microbiome leads to inflammation and bone loss; osteoclasts are activated to resorb alveolar bone and this loss of bone ultimately leads to destruction of the supporting structures of the tooth within the crypt (107). Chronic and severe periodontal disease is characterized by “red complex” bacteria including *Porphyromonas*
gingivalis, Tannerella forsythia, and Treponema denticola (82). Eight bacterial taxa (including Prevotella micra and Fusobacterium alocis) and four bacterial clusters were identified more frequently and at higher abundance from patients with periodontal disease compared to salivary samples derived from patients within the controlled cohort (108, 109).

Dental caries exists as another state of dental disease caused by microbial dysbiosis among the dental biofilm. Dental caries, more colloquially referred to as “cavities,” are defined as dissolution of the tooth caused by the production of acidic by-products by oral bacteria as the result of fermenting dietary carbohydrates (110). Dental caries are initiated by a disturbance in homeostasis between minerals of the tooth and microbial species within the dental biofilm (111, 112). Acidic by-products of acidogenic bacteria such as Streptococcus mutans and Lactobacillus cause the local pH to decrease. This decreased pH results in subsequent loss of calcium, carbonate, and phosphate from the dental enamel (113, 114). Enhanced consumption of dietary carbohydrates and the resulting decrease in local pH causes saliva to lose its protective buffering capacity. The continued reduction in pH caused by the production of acidic by-products leads to the progressive erosion of the dental enamel, cementum, and dentin. Under these low pH conditions, the oral microbiota shifts to show a decrease in overall species richness and an increased population of aciduric species such as S. mutans, Lactobacilli, Propionibacterium, and Scardovia (115, 116).

Community profiles of dental caries sites show reduced species diversity and an altered salivary microbiome profile (108, 109). Additional species such as Veillonella also show association to dental disease, as these species consume the lactic acid products
produced by aciduric species. Additional bacteria associated with dental caries include but are not limited to *Bifidobacterium, Neisseria,* and *Selenomonas.* Human Oral Microbiome Identification Microarray (HOMIM) profiles of dental caries show reduced diversity of five bacterial taxa including *Veillonella parvula, Veillonella atypica, Megasphaera micronuciformis, Fusobacterium periodontium,* and *Achromobacter xylosoxidans.* Alternatively, two bacterial taxa (*Solobacterium moorei* and *S. salivarius*) and three bacterial clusters (*S. parasinguinis I and II, S. salivarius,* and *S. sinesis* species clones) were present at significantly higher levels (82). Although tooth decay is a reversible process if treated with sufficient oral hygiene practices, cavitation will ultimately ensue if demineralization of dental enamel is allowed to continue (110).

Metabolic Capabilities of the Oral Microbiome

The characterization of health or pathology within the oral cavity can be defined by the microbial community composition as well as by active metabolic capabilities. Conserved metabolic changes are observed in the transition from health to disease. Transcriptional profiling mechanisms are employed to determine variances in bacterial behavior and metabolism as a result of changes in community composition and pH (117). Oral microbial communities are impacted by the frequent rise and drop in the local pH of the oral cavity. Carbohydrate pulses within the oral cavity result in a decrease in the local pH. This decrease in pH is attributed to microbial fermentation of dietary-derived carbohydrates. A variety of alkalogenic microbial responses exist to restore homeostasis following a carbohydrate pulse (118).

The “Stephan Response” describes the microbial processes that restore the local pH to homeostatic resting conditions following a carbohydrate pulse. The Stephan
Response involves a variety of alkali-generating pathways that vary temporally in regard to their timing in restoring pH homeostasis. The temporal regulation of microbial pathways occurring during catabolism of dietary-derived sugars. Metabolites belonging to pH-tolerant and alkali-generating pathways, including arginine, ornithine, citrulline, glutamate, serine, threonine, and urea, varied dramatically throughout the course of homeostatic restoration (118). The genes encoding metabolic pathways related to these metabolites displayed differential transcription activities in response to the increasing pH during the Stephan Response. For example, the arginine deaminase system was noted to be important under neutral pH conditions, urease activity was noted to be most active under low pH conditions, whereas the activities of glutamate dehydrogenase and serine deaminase became more pronounced during the pH recovery phases. The primary metabolites detected in the pH neutralizing process included dihydroxy acetone, 2-hydroxyisolavéric acid, and alkaline amino acids.

The Ecological Plaque Hypothesis describes the significance of environmental influences (i.e. diet) to promoting microbial dysbiosis and subsequent dental disease. This hypothesis states that potentially cariogenic bacteria naturally occur among dental biofilm communities but maintain a relative species abundance characteristic of a healthy microbiome. An increase in the availability of dietary-derived fermentable carbohydrates decreases the local pH and shifts population dynamics to favor the metabolic capabilities and colonization of aciduric and acidogenic species. Substantial changes in the local environment changes the competitiveness of microbes within the dental biofilm, thus leading to an overabundance of species that are most suited to inhabit the changed environment. The changed environment results in overall decreased species diversity and
decreased relative species abundance of commensal oral bacterial to pathogenic bacteria, thus causing states of cavitation and inflammation to ensue (94).

Community profiling alone does not describe the overall health or pathogenicity of the oral biofilm, as the metabolic capabilities of predominantly commensal microbes can change to become pathogenic in the face of environmental challenge. An example of this is observed when *Streptococcus oralis*, a commensal primary colonizer of the dental biofilm, becomes displaced from the oral cavity following trauma or invasive dental procedures. *S. oralis* has been associated with endocarditis as a major etiologic agent of the disease when displaced from the oral cavity. Dietary-derived carbohydrate sources become unavailable when *S. oralis* enters systemic circulation, and metabolic adaptions occur as a result. In the absence of dietary-derived carbohydrates, serum glycoproteins provide a source of carbon and nitrogen. *S. oralis* strains grown in the presence of α-acid glycoprotein possessed the metabolic capacity to degrade glycan chains and to utilize N-acetyl sugars as a source of carbon via action of sialidase and N-acetylg glucosaminidase enzymes. Alternatively, when grown in glucose-supplemented media, the action of these enzymes decreased, indicating that glucose catabolism serves as the preferred metabolic pathway of *S. oralis* (119).

Oral Pre- and Probiotics Aid in Restoring Microbial Homeostasis

Dental disease is related to dysbiosis among polymicrobial interactions and is thus multifactorial in nature, and not necessarily attributed to a single species. Current methods of dental disease treatment and prevention focus largely on treatment and interception of existing dental disease and neglect the need for noninvasive methods of dental disease prevention that strive predominantly to restore microbial homeostasis.
Dental caries, although reduced in prevalence among many developed countries, still exists as a major public health issue (120). Current treatment and prevention methods for dental caries focus on the remineralization of lost dental enamel as opposed to the restoration of microbial homeostasis. The lesions characteristic of the initial stage of dental enamel demineralization, or incipient carious lesions (ICL), is defined by an intact enamel surface with net loss of mineral from the enamel surface. Current methods of ICL treatment focus on remineralization of lost dental enamel, a process through which calcium and phosphate ions are redeposited into demineralized enamel to create a net mineral gain (121). Fluoride ions are used in the remineralization of ICLs by adsorbing to the dental enamel surface and attracting calcium ions. Additionally, fluoride ions replace \( \text{OH}^- \) ions that comprise hydroxyapatite \([\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]\) to produce fluorapatite \([\text{Ca}_{10}(\text{PO}_4)_6\text{F}_2]\) which demonstrates greater resistance to cariogenic challenges (122, 123).

Effective prevention of microbial dysbiosis and subsequent dental disease has shifted to attaining states of microbial community homeostasis and encouraging the establishment of commensal microbial species (94, 124, 125). Such states of microbial homeostasis may be attained through use of oral pre- and probiotics that seek to establish healthy biofilm communities that promote the initial colonization of commensal microbial species and thus the subsequent establishment of healthy oral biofilm communities overall. Probiotics are recognized as living microbial organisms that are directly administered to the patient in an attempt to introduce healthy microbial species to an environment. Prebiotics are non-digestible substances that impact existing microbial communities by supporting proliferation and viability of specific species. Prebiotics
subsequently induce probiotic effects in that they support the growth of indigenous commensal bacteria associated with states of health.

The effects of pre- and probiotics seek to alter the composition and enzymatic activity of the microbiome in such a way that benefits the host (126, 127). Probiotics infer a beneficial effect via many mechanisms, including but not limited to competitive inhibition of pathogenic microbes, modulation of the host immune response by reducing production of pro-inflammatory cytokines, as well as inhibiting the growth of pathogenic species by producing metabolic by-products that inhibit or are antagonistic to their growth. The effect of prebiotics is more or less indirect, as prebiotics encourage the growth of beneficial members of the existing microbial community rather than providing immediately observed benefit (124).

Impact of Diet on the Oral Microbiome

The microbial community population is heavily influenced by host diet composition, as is the microbiome of the GI tract. Variances in diet composition contribute to the establishment of differing microbial communities in terms of relative species abundance as well as population dynamics. Some diets are associated with a microbiota characteristic of a healthy oral environment while others show association with microbial communities that correlate to states of dental disease. Diets rich in carbohydrates are linked to increased risk of periodontal disease and dental caries. The increased association of dental disease to a diet high in carbohydrates is due to the metabolic byproducts produced by bacteria residing on the dental surface. Bacteria within the dental biofilm ferment carbohydrates derived from the host diet and subsequently produce acidic metabolic byproducts. The acidic by-products of acidogenic
biofilm species decrease community diversity as well as contribute to demineralization of the dental enamel surfaces. Acidogenic microbes produce acidic by-products that change the surrounding environment such that the proliferation and occupation of commensal bacteria is disrupted. This cycle exists as a positive feedback mechanism in which the presence of excess acidic by-product ultimately leads to the proliferation of aciduric and acidogenic species and results in an even more exaggerated production of acidic by-products (86). If the pH within the oral biofilm drops to a critical level of 5.5, the dental enamel surface begins to demineralize (81). Spreadbury’s “dietary carbohydrate-density” hypothesis explains this, as it relates the density of dietary carbohydrates to the incidence of inflammatory oral bacteria (128).

Oral microbiome composition has been shaped by sociocultural changes that have occurred throughout our recent evolutionary history, especially among the onset of the Neolithic and Industrial eras (129-131). The modification of ancestral diets has been suggested as a causative factor in the emergence of modern chronic disorders, including dental disease. It has been suggested that the human oral microbiome is maladapted to current dietary composition, thus leading to microbial dysbiosis and subsequent disease. This is congruent with the spread of oral polymicrobial diseases that have emerged recently on an evolutionary scale (94, 132). The changes in diet composition that occurred during the shift from pre-agrarian and post-agrarian communities highlights the significance of diet to oral microbial composition. Dental caries in humans became measurably greater in post-agricultural societies due to the significant increase in carbohydrate consumption, as major shifts in species composition within the oral microbiome have been identified that coincide with the Pre-agricultural-Neolithic
transition (133). Dental calculus, developed from the mineralization of dental biofilm with calcium phosphate, has proven a resourceful tool in analyzing prehistoric human microbiota. Genetic analysis of preserved dental calculus has been used to explore the impact of diet on health and disease on prehistoric populations. A study of calcified dental plaque samples from the Pre-agricultural to Neolithic eras demonstrates a shift in microbial community composition and decreased microbial diversity at this time. Increased consumption of carbohydrates at the start of the Neolithic period is associated with a notable increase in the incidence of oral pathology.

The introduction of refined sugar to our diet at the advent of agriculture prompted certain bacteria within the dental biofilm to metabolically adapt to these post-agricultural environmental pressures. For example, *Streptococcus mutans* evolved to thrive under new dietary conditions introduced in the Neolithic era by adopting a novel method of carbohydrate metabolism alongside an increased resistance against the acidic by-products of this new metabolic pathway (134). These expanded metabolic and defensive capabilities by *S. mutans* as well as other aciduric and acidogenic bacterial species led to their increased prevalence within the oral cavity, and ultimately prompted states of microbial dysbiosis to ensue. It is the adaption of aciduric and acidogenic bacterial species to the changed diet of the Neolithic era that is suspected to be the causative factor of the emergence of dental disease (79, 133).

The incidence of dental caries and periodontal disease is markedly lower among pre-agrarian hunter-gatherer societies and earlier hominins (2, 133, 135). It is of importance to note that extensive dental wear existed prior to the introduction of agriculture. However, anthropologists consider human tooth wear as a natural
phenomenon in which teeth, although worn, remain functional throughout life. Wear only becomes pathological if pulpal exposure or premature tooth loss occurs (136). Dental wear is a reality for dental structures. From an evolutionary perspective it can be argued that wear acts as a selective force to shape the anatomy of the teeth as well as the properties of the dental tissues. Masticatory efficiency is reliant upon the anatomical relationship that exists between different wear characteristics of dentine and enamel together with the overall wear process (137). It has additionally been documented that the stomatognathic system is responsive to progressive wear and is able to adapt, suggesting a dynamic craniofacial complex (138, 139).

The term “dental wear” is often wrongly interchanged with “dental disease”; however, variances exist between these terms (137). Dental wear refers both to chemical or mechanical damage, and includes erosion, attrition, abfraction, and abrasion (140). Attrition, abfraction, and abrasion are forms of mechanical wear, whereas erosion is caused by chemical action. Erosion is the gradual and irreversible dissolution of dental tissue induced by acidic agents, produced as the metabolic by-products of acidogenic bacteria (141). Attrition is wear defined by contact between teeth in the absence of food, and is characterized by a facet that is matched by a corresponding facet on a tooth in the opposing arch (142). Abfraction occurs when the tooth is subjected to extreme stress and fatigue in a particular area that is far away from the force application point that occurs during occlusal and parafunctional loading, and is microscopically identified by multiple cavitation and overlapping furrows, as well as parallel striations that alternate with protruding crests (143, 144). Abrasion is caused by the interaction between exogenous
objects and substances, including food (137). Lesions induced by dental enamel erosion present with distinctive rounded edges and may also be characterized by chemically eroded “honeycomb” structure that occurs when prismatic enamel is eroded and interprismatic enamel protrudes. Abrasion alternatively is defined by smooth enamel that contains striations of varying depths, longitudinal and transverse grooves, scratches in lesions caused by light pressure, and pitting in lesions caused by greater pressure (144).

Abrasion has been noted to exist among hunter-gatherer populations for thousands of years. However, the prevalence of erosion in early pre-agricultural populations proves insignificant. Non-carious cervical lesions are characterized by loss of tooth structure occurring at cemento-enamel junction that are unrelated to dental caries. The three mechanisms that cause such lesions include mechanical stress, friction, and biocorrosion (non-carious chemical wear) (145). Non-carious cervical lesions have not been identified extensively within these pre-agricultural populations and are therefore understood to exist as a modern category of pathology (137). The introduction of dental disease observed alongside the advent of agriculture approximately 10,000 years ago has been attributed to an increased consumption of carbohydrates that occurred at this time, and the resulting metabolic adaptions of microbial species to this diet that caused increased production of metabolic by-products and subsequent states of disease.

Evolution of Human Diet and Dental Disease

The effects of carbohydrate density on the incidence of dental disease demonstrate how dietary changes stemming from the Neolithic and Industrial periods have affected oral health over time. The introduction of dental disease following the shift to a post-agrarian diet is not the only state of disease that has been linked to this dietary
shift, however. It has been predicted that the rise of other chronic human diseases might also be attributed to the development of agriculture as well as the onset of the modern industrialized era (133, 146). The basis for this argument is the notion that, on an evolutionary time scale, the pre-agricultural human genome has not had sufficient time to adjust to the dietary and lifestyle changes introduced throughout societal advances over the last several centuries to millennia of human history.

For example, modern food staples are drastically different than those belonging to the Pre-agricultural diet, and even to diets from a century ago. The dietary changes attributed to the rise of the Neolithic, and the more modern Industrial age, drastically changed seven nutritional aspects of the Pre-agricultural diet. These changes include but are not limited to glycemic load, fatty acid composition, sodium content, and fiber content (131). Many studies have sought to understand the beneficial effects of reverting back to a pre-agricultural diet on the incidence of chronic disease in humans (146, 147). As with other chronic diseases such as diabetes mellitus and atherosclerosis, the effects of both the pre- and post-agricultural diet on the incidence of dental disease has been observed to better understand the effects of dietary nutritional content on oral health. For example, an inverse relationship exists between the progression of periodontal disease and the addition of dietary fiber from fruits and vegetables. Interestingly, a stronger inverse relationship was noted between fruit consumption and periodontal disease, although fruits intrinsically have higher amounts of sugar than do vegetables. It was proposed that the nutrients found in fruits such as vitamins A, E, and C, as well as various phytochemicals such as isoflavones, resveratrol, and catechols may additionally act as
antioxidants, antibacterial agents, or promote enhanced cell-mediated immunity (148-151).

Importantly, in addition to nutritional changes, substantial changes in dietary texture also resulted from abandonment of the pre-agricultural diet, especially in regard to fibrous content. Many staples of the Western diet, and specifically the U.S. diet, such as refined sugars, vegetable oils, and dairy products, are devoid of fiber. These staples constitute nearly 50% of the U.S. diet alone, however. Refined grains that are devoid of fiber make up 85% of the U.S. diet, and contain nearly 400% less fiber than whole grains. Fruit contains twice the fiber found in whole grains, and non-starchy vegetables contain eight times the amount of fiber found in whole grains (131). The diet of humans up until the origin of agriculture was widely comprised of fiber-rich fruit and vegetables. Throughout the Neolithic and the industrialization periods that followed, many of these pre-agricultural staples were replaced with products devoid of fiber. The advent of agriculture that emerged at the start of the Neolithic era is responsible for a significant decrease in fibrous content consumed from this time onward (131, 152, 153).

The ratio of carbohydrate consumption and its affect to human health has been noted previously (154-156). Many present-day diet-related diseases are relatively absent in hunter gatherer societies, and, because of this, understanding the carbohydrate composition of such non-Western diets is of particular interest (157). While it is often assumed that diets of hunter gatherer societies were and are devoid of carbohydrates, it has been estimated that pre-agricultural carbohydrate consumption ranged from 35-65% of total daily energy intake (158). Modern day hunter gatherer carbohydrate consumption ranges from 3%-50% of total energy intake (157). Today, the recommended carbohydrate
intake is 45-65% of total energy daily, with 25% of these carbohydrates derived from added sugars. Pre-agricultural carbohydrates were derived primarily from fruits and vegetables, whereas 85% of modern carbohydrates are derived from cereal grains (158). The plant-based carbohydrate component of present day hunter gatherer diets consists of minimally-processed plants having a low glycemic index and a high fiber content (146, 152, 159). Refined sugars and grains that make up a large proportion of the modern Western diet and are alternatively characterized by a high glycemic index and low fiber content. The low incidence of systemic disease among hunter gatherer societies should not be attributed to a lack of carbohydrate consumption, but rather by differences in the quality of carbohydrates in comparison to the Western diet (157).

Pre-agricultural diets accrued more fiber from fruits and vegetables compared to the Westernized diet, in which most fiber alternatively derives from cereal grains (131, 146). The notion that fiber offers benefits to the body is not a novel paradigm, as many studies have studied the effect of fiber to host health and wellbeing. A diet rich in fibrous fruits and vegetables has been observed to lower risk of stroke, cancer, and cardiovascular disease, as well as offer many benefits to the GI system (160-164). Additionally, fiber has been noted to provide health benefit via lowering serum glucose levels, blood pressure, lipids, and body mass index (165-167). For example, greater consumption of dietary fiber has been noted to result in a marked increase in intestinal health. In a study on the impact of fiber to intestinal health, a reduction in colon cancer prevalence was observed in Africa, in which a diet “high in roughage” is consumed. Colon cancer and constipation are rare in Africa, whereas they are common diseases observed in Westernized countries (168, 169). A diet rich in fibrous fruits and vegetables
offers many health benefits to the body and may also promote oral health by providing mechanical action against accumulating plaque as well as promoting increased salivary flow via greater chewing efforts and stimulation of the parotid salivary gland (170, 171).

Dietary Texture and Dental Disease

The advent of agriculture that emerged at the start of the Neolithic era is responsible for a significant decrease in fibrous content consumed from this time onward (131, 152, 153). This decrease in dietary fiber, in addition to the changed nutritional content of foods, may have contributed to the emergence and progression of dental disease. Dietary fiber has demonstrated to produce beneficial outcomes to oral health even in the absence of sufficient oral hygiene practices. The incidence of dental diseases has often been associated with various socioeconomic factors including but not limited to low educational level, income, and poor housing conditions. This traditional association was challenged, however, in a study on the association of dental health status and dietary fiber intake among various socioeconomic settings (rural, marginal-urban, and urban) in central Mexico. In this study, the community that demonstrated better dental status was the rural community, a setting that is characterized by lower socioeconomic status. The rural community differed from the urban and marginal-urban communities in that this community demonstrated the highest fiber intake. Although the rural community maintained lower socioeconomic status, they were characterized by a diet high in fiber content (172).

Additionally, a study comparing the oral health of Hadza village and hunter-gatherer societies (located within a 4000km² area around Lake Eyasi in Northern Tanzania) recognized a similar outcome. Women in the village groups that consume a
maize-dominated diet showed increased incidence of dental caries in comparison to women in the hunter-gatherer group that consume primarily a diet comprised of wild foods (173). Hadza women consume fiber-rich tubers annually. Tuber and various other underground storage organs (USOs) are starchy and carbohydrate dense, but they also contain a high fiber content. Among the Hadza tubers consumed, the edible fraction of the USOs varied between tuber species between 20-75%. Hadza women use the same sites for digging up tubers and did not discriminate between species. As a result, some of the tubers consumed by Hadza women are comprised largely of inedible material. This inedible material, expectorated as “quids” following completed chewing, may provide an abrasive component that acts to cleanse the teeth of the Hadza people even in the absence of oral hygiene practice (174).

A majority of studies that demonstrate the relationship of diet texture to increased incidence of dental disease are those comparing the oral health of animals in captivity to that of those living in the wild. Animals consuming an unnaturally soft diet in captivity show an increased risk of dental and periodontal disease compared to those living in the wild. The effect of captivity on the oral health of the black-footed ferret has been demonstrated by comparing the dentition of the wild and captive black-footed ferrets. It was observed that a greater incidence of calculus accumulation and periodontal disease occurred among the captive group (175). This suggests that greater incidence of dental disease among the captive groups can be related to their unnaturally soft diets. Animals in captivity are provided with a diet that offers the same nutritional requirements as would be met in the wild. While these diets fulfill nutritional requirements, they differ in mechanism of delivery. For example, diets fed to captive carnivores primarily consist of
ground meats supplemented with vitamins (176). These diets offer the same nutritional requirements as the wild diet but are devoid of the mechanical challenge that would otherwise be provided via the shearing, crushing, and grinding of animal tissues, such as bone, cartilage, and other skeletal connective tissues (177-179).

The significance of diet texture to oral health has been further demonstrated by supplementing the soft diet of ferrets in captivity with small pieces of bone. The addition of bone prevented accumulation of calculus as well as removed calculus that had already formed. To control for bone nutritional content as a potential cause of reduced plaque formation, bone was crushed and added to the diet to eliminate the possibility of the nutritional content of the bone disrupting plaque formation as opposed to texture (180). Animals fed the diet with crushed bone developed similar results to animals fed a wholly soft diet, thus demonstrating that the material properties of in-tact bone tissue were the factor that was able to disrupt accumulation of plaque. It has additionally been noted that abrasive commercially produced diets decrease evidence of periodontal disease and tartar accumulation in domestic pets compared to those fed commercially produced soft diets (181-183). Current animal products designed to promote oral health utilize dietary texture components to do so. Dental hygiene chews for domestic dogs have been shown to reduce the accumulation of plaque and calculus, and to lower the incidence of gingival disease (184-187).

Applications of Metagenomic Analysis in Dentistry

Metagenome analysis has emerged as an important tool in understanding microbial community interactions and population dynamics. The metagenomic approach is especially useful to understanding oral microbial interactions for many reasons,
including the inability to culture many oral bacteria with traditional lab techniques (188). However, the primary significance of metagenomic analysis to understanding oral bacteria lies in its ability to study the oral microbiome in its entirety and to allow insight into microbial community dynamics in states of health and disease. Many states of dental disease cannot be attributed to a single organism, but instead result from polymicrobial factors (83, 189) The metagenomic approach provides a holistic understanding of the oral microbiota that demonstrates the role of polymicrobial interactions and thus the multifactorial causes leading to states of dental disease. It is presumed that nearly half of all oral bacteria are unaccounted for due to the inability to perform in vitro studies on individual bacteria that normally reside within intricate and complex biofilm communities.

Recent advances in sequencing technology and molecular biology have allowed for a more comprehensive understanding of the oral microbiome as well as the complex processes leading to microbial dysbiosis and subsequent dental disease. These advances utilize molecular approaches that capture in vivo conditions of the oral microbiome. In this way, the significance of traditionally non-cultivable species is accounted for. Molecular techniques include the use of PCR amplification and sequencing of bacterial 16S rRNA derived from samples collected in vivo (9, 56, 82). Advances in molecular biology have aided in the advancement of dental research in that they enable the culture-independent study of the oral microbiome, thus providing a more comprehensive understanding of the oral microbiome within in situ conditions.

The most common culture-independent technique used to analyze the oral microbiome utilizes 16S rRNA gene community profiling (96). Carl Woese identified the
16S rRNA gene as universal taxonomic marker that can be used to determine taxonomic relatedness and variation. Ribosomes are functionally and universally preserved molecules as they are a functional necessity among all known domains of life. Bacterial ribosomes are comprised of rRNA subunits, 5S, 16S and 23S. The 16S rRNA marker gene is advantageous to understanding taxonomic variation and relatedness in that it contains both conserved and variable regions that allow insight into ancient relatedness as well as more recent lineage variation among species (190). The hypervariable regions present in the 16S rRNA gene are unique to microorganisms and can be used to identify species residing within the biofilm. 16S rRNA genes can be extracted from samples collected from the oral cavity, amplified, sequenced, and subsequently compared with databases such as the Human Oral Microbiome database (HOMD) (191). The HOMD links sequence data with phenotypic and phylogenetic information regarding organisms found within the mouth. Metagenomic and metatranscriptomic approaches provide insights into the genetic composition of the oral microbiome as well as the genes that are being actively transcribed, respectively (192). Together, community profiling, metagenomics, and metatranscriptomics have advanced our understanding of the collective genome and metabolic actions of the oral microbiome (125).

The identity of certain microbial species as the causative agents of dental disease is an inexact conclusion that does not explain the polymicrobial interactions that lead to states of microbial dysbiosis that ultimately result in pathological conditions. To understand the cause of dental disease, an in vivo approach must be used to replicate the true activities of microbial communities among the dental enamel surface. Studies utilizing metagenomic approaches not only seek to identify pathological agents of dental
disease, but also the microbial communities associated with states of oral microbial homeostasis and health (83). This study utilizes metagenomic analysis to better understand the influence of dietary texture on microbial population dynamics within the oral biofilm, specifically with respect to the oral biofilm present on the dental enamel surface.
MATERIALS AND METHODS

The Murine Dental Microbiome

This study promotes the use of mice as an experimental model to study dental disease, as it utilizes mice to create a controlled *in vivo* study. Traditionally, controlled studies relating to dental-related projects are difficult to achieve *in vivo* due to the vast number of variables that exist, including but not limited to the inability to account for subject compliance as well as the inability to attain a more or less same pre-existing microbiome among subjects. Controlled studies usually entail the use of culture methods that do not capture the true dynamics of the oral microbiome (56). The etiology of dental disease is polymicrobial in nature, and thus a controlled *in vivo* study should be utilized to understand the multifaceted processes leading to states of microbial dysbiosis and disease. In this study, mice were exposed to the same dietary and environmental conditions prior to the study as well as are able to be carefully followed during the course of the experiment. This allows a similar pre-existing microbiome to be attained among subjects as well as ensures compliance of dietary regimen among subjects.

Comprehensive metagenomic studies of the murine oral microbiome are lacking. To our knowledge, no study of the murine dental microbiome has been completed to date. We identified three previous studies that investigated the oral microbiome of murine models. Various studies have characterized the oral microbiota of mice (193-195), however these studies are based on cultivation methods. It is now accepted that microbial community
analysis of the oral cavity should rely on culture-independent studies that utilize molecular identification methods (83, 196). Comprehensive studies on the oral microbiome of rats and mice have been performed with regard to the dorsal surface of the tongue, gingival tissues, oral mucosal surfaces, palate, and incisors (196-198).

Despite the advances these studies have made in providing a greater characterization of the oral microbiome, these studies do not specifically characterize the dental microbiome of mice. The surfaces within the mouth differ widely from one location to another, and the non-shedding nature of the dental surfaces makes this stratum unique within the oral cavity for microbial growth. Thus, it is important to characterize the dental microbiome in isolation from other sites within the oral cavity (53, 199). To aid in future studies of the dental microbiome, we aimed to characterize the dental microbiome of mice when exposed to various diet types. Our study only provides information regarding dental (mandibular) surfaces of experimental mice rather than the entire oral cavity. The murine dental microbiome was characterized in this study to determine and compare microbial diversity and taxa abundance in response to different diet types.

Current sampling methods used to study the dental microbiome fail to isolate the dental microbiome in a specific manner, perhaps causing bias and a false-characterization of the dental microbiome. Considerable differences in bacterial diversity and composition exist between individual sites and surfaces of the oral cavity. It is well established that microbial community composition differs from one intra-oral location to another, as various sites within the oral cavity harbor distinct microbiota (55, 200). Despite this, many studies investigating the dental microbiome utilize samples from
selected teeth, salivary samples, or swabs of the dorsal surface of the tongue or inner cheek (55, 95, 201). Investigating microbial diversity within the oral cavity, specifically with regard to the dental surfaces, may allow for a better understanding of the composition of microbiota in health and disease (10, 96). Precise and appropriate sampling methods are needed to relate specific microbial profiles within the mouth to states of dental health and disease.

Study of the dental microbiome using sampling methods that are specific to a defined tooth or dental site do not provide a comprehensive understanding of the dental microbiome, as bacterial community composition can differ among different teeth and even sites among the same tooth. The microbiota of different dental surfaces of all teeth was collected and compared to one another, as well as to the microbiota of saliva, to determine whether current sampling methods are representative of the microbiota of the site of disease (202, 203). It was observed that considerable differences in bacterial diversity existed between different teeth, as well as between the surfaces of teeth and saliva. Additionally, bacterial diversity differed between varying sites of the same tooth, demonstrating the diverse micro-niches that exist within the oral cavity even among the same anatomical location (202).

Bacterial diversity differs not only when comparing dental surfaces to other oral surfaces, but also between various types of teeth (canines, molars, etc.) as well as between various surfaces of the same tooth (lingual, buccal, etc.). Collecting plaque samples from the same site of the same tooth of different subjects may not allow for a complete characterization of the dental microbiome in its entirety. This study isolates the dental microbiome from other sites in the mouth to study the dental microbiome
specifically, as well as utilizes whole-jaw extraction to account for the entirety of the dental microbiome.

This study additionally seeks to assign a quantitative value to the effects of dietary texture on dental health and disease. Currently, studies relating to the effects of dietary texture on the oral microbiome have been limited to observation of resulting phenotypic discrepancies. These phenotypic outcomes include but are not limited to dental enamel erosion and wear (144). While these observations are beneficial to the study of dietary texture on dental disease, they are qualitative in nature and neglect to account for the effects of dietary texture on the microbial level. Subjecting samples to metagenomic analysis provides a quantitative value to the progression of dental disease due to variances in dietary texture. By observing changes in microbial diversity and relative abundance, a quantitative value can be assigned to better understand the effect of dietary texture on dental health and disease.

Animals and Dietary Groups

Three-week-old female mice (N=28) of the ICR/CD-1 strain (Charles River Labs, Wilmington, MA) were housed in standard shoebox rodent housing throughout the duration of the experiment. Animal usage was approved by Mercer University’s Institutional Animal Care and Use Committee (IACUC #A1609015). Animals were randomized into four dietary groups (n=7 each) that contrasted the presence or absence of two additional dietary ingredients [Control (C), Control+Sugar (S), Control+Fiber (F), and Control+Sugar+Fiber (SF)]. DietGel 76A (Clear H2O, Westbrook, ME) was used as the base (C) for all diet types. Animals were raised on the same dietary regimen prior to dietary group assignment to establish a similar oral microbiome among all subjects. The
S and SF diets each contained an additional 6.5g sucrose. The F and SF diets each contained an additional 4.2g lignin. This experimental design allows for each dietary ingredient to be tested alone and as they interact with one another to drive variation in microbial diversity and abundance across the sample space. Mice were raised on their respective diets for approximately 60 days as to allow adequate time for sufficient oral biofilm accumulation. Mice cages were cleaned and refreshed weekly over the course of the study. To establish a homogenous ambient microbiome for all groups a small amount of litter from each cage was mixed together and redistributed during the time of weekly cleaning. In this way, any changes in oral microbial communities could be attributed to dietary differences alone as opposed to differences in environmental microbial communities.

Sample Collection

After 60 days of a specific dietary regimen, mice were euthanized using CO₂ overdose (~3.75 months old). The lower jaw of each specimen was collected using sterile methods under controlled airflow. Careful attention was given not to disturb the dental surface. Jaws were placed in a 1.0ml collection tube and immediately stored at -80°C.

DNA Isolation and amplification

Bacterial genomic DNA was extracted from the jaws using DNeasy PowerBiofilm Kit (Qiagen Inc., Maryland, USA) per the manufacturer’s instructions with the following modification: cell lysis was conducted by bead beating the extracted jaws for 5 minutes. Samples were subsequently nanodropped to determine DNA concentrations and the 260/280 ratios for each sample. Following genomic DNA extraction and quantification, all samples were prepared for 16S amplification and sequencing at the University of
Illinois-Chicago Research Resources Center. The V3 region of 16S genes was amplified using the 515f/806r primer pair. Size selection was performed to eliminate mitochondrial 16S and eukaryotic 18S amplicons.

Statistical Analyses

In this experiment we have measured effects caused to the mouse host-associated oral (mandibular) microbiome in response to sugar (sucrose) and fiber (lignin). These effects represent dependent variables measuring differential diversity and differential abundance between 28 mice raised from weaning to maturity on differing dietary regimes. The dietary regimes in this study represent two co-varying independent variables, presence or absence of sugar and the presence or absence of fiber. Thus, four dietary groups can be permuted that describe a classic 2x2 experimental design (C, S, F, and SF). The strength of this design is that the two covariate independent variables (sugar and fiber) can be examined by the effect that each is having on the dependent variable measured (diversity or abundance). Each independent variable can be investigated to determine if it significantly affects the variation in diversity or abundance across the experimental sample space by itself, but also whether sugar and fiber might significantly interact together to impact diversity and abundance.

To measure within sample diversity (alpha and beta diversity) a type of Generalized Linear Model (GLM) statistical procedure was used that yields three sources of potential variation, presence or absence of sugar, presence or absence of fiber, and the third source is the interaction between these first two sources. These measures for alpha diversity represent the Shannon Index, which is a measure of richness and evenness of microbial taxonomic diversity within each mouse sample. For any significant effect that a source is
having on alpha diversity there will be a P-value <0.05 and pair-wise testing (Kruskal-Wallis followed by Mann-Whitney tests) to determine exactly how dietary grouping either increases or decreases alpha diversity of the mouse oral microbiome. These measures for beta diversity represent the pairwise dissimilarity of microbial taxonomic units observed between each sample. For any significant effect that sugar, fiber, or their interaction is having on beta diversity there will be a significant $R^2$ value and $P<0.05$ using an ADONIS procedure. ANOSIM results also test whether sugar or fiber significantly affected beta diversity (sample dissimilarity) using an $R$ value and $P<0.05$, but it cannot discriminate whether interaction between these covariates was observed.

To measure within sample abundance (read Counts Per Million, or CPM) a type of GLM was used that yields three sources of potential variation, presence or absence of sugar, presence or absence of fiber, and the third source is the interaction between these first two sources. For any significant effect that a source is having on microbial abundance there will be a P-value <0.05 and pair-wise testing to determine exactly how dietary grouping either increases or decreases log transformed fold change differences in CPM. Such differences will allow identification of specific effects associated with the mouse oral microbiome at the phylum and the genus level. Figures in this document are provided in color.
RESULTS

Development of a Dental Model System

This study design offers a new approach to understanding the dental microbiome in that it successfully isolates it from other sites within the oral cavity using a controlled *in vivo* model. Here, mice underwent full-jaw extraction and subsequent DNA isolation and PCR. During the process of jaw extraction, the dental microbiome remained isolated and undisturbed. While some epithelial (gingival) and connective tissues remained attached to the jaws, muscular tissues were not included. Bacterial primers were used to both exclude any eukaryotic DNA of the murine mandibular tissue as well as to amplify the microbiome from the dental surfaces alone. This is relevant to advancing the study of the dental microbiome because the oral biofilm of the dental surface remained undisturbed during sample collection. This means the dental surface microbiome was successfully separated from other intraoral microbial communities. This is significant because each surface within the mouth harbors distinct microbial communities (9, 82). Previous studies have been limited to the study of the microbiome of the oral cavity in its entirety due to difficulty in attaining sufficient as well as representative metagenomic DNA of the dental microbiome specifically. To study the microbial communities having specific contribution to dental disease, a method that isolates the dental surface from other oral surfaces must be utilized.
Effect of dietary variability on microbial diversity

We used in-depth 16S sequencing to analyze the composition of the dental microbiome in response to four different diets in mice. The 16S gene hypervariable region (V3) was amplified from whole jaw extractions in the study cohort. Illumina sequencing of the 16S PCR products produced a total of 700,000 sequences with an average of approximately 25,000 sequences per animal, post-quality filtering. Altogether, the mouse dental microbiome for each diet contained a diverse array of bacteria from 10 phyla (Figure 2a). As expected, the complex microbial communities found in the dental microbiomes varied across individuals on the same diet at the phyla level (Figure 2b). The three most abundant phyla in the C group included Firmicutes (71%), Actinobacteria (13%), and Bacteroidetes (11%). Overall, the microbiomes of the mice were dominated by the phyla Firmicutes (85% in S, 78% in F, 71% in C and 47% for SF). The Bacteroidetes and Actinobacteria were the next most dominant phyla for the C, S, and F diets, while Proteobacteria and Bacteroidetes dominated secondarily for the SF diet. The abundance of these taxa suggests that they form the interactive and metabolic framework of the mouse dental microbiome. However, it is interesting to note the variance in abundance among these phyla among each dietary group. The other phyla were present in low abundance (less than 4%) in samples across all diets.
**Figure 2a.** Stacked bar chart of major phyla represented for each dietary cohort. Only the top 10 phyla represented are shown here for illustrative purposes. The five most abundant phyla represented for each dietary type included Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria. Overall, each dietary regimen was dominated by Firmicutes. Bacteroidetes and Actinobacteria were the second most prevalent phyla represented for the C, S, and F diets. Alternatively, Bacteroidetes and Proteobacteria were the second most abundant phyla represented for the SF diet.

**Figure 2b.** Stacked bar chart of major phyla showing counts of bacterial taxa per each sample. Variances in bacterial counts were observed at the phylum level across individuals. Sample numbers are associated with the following diet groups: 1-7 (S), 8-14 (F), 15-21 (C), 22-28 (SF).
The mouse dental microbiome for each diet contained a diverse array of bacteria from 20 dominant genera (Figure 3a). Additionally, the complex microbial communities found in the dental microbiomes varied across individuals on the same diet at the genus level (Figure 3b). The top 10 genera having the average greatest abundance among each diet type included Streptococcus (17%), Staphylococcus (17%), Lactobacillus (13%), Ruminococcaceae;Other (9%), Enterococcus (9%), S24-7;Other (6%), Aggregatibacter (4%), Bacteroides (4%), Corynebacterium (3%), and Unassigned;Other (2%), post-quality filtering. The top three genera represented in the C group included Streptococcus (27%), Staphylococcus (21%), and Lactobacillus (15%). The S group was also dominated by these genera Staphylococcus (28%), Streptococcus (26%), and Lactobacillus (18%). The F group was dominated by Staphylococcus (28%), Enterococcus (22%), and Lactobacillus (17%). The SF group was dominated by Ruminococcaceae;Other (21%), Aggregatibacter (20%), and Streptococcus (18%) (Figure 3b). For the S group, five genera had abundance >3%. The C group had six genera with abundance >3%. The F and SF groups had seven genera with abundance >3%. The dominance of fewer genera in the S group reflects a decrease in community evenness. The increased number of genera having abundance >3% in the F and SF group alternatively reflects an increase in community partitioning and evenness. This observation is reflected in pairwise comparisons of community evenness using Pielou analysis, in which the interaction of sugar and fiber demonstrates significantly greater community evenness than effect of sugar alone (P=0.007) at the phylum level.
Figure 3a. Stacked bar chart of the 20 major genera showing counts of bacterial taxa in each dietary cohort at the genus level. The top 10 genera having the average greatest abundance among each diet type included *Streptococcus*, *Staphylococcus*, *Lactobacillus*, *Ruminococcaceae;Other*, *Enterococcus*, *S24-7;Other*, *Bacteroides*, *Aggregatibacter*, *Unassigned;Other*, and *Corynebacterium*.

Figure 3b. Stacked bar chart of the 20 major genera represented in each dietary cohort within each sample. Variances in bacterial counts were observed at the genus level across individuals. Sample numbers are associated with the following diet groups: 1-7 (S), 8-14 (F), 15-21 (C), 22-28 (SF).
The results obtained from this experimental procedure indicate that the dietary variables tested significantly affect the microbial composition (diversity abundance) of mouse host-associated oral-mandibular samples (N=28). Alpha diversity was found to be affected at the phylum level and not the genus level. The alpha diversity was significantly increased by the presence of fiber (GLM; Pr>F = 0.021) but not sugar (GLM; Pr>F = 0.978) (Table 2). Interestingly, sugar only seemed to have an effect on Shannon Index when combined with fiber as a statistical interaction (GLM; Pr>F = 0.006). The Kruskal-Wallis test is roughly consistent with this observation showing that fiber (P=0.039) and not sugar (P=0.783) show differences in Shannon Index between dietary groups (Table 3). Pairwise comparisons reveal that the SF group has a significantly more diverse microbiome compared to the S group alone (Figure 4).

Pielou analysis and species counts were used to determine if changes in Shannon Index values were driven by changes in community evenness or richness, respectively. Pielou analysis revealed that community evenness was affected at the phylum level and not at the genus level. At the phylum level, community evenness was not significantly increased by the presence of sugar (GLM; Pr>F=0.995) or the presence of fiber (GLM; Pr>F=0.072). Sugar only seemed to have an effect on community evenness when combined with fiber as a statistical interaction (GLM; Pr>F=0.005). The Kruskal-Wallis test is roughly consistent with this observation showing that neither sugar (P=0.963) or fiber (P=0.142) significantly increase community evenness. Pairwise comparisons reveal that the SF group displays increased community evenness compared to the S group alone (P=0.007).
Table 2. GLM analysis of alpha diversity. A significant difference in alpha diversity was found at the phylum but not genus level. No significant change in alpha diversity was observed at the genus level. Significant differences in alpha diversity were found for the F and SF groups.

Table 3. Mann-Whitney pairwise comparison of alpha diversity. Mann-Whitney pairwise comparisons revealed a significant change in alpha diversity between the SF group and S groups (P<0.01). This indicates that fiber has a significant impact on alpha diversity in the presence of sugar.
Analysis of species counts revealed that community richness was affected at the phylum level and at the genus level. At the phylum level, community richness was significantly increased by the presence of fiber (GLM; Pr>F=0.013) but not by the presence of sugar (GLM; Pr>F=0.596) or the interaction of sugar and fiber (GLM; Pr>F=0.790). The Kruskal-Wallis test is consistent with this observation showing that fiber has a significant effect on community richness (P=0.005) and sugar does not (P=0.849). Pairwise comparison revealed that the SF group community displays significantly greater community richness than the S group (P=0.047) alone. At the genus level, community richness was significantly affected by the presence of sugar (GLM;
Pr>F=0.002) and fiber (GLM; Pr>F=0.013), but not by the interaction of sugar and fiber (GLM; Pr>F=0.752). Kruskal-Wallis analysis is consistent with the observation for sugar (P=0.005 and fiber (P=0.027). Pairwise comparison revealed that the C group has significantly greater community richness than the S diet alone (P=0.009). Pairwise comparison revealed that the F diet has near-significant greater community richness than the C diet (P=0.055).

Additionally, the dietary variables tested significantly affect the beta diversity of mouse host-associated oral-mandibular samples (N=28) at the phylum level and at the genus level, however the strength of this association is greater at the phylum level (Figure 5a and 5b). For the phylum level the ADONIS value for fiber (R²=0.321; Pr>F = 0.002) shows a significant effect on increasing sample microbial dissimilarity but both sugar and the interaction between sugar and fiber did not. The ANOSIM value for fiber also corroborates this finding (R=0.382; P=0.001) while the value for sugar does not (R=0.033; P=0.232) (Table 4). The same effect was observed at the genus level but the strength of fiber as an effect on the dissimilarity between samples (R²=0.178; Pr>F=0.006) is weaker and non-existent in sugar, or their interaction, as a source of variation (Table 5).
Table 4. ADONIS and ANOSIM analyses of Beta diversity at the phylum level. ANOSIM analysis revealed a significant change Beta diversity at the Phylum level for the fiber diet alone. ADONIS analysis also revealed that Beta diversity was significantly impacted by the presence of fiber.

Table 5. ADONIS and ANOSIM analysis of Beta diversity at the Genus level. ANOSIM analysis revealed that a significant change in Beta diversity was observed at the Genus level for the fiber diet alone. ADONIS analysis also found that Beta diversity was significantly impacted by the presence of fiber at the genus level.
Figure 5a. Multi-dimensional scaling (MDS) plot demonstrating changes in Beta diversity at the phylum level. ADONIS and ANOSIM analyses revealed that the presence of fiber alone had a significant impact on Beta diversity. The presence of fiber (blue) shows significant community dissimilarity from the presence of sugar (red).

Figure 5b. Multi-dimensional scaling (MDS) plot demonstrating changes in Beta diversity at the genus level. ADONIS and ANOSIM analyses revealed that the presence of fiber alone had a significant impact on Beta diversity. The presence of fiber (blue) shows significant community dissimilarity from the presence of sugar (red). Community dissimilarity is more significant at the phylum level than at the genus level, however.
Effect of dietary variability on microbial abundance

The abundance analyses include two strategies for comparing standardized taxonomic unit counts as counts per million (CPM). The complete omnibus analysis is a strategy for identifying which taxa have their abundance (CPM) affected by either of the two independent variables (sugar and fiber) or their interaction. A second strategy is to use pair-wise testing between each dietary group to determine how group differences report as fold changes in abundance in that pair-wise comparison. The omnibus results indicate that at the phylum level there are no significant effects on abundance caused by sugar, fiber, or their interaction.

The omnibus results at the genus level indicate that Aggregatibacter is significantly affected by sugar (GLM Qvalue <0.01) and by fiber (GLM Qvalue <0.05), Streptococcus is affected by fiber alone (GLM Qvalue<0.05), Enterobacteriaceae;Other is affected by fiber (GLM Qvalue<0.05) and by the interaction of sugar and fiber (GLM Qvalue<0.01), Enterococcus is affected by the interaction of sugar and fiber alone (GLM Qvalue<0.01), Planococcaceae;Other is affected by the interaction of sugar and fiber alone (GLM Qvalue<0.01), and Staphylococcus is affected by the interaction of sugar and fiber alone (GLM Qvalue<0.05) (Figure 6). These omnibus results allow the reader to focus attention on those taxa that showed overall differences and examine them more closely for pair-wise differences between each dietary group. However, observation of individual sample OTUs may indicate that significant QV values assigned to some of these genera may be due to outlying individual samples as opposed to a dietary cohort. It is a statistical reality that pair-wise differences may get detected in the absence of omnibus differences. When this happens, it indicates that the pair-wise difference
detected may be a minor taxonomic change compared to those changes in abundance marked by both pairwise differences and omnibus differences. Thus, the latter are the focus of exploring whether fold change in abundance was a meaningful (i.e., statistically significant) difference as a result of the experimental treatment with either sugar or fiber, or whether the pair-wise difference in fold change abundance was not statistically significant.

**Figure 6.** Counts per sample of the top 20 genera for each dietary cohort. Genera having a significant QV values included *Aggregatibacter, Streptococcus, Enterobacteriaceae;Other, Enterococcus, Planococcaceae;Other, and Staphylococcus*. Planococcaceae;Other is not included in the top 20 genera, suggesting that its associated significant QV value is driven by an outlying individual harboring high numbers of this taxon.
Significant differences were observed for pairwise comparisons between each dietary group. A large majority of taxa having significant pairwise comparisons (QV<0.05) were identified for the S vs. SF comparison. These taxa included *Aggregatibacter*, Enterobacteriaceae;Other, *Corynebacterium*, *Enterococcus*, *Dorea*, *Blautia*, *Coprococcus*, *Akkermansia*, *Parabacteroides*, *Bacteroides*, Planococcae;Other, *Proteus*, *Staphylococcus*, rc4-4, and *Aldercruzia*. Two of these genera that were not included as also having significant QV values for the omnibus results include *Akkermansia* and *Corynebacterium*. *Corynebacterium* was associated with a significant P value for the omnibus results, however (P<0.01). *Corynebacterium* decreased significantly with the SF group in comparison to the S group (QV<0.05). For the S group, 516 OTUs (mean value for all seven animals) were observed for *Corynebacterium*, accounting for 2% of total reads identified for the S group. Alternatively, 126 (mean count per dietary group) OTUs were observed for the SF group, accounting for <1% of total reads for this dietary group. The significant decrease in *Corynebacterium* that occurred with the SF group demonstrates that fiber has a significant impact on decreasing this taxon in the presence of sugar. This discrepancy between the S and SF groups is of interest because *Corynebacterium* is notable for its extensive involvement in dental biofilm formation (87).

*Akkermansia* was not associated with significant QV or P value for the omnibus results. *Akkermansia* increased significantly with the SF group in comparison to the S group (QV<0.01). For the S group, 117 OTUs were observed for *Akkermansia* and these reads accounted for <1% of total reads for this dietary group. For the SF group, 5068 OTUs were observed for *Akkermansia* and 3% of total reads for this dietary group.
derived from this taxon. The significant increase in *Akkermansia* that occurred with the SF group indicates that, in the presence of sugar, fiber has a significant effect on increasing the abundance of this taxon. The increase in *Akkermansia* associated with the addition of fiber is of particular interest because it has relevance to the colonic microbiota. Non-digestible fibers provide metabolic substrates for colonic bacteria. The mucus layer that overlies the colonic epithelium has a high polysaccharide content and may also act as a metabolic substrate for certain microbes in the absence of fiber as a substrate. The colonic mucous barrier is critical for maintaining health, and disruption of this barrier causes increased pathogen susceptibility and has been associated with inflammation, colon cancer, ulcerative colitis, and colitis (204, 205).

In the absence of fiber, changes in abundance of mucin-degrading bacteria, including *Akkermansia muciniphila*, have been observed. Depletion of dietary fiber allows this subset of bacteria to increase in abundance due to their ability to express mucin-degrading CAZymes and to outcompete other bacteria by utilizing mucin as a metabolic substrate. Degradation of the mucus layer by fiber-deprived gut microbes is associated by increased contact of luminal bacteria to the intestinal epithelium, and thus a potential threat to host health. Alternatively, colonic mucus thickness is associated with a diet that is high in dietary fiber (204). In our study, *Akkermansia* increased significantly from the S diet to the SF diet. This increase in *Akkermansia* supports previous studies that demonstrate the utilization of fiber as a metabolic substrate for this taxon. Additionally, this finding is significant because it demonstrates similarities in microbial responses to dietary changes among the intestinal and oral microbiota and could ultimately lead to a
greater understanding of the association of oral health to gastrointestinal and systemic health.
DISCUSSION

Murine Oral and Dental Microbiomes

Studies of the murine oral microbiome are lacking, and those specific to the microbiome of the murine dental surfaces do not exist to our knowledge. The most abundant genera OTUs derived from microbial communities of the murine gingival and mucosal surfaces are *Streptococcus, Staphylococcus, and Lactobacillus* (197). In the study presented here, the dominant genera in the control diet included *Streptococcus, Staphylococcus, Lactobacillus* and *Corynebacterium*. *Corynebacterium* have recently been acknowledged as significant to the formation of the dental biofilm, as they provide filaments upon which subsequent bacterial adhesion is directed.

“Hedgehog” biofilm structures form on the surfaces of the tooth, and are comprised of a base, annulus, and perimeter. Early colonizers such as mitis Streptococci and *Actinomyces* occupy the base. *Corynebacterium* attaches to these early colonizers and provides extended filaments throughout the structure upon which additional bacterial attachment sites are created. At the periphery, the *Corynebacterium* filaments are surrounded by Streptococcal and *Porphyromonas* species (86). *Corynebacterium* are not recognized as a top taxon among the murine oral soft tissues, suggesting that this taxon is more specific to dental surfaces than to soft tissue surfaces. This observation may reflect that the unique non-shedding surface of the dental surface facilitates *Corynebacterium*
adherence and formation of hedgehog structures that are the cornerstone of dental biofilm communities.

Significant genera

_Aggregatibacter_. The omnibus results at the genus level indicate that _Aggregatibacter_ is significantly affected by sugar (QV<0.001) and fiber (QV<0.05). The species level of _Aggregatibacter_ was not identified by the 16S rRNA analysis used here. However, _Aggregatibacter_ species such as _Aggregatibacter actinomycetemcomitans_ are found readily within the oral cavity. _A. actinomycetemcomitans_ is a slow-growing, nonmotile, facultative anaerobic, gram-negative bacterium that resides among the gingival crevice in the oral cavity and is associated with localized aggressive periodontitis. Within the gingival crevice _Aggregibacter_ occupies moderate pocket depths that have exposure to oxygen. It has been noted that _Aggregibacter_ demonstrates enhanced growth in the presence of oxygen (205, 206).

_Aggregatibacter_ displays limited enzymatic capabilities in terms of carbon source utilization. Carbon sources used by _Aggregatibacter_ include glucose, mannose, fructose, maltose, and lactate (207). Interestingly, _Aggregatibacter_ practices niche partitioning when competing for carbon sources against faster-growing bacterial species within the oral cavity. Under these conditions, _Aggregatibacter_ preferentially uses lactate as a carbon source as opposed to other carbon substrates to decrease competition with other bacteria. Lactate is transported into _Aggregatibacter_ via lactate permease, and subsequently is converted to pyruvate by lactate dehydrogenase. An increase in pyruvate inhibits autophosphorylation of the E1 transport protein, which leads to a reduced activity of the phosphoenolpyruvate-dependent phosphotransferase system (PTS). The PTS
system is needed for the transport of mannose, glucose, and fructose, while the lactate permease transporter is proton-driven (208). *Aggregatibacter* demonstrates faster growth and yields higher cell counts when grown in the presence of glucose. However, when provided with glucose, fructose, and lactate, *Aggregatibacter* prefers to metabolize lactate despite yielding slower growth (205).

Aggregatibacter significantly increased in the presence of sugar. Organisms within biofilm communities are partitioned to support metabolic compatibility between organisms. *Aggregatibacter* is often associated with mitis group Streptococci that metabolize carbohydrates to yield lactate. Association with these Streptococcal species allows *Aggregatibacter* to utilize the end product of Streptococcal metabolism as a carbon source. Mitis group Streptococci are characterized as early colonizers of the dental biofilm and utilize carbohydrates to produce lactate. Interestingly, a significant increase in *Aggregatibacter* also occurred in the presence of fiber. A decrease in Streptococcal species may decrease the availability of the preferred lactate substrate of *Aggregatibacter*. Although *Aggregatibacter* prefers lactate utilization, decreased competition for other carbohydrate sources and a decrease in lactate availability may promote utilization of the PTS pathway in *Aggregatibacter* that is associated with increased growth rates (205). Lactic acid is produced by Streptococci and Lactobacilli in the oral cavity and provides a major energy source for *Aggregatibacter actinomycetemcomitans* and Veillonellae (209). Lactate production and consumption was not measured in this study, however.

*Streptococcus*. Streptococcal species in the oral cavity consist of cariogenic mutans Streptococci (*S. mutans* and *S. sobrinus*) and non-cariogenic viridians
Streptococci (\textit{S. sanguinis, S. oralis, S. gordonii}). Cariogenic mutans Streptococci attach to glucan-coated surfaces and metabolize sugar to produce extracellular polysaccharides and acidic products (210). \textit{Streptococcus} was significantly affected by fiber at the omnibus level (Q<0.05).

In our study the fiber component used was lignin, a complex aromatic heteropolymer that acts as a constituent of the plant cell wall. Most microbes do not readily metabolize lignin; the best-known microbes associated with lignin degradation are fungi that decompose wood, as well as soil microbes such as Actinomycetes. Lignin was used here to serve solely as an abrasive component, as no microbes within the oral cavity are known to catabolize lignin to date (211). In this way, changes in microbial abundance and diversity observed among the F group were due strictly to changes in dietary texture without possible conflating effects due to microbial metabolism. Thus, the significant increase in Streptococcal species associated with the F group is not due to an introduction of an additional metabolic substrate by the fiber, but possibly by the abrasiveness introduced with this diet. The use of abrasive components has been widely used to reduce dental disease in animals. For example, dental chews containing a textured component have been utilized in domestic dogs to reduce dental plaque via mechanical action on dental surfaces. Addition of dental chews to the canine diet significantly reduces plaque and calculus accumulation as well as the incidence of gingivitis (212).

Dental biofilm initiation by early colonizers requires the presence of the dental pellicle. The dental pellicle is a thin film coating the dental surfaces that is composed of host salivary glycoproteins. Gibbs Free Law of enthalpy governs this initial attachment, as greater free energy is released upon glycoprotein binding to the dental enamel surface.
The surfaces of mitis group Streptococci are coated with various adhesins that are specific for salivary proteins and glycoproteins that are present on the dental enamel surfaces (208). Once early colonizers attach to the dental surfaces, they excrete exopolysaccharides that allow the bacteria to remain attached to the pellicle and to one another (213).

Early colonizers such as mitis Streptococci and Actinomyces occupy the base of hedgehog structures, described in the previous section. These early colonizers allow the attachment of Corynebacterium, which promotes subsequent biofilm community colonization (86). The significant decrease in Streptococcal species observed with the F group may be associated with the abrasive action of the lignin against dental surfaces; this abrasive action is presumed to disrupt bacterial attachment to the dental pellicle or adhesion of the dental pellicle to the dental enamel surfaces. However, identification of Streptococcal bacteria at the species level is needed to support this assumption, as Streptococcal species (such as S. mutans) also occupy the periphery of the biofilm.

Enterococcus. Enterococci are facultative anaerobic, Gram-positive cocci that are commensal organisms of humans in the gastrointestinal tract. Two species of Enterococcus (E. faecalis and E. faecium) have been isolated in low numbers form the oral cavity. E. faecalis has been associated with root canal infections (210). Genes for carbohydrate metabolism are the most common genes found in the genome of Enterococcus, with the pentose phosphate pathway and the glycolysis/gluconeogenesis pathways representing the core genes represented for carbohydrate metabolism. Other core genes identified for Enterococcus demonstrate metabolic capabilities for fructose, mannose, lactose, and galactose catabolism (214).
A significant decrease in *Enterococcus* at the omnibus level was observed for the interaction between sugar and fiber in the experiment (QV<0.01). Enterococcal species have been previously observed to inhibit Streptococcal colonization among the mutans Streptococci group, as well as to reduce overall biofilm accumulation. It is interesting to note that Enterococcal species cultured with viridians Streptococci resulted in reduced total biofilm mass but not when cultured in isolation (210). This observed decrease in total biofilm mass for combined viridians Streptococci and Enterococci culture supports our finding that Enterococcal species significantly decreased when independent variables sugar and fiber interact.

*Staphylococcus*. Staphylococci are Gram-positive bacteria that are ubiquitous among the human body. However, opportunistic infection by Staphylococci has been noted extensively, especially in terms of nosocomial infection (Ferreira et al., 2013). Staphylococci species primarily colonize the skin and are transient colonizes of mucus membranes. However, a study of isolation frequencies of oral Staphylococci revealed that 99 clinical isolates were readily identified, with *S. aureus* and *S. epidermidis* most frequently isolated (215). Staphylococci have been recognized as regular colonizers of the oral cavity, especially among periodontal pockets, and have been linked to periodontal infection (216).

A recent study characterizing the murine oral microbiome lists two species of Staphylococci (*S. xylosus* and *S. nepalensis*) as species observed to have the most abundant OTUs among samples derived from murine gingival tissues and oral mucosal surfaces (197). *S. aureus* is a facultative anaerobe that possesses genes for the Embden-Meyerhoff-Parnus, Triboxylic acid cycle, and pentose-phosphate pathways for
carbohydrate metabolism. Oxygen deprivation acts to alter transcription of genes for aerobic metabolism and to promote a switch to anaerobic metabolism. Under anaerobic conditions, lactate is the main end product of Staphylococci metabolism (217). Staphylococci species decreased significantly with the SF diet and this is consistent with the observed statistical interaction between independent variables sugar and fiber (QV<0.01). This significant decrease may be due to increased competition for dietary derived carbohydrates.

Planococcaceae;Other and Enterobacteriaceae;Other. The final two significant differences identified from this study are for the Families Planococcaceae;Other and Enterobacteriaceae;Other of the phyla Firmicute and Proteobacteria, respectively. However, these families were noted for significance based on a single animal in a diet group that harbored unusually large numbers of OTUs. Planococcaceae;Other was significantly affected by the interaction of sugar and fiber (QV<0.01). The outlying animal for Planococcaceae presented with 769 OTUs from the S group, while the average for the remaining six animals was only 25 OTUs. Enterobacteriaceae;Other was significantly affected by fiber (QV<0.05) and the interaction of sugar and fiber (QV<0.01). Similarly, for Enterobacteriaceae;Other, the outlying animal presented with 2015 OTUs from the SF group while the average for the remaining six animals was only 4.5 OTUs. Nevertheless, we must consider whether a larger study might support these results. Planococcaceae;Other was not included in the top 20 species for total OTUs and Enterobacteriaceae;Other was the final group listed in the top 20 genera, suggesting that their impact on the dental microbiome is limited. This is supported by the lack of
information in the literature for the occurrence of these families in the oral cavity of vertebrates.

Future Directions

The study of microbial communities directly from their natural environments is achieved using culture-independent approaches. Metagenomics allows the study of microbial community structure, phylogenic composition and relatedness and diversity, as well as the metabolic capabilities and functional diversity that exist within a community. Here, 16S rRNA analysis was utilized to obtain a better understanding of how dietary variance alters microbial communities on the murine dental surfaces in terms of relative population dynamics, including abundance and diversity. 16S rRNA analysis achieves identification of genetic diversity within a microbial community and is a fundamental and rudimentary process in understanding microbial communities. The 16S rRNA genes can be used to determine the presence of various taxa by identifying differential sequence diversity that distinguishes bacterial species. The 16S gene contains nine hypervariable regions that provide enhanced specificity. However, the V2, V3, V6 hypervariable regions provide maximum specificity in identification of bacterial lineage and identification (218). Here, the V3 region was used in 16S analysis to determine the microbial species present.

An identification of microbial species within a community is essential for understanding community dynamics. However, a functional characterization of the microbial community is also needed to fully understand the metabolic interactions taking place within the community. Whole genome shotgun sequencing (WGS) metagenomic approach allows for the characterization of the functional capabilities of microbial
communities. Metagenomic approaches for characterizing microbial community metabolic function utilize a “best-BLAST-hit” approach. This approach searches individual short reads from a sequenced community against a previously characterized reference database via BLAST (219).

While our study achieved determining alpha and beta diversity as well as microbial abundance profiled by utilization of a 16S metagenomic approach, whole metagenome sequencing is needed to understand the metabolic potential of the microbial communities identified, as well as to possibly provide a metabolic explanation for the changes observed with the introduction of varying diet types. Whole metagenome sequencing will be performed in future studies to provide a metabolic perspective for the changes observed among each dietary group. Whole metagenome sequencing will also be used to more specifically identify the bacteria affected at the species level. Streptococcal species occupy both the primary layer as well as periphery of the dental biofilm and identification at the species level will provide greater insight relating to the impact of diet on these groups specifically. This will be especially useful to understanding the extent to which the abrasive action of lignin disrupts the biofilm, from either the periphery of the biofilm or impact to the initial layer.

Sample size is critical to capturing microbial community structures. It has been noted that variation in sample sizes significantly influence observed microbial community structure in terms of richness, evenness, diversity, and dispersion among all replicates. Greater richness, evenness, and diversity values, as well as lower replicate variability, have been noted among larger sample sizes (220). The study presented here included seven replicates per dietary regimen. However, discrepancies in observed
microbial community dynamics could change if replicate number was increased. Taxa that revealed near-significant changes in our study (having significant PV values but not QV values) in terms of microbial abundance included *Corynebacterium* Lachnospiraceae;Other, Clostridiales;Other;Other, *Proteus, Ruminococcus, Peptococcaceae, rc4-4*, and *Aldercruzia*. These taxa may emerge as significant when more replicates are utilized. The small number of replicates used may distort results regarding significant taxa and may obscure possibly significant taxa. A greater replicate pool will be used in future studies to fully elucidate and affirm the findings of this experiment.

Future studies will also aim to understand a possible association among the dental and GI microbiomes. Previous studies have identified specified GI and oral bacterial profiles to exist in cases of local and systemic disease (221). However, a direct association has yet to be identified. We would like to observe how microbial diversity and abundance respond to the same dietary regimen among both the GI and oral microbiomes. In addition to observing specified bacterial profiles among each site in response to these dietary regimens, we would also like to observe if similar significant increases or decreases exist for the same phyla and genera. This will allow a greater understanding of not only a possible association among these sites but may also demonstrate the impact of the oral microbiome to the GI microbiome.
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