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The Influence of Infant Formula on the Growth of Commensal and Pathogenic Streptococcus Species in the Infant Oral Cavity

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Objective: The objective of this thesis was to determine if a probiotic-supplemented infant formula (Nutramigen Enflora) and non-probiotic infant formula (Enfamil NeuroPro) differentially influence the growth of the commensal *Streptococcus mitis* and the opportunistic pathogen *Streptococcus mutans*.

Committee

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THE INFLUENCE OF INFANT FORMULAE ON THE GROWTH OF COMMENSAL AND PATHOGENIC *STREPTOCOCCUS* SPECIES IN THE INFANT ORAL CAVITY

by

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ABSTRACT

The oral microbiome is a complex community of microorganisms that can both reflect and greatly influence the health of the human host. A number of diseases are associated with dysbiotic oral microflora in infants and children, including dental (e.g. dental caries, gingivitis, and periodontal disease), and gastrointestinal diseases (e.g. pediatric appendicitis, celiac disease, and pediatric inflammatory bowel disease). A variety of factors can influence the composition of the oral microbial community in infants, including gestation length, mode of delivery, feeding method, and diet. This study focuses on the effects of diet on the growth of a commensal bacterium (*Streptococcus mitis*) and pathogenic bacterium (*Streptococcus mutans*) commonly found in the oral cavity of infants. A culture-dependent model was utilized to test the effects of one infant formula supplemented with a probiotic (*Lactobacillus rhamnosus*) (Nutramigen Enflora) and one infant formula without probiotic supplementation (Enfamil NeuroPro) on the growth of the commensal and pathogen. The growth of the commensal and pathogen were assessed by measuring colony forming units (CFUs), pH levels of culture media and by performing a Snyder's media test (a measure of acidogenesis and pathogenicity) over time. Results indicate that the probiotic-supplemented formula may selectively inhibit the growth of the pathogen and aid in producing more favorable conditions for the commensal. The additional health benefits to the gut conferred by the probiotic may make Nutramigen Enflora the preferred infant formula for overall health. The results of this study may assist parents in selecting alternatives to breastmilk that will support the proper development of the infant oral microbiome by favoring the growth of commensal bacteria.

Table of Contents

INTRODUCTION

The human microbiome is defined as the totality of microorganisms which inhabit the human body (Huttenhower et al. 2012). These microbial symbionts are intimately intertwined with both systemic and specific bodily function (Gilbert et al. 2018), and each niche in the human body nurtures a unique community of microbes. The term supraorganism describes the totality of microbial inhabitants and the human hosting them which interact with each other and operate as a whole, with the microbial inhabitants contributing traits that humans alone lack (Turnbaugh et al. 2007). Organisms from various domains comprise the microbiome, including bacteria, fungi, archaea, and viruses (Wade et al. 2013). The bacterial constituents are the most well-studied component of the human microbiome, and while some research on disease-inducing fungi exists, the archaeal, fungal and viral constituents of the human microbiome have yet to be thoroughly investigated (Wade et al. 2013).

Recent estimates indicate that there are about as many bacterial cells as there are human cells in the body (Sender et al. 2016), while the number of viruses may equal or exceed the number of human cells (Reyes et al. 2010). The gut alone harbors about a thousand species of bacteria with a combined total of 2 million genes, which is 100X the number of genes in the human genome (Turnbaugh et al. 2007). While it is known that the microbiome is a key factor in maintaining health and in the development of many disease states, the specific human-microbe interactions that lead to healthy or disease states are not yet well understood (Huttenhower et al. 2012; Gilbert et al. 2018). The opportunity for significant advancements in understanding the underlying mechanisms regarding the microbiome's role in human health has led to the increase in

research dedicated to uncovering these links, pioneered by the Human Microbiome Project.

Overview of the Human Microbiome Project: Current Understanding of the Human Microbiome and Emergent Questions

The Human Microbiome Project (HMP) was conceived in 2007 by the National Institute of Health (NIH) as a response to advances in knowledge of the human microbiome. Findings from early research on the human microbiome led to heightened interest in the subject and ultimately the creation of the HMP. One of these initial findings that spurred the inception of the HMP was the intimate role that microorganisms play in the function of the human host. Because the microbiome and human host essentially work as a single organism, the genome of the microbiome can be thought of as an extension of the human genome, adding functionality where the human genome lacks. Thus, the characterization of the human microbiome and the supraorganism's genome is an essential step in understanding human physiology. This is the main objective of the HMP: to characterize the microbiota that inhabit the human body and understand the intra- and inter- specific interactions between the human host and its native microflora (Turnbaugh et al. 2007).

The goal of the first phase of the HMP was to characterize the microbial communities inhabiting various environments within the human body. Samples from body sites such as the nose, mouth, gut, skin, and vagina were taken from healthy adult subjects and analyzed to find the composition of the communities inhabiting these different microenvironments and elucidate the microbial metabolic functions (Proctor et al. 2019). The results of the control population were then compared to those of

individuals with specific diseases, such as inflammatory bowel diseases, to find any correlations between host disease states and microbiome composition. The results of the first stage of the HMP include an open-access database of nucleotide sequences of microbes found within the human body (http://hmpdacc.org), in addition to a wealth of descriptive knowledge of the human microbiome and improved methods for microbiome-related research (Proctor et al. 2019).

This first phase of the HMP found that host phenotype did not perfectly correlate with microbiome composition, rather that the specific molecular functions of the constituent microbes were more likely to predict the host's phenotype (Huttenhower et al. 2012). This finding prompted the second phase of the HMP, with a goal of delving further into the specific molecular interactions between host, microbial metabolism, and immune function and to investigate how these interactions change over time. These questions are being explored by studying the interplay between host and microbiome in three conditions that strongly correlate with shifts in the microbiome: preterm birth, inflammatory bowel diseases like Crohn's disease and irritable bowel syndrome (IBS), and diabetes. This second phase of the HMP involves longitudinal studies of individuals with these diseases in an effort to understand the interactions between the human microbiome and the disease states. In order to answer these questions, the HMP investigated host factors including host genetics, metabolome, epigenetic influences and immune function and how they relate to variable states of the microbiome. Microbiome community composition and metabolic function were investigated to characterize the human microbiome during these states of health and disease.

Whereas the first stage of the HMP sought to catalog diversity, this second phase seeks to understand specific changes in alpha and beta diversity within the context of health and disease. For example, it was found that women who had a vaginal microbiome dominated by *Lactobacillus crispatus* were less likely to give birth preterm and that vitamin D deficiency was correlated with the presence of taxa including *Sneathia amnii and Prevotella spp*, both of which were more prevalent in women giving birth preterm (DiGuilio et al. 2015; Jefferson et al. 2019; Zhou et al. 2017). It was also found that the presence of pro-inflammatory cytokines in the vagina were linked to the presence of those dysbiotic taxa associated with preterm birth (Proctor et al. 2019). These findings suggest that an increase in pro-inflammatory microbes could negatively affect the body on a systemic level, leading to negative health outcomes such as preterm birth. A study comparing patients healthy patients and those with IBD reported that increased serum antibody levels, altered transcription in some microbes, and shifts in bile acid levels were correlated with IBD (Lloyd-Price et al. 2019). It was also found that healthy patients had a more stable gut microbiome and immune response compared to patients with IBD, whose gut microbiome composition and immune response tended to fluctuate more widely (Lloyd-Price et al. 2019). The results of these studies suggest that there are links between the disease states and the community composition of the microbiome. However, more studies are needed to investigate the underlying factors that drive these observed correlations.

One immediate take away from the preliminary results of the second phase is that there is no definitive model healthy microbiome. A healthy microbiome can exist in a multitude of different forms among individuals, indicating that even more wide-range

studies are required for understanding the roles of the microbiome in human health (Proctor et al. 2019). Another important finding is that microbe-host interactions occur within specific times and locations, and that these interactions can have effects that are both localized and systemic. For example, in a study following both patients with type-1 diabetes mellitus (T1D) and healthy individuals, it was found that an altered gut microbiome was associated with impaired immune response in insulin-resistant individuals experiencing a respiratory viral infection, showing that shifts in a localized region (the gut microbiome) can have implications for systemic bodily functions (the immune response) (Zhou et al. 2019). These are only the first few questions at the beginning of a long process of discovery of the intricacies of the connections between the human host and its microbiome. The next goals on the horizon for the HMP include answering questions such as how initial microbiome acquisition impacts the development of the immune system, and other descriptions of how the microbiome changes over time, including causation with respect to the development of disease states (Proctor et al. 2019).

Niches within the Human Microbiome

The first phase of the HMP demonstrated that the various niches within the human body have distinct microbial communities (Turnbaugh et al. 2007). A multitude of species cohabitate in each respective niche, such as the oral cavity or the gastrointestinal tract. Each microenvironment provides a unique set of substrates and nutrient sources, which influences the kinds of microbes that inhabit that space. For example, gut microbiota assists in digestion and uptake of nutrients, vaginal microbiota produces hydrogen peroxide to maintain pH, and commensal skin microbiota compete

with opportunistic pathogens (Shafquat et al. 2015). It is generally thought that each of these communities has a particular ratio of the constituent organisms that maintains homeostatic balance both within that community and between the host and the microbial symbionts. However, microbiome composition is highly variable between individuals, and thus the indicators of dysbiosis are more nuanced than a simple shift in composition ratios (Gilbert et al. 2018). In fact, more recent research indicates that it may not only be the species composition of the oral microbiome, but the array of metabolic byproducts produced by the oral microbiota that influences human health. This "microbial metabolome" interacts with the host on a physiological level by modulating immune, endocrine, and nervous system function (Gilbert et al. 2018). However, it is clear that there are species associated with dysbiosis and various disease states and species that are associated with health (mentioned later in this paper). These associations are not a clear-cut binary, but rather a spectrum on which a species can increase or decrease in virulence depending on environmental factors (Huttenhower et al. 2012).

Results from the HMP in 2012 revealed characteristics of 18 different ecological niches within the microbiome (Huttenhower et al. 2012). These 18 sample sites were representative of four main body sites: the oral cavity, the gastrointestinal tract, the skin, and the vagina. The results of this study showed that the oral cavity was dominated by *Firmicutes* and *Proteobacteria*, the gastrointestinal tract by *Bacteriodetes,* the skin by *Actinobacteria,* and the vagina by *Firmicutes*. The most prevalent species in gut was *Prevoletella copri*, while the oral cavity was dominated by *Streptococcus mitis*. Species that dominated the skin include *Propionibacterium acnes, Corynebacterium*

kroppenstedtii, and *Corynebacterium accolens*. The most prevalent species found in the vagina include *Lactobacillus crispatus*, *Lactobacillus iners*, *Lactobacullis gasseri, Prevotella amnii*, and *Lactobacillus jensenii*. The oral cavity had the greatest alpha diversity, whereas the vagina had the lowest alpha diversity. The skin showed the greatest beta diversity, with the oral cavity and vagina having more similar compositions between individuals. It was also found that the closer the physical locations of the body sites were to each other, the more similar their compositions were (Huttenhower et al. 2012).

Species Interactions between the Human Host and Microbiota

Symbiosis, the cohabitation of different organisms within an environment, describes the relationship between a human host and microbiota. The dynamics of this relationship vary on a spectrum from mutualistic to parasitic interactions. In a mutualistic relationship, both parties benefit from the cohabitation. In a parasitic relationship, the microbe benefits while the human host is harmed. Commensalism describes the midway point of the spectrum, in which the interaction is neither beneficial nor harmful for the host (Eloe-Fadrosh et al. 2015). According to Foster et al. 2017, the interactions between the host and the human microbiome can be categorized as microbe-to-host, host-to-microbe, and microbe-to-microbe. Microbe-to-host interactions describe how the microbiome impacts human health and has been the most well-studied of the three types of interactions, while the host-to-microbe and microbe-to-microbe interactions have more impact on the development of the human microbiome. The human host exerts pressure on the microbiota in order to control the multitude of microbes inhabiting the body, ensuring that the microbes do not cause significant harm. Microbes influence

the structure of the microbiome through microbe-to-microbe interactions, including interspecific competition, in which commensal species typically outcompete the opportunistic pathogens (Foster et al. 2017). Thus, the interplay between the microbiome's survival and the human host's selective cultivation of the microbiota shapes the community structure of the human microbiome.

There is evidence that animals and their microbiota have co-evolved to maintain mutualistic and commensal relationships. One example of this intimate link between host and microbiome is that a significant portion of metabolic functions are carried out by symbiotic microorganisms (Cho & Blaser 2012). In fact, a study showed that germ-free mice lacked most of the metabolites that normal mice possessed, indicating that the microbiome plays a major role in metabolism (Vrieze et al. 2010). When environmental conditions are ideal, the commensal microbes will be favored and contribute to host health by competing with opportunistic pathogens (Shafquat et al. 2015). One study, conducted as a part of the Human Microbiome Project, aimed to characterize the composition of the human microbiome in healthy adults and showed that all healthy individuals lacked bacteria with known pathogenic potential to humans above 0.1% abundance (Huttenhower et al. 2012). The only bacterial species associated with pathology found in the healthy microbiome were opportunistic pathogens, such as *Propionibacterium acnes* and *Staphlyococcus aureus* on the skin, *Streptococcus mitis* and *Rothia mucilaginosa* in the oral cavity, *Alistipes putredinis* and *Bacteroides vulgatus* in the gut, and *Bifidobacterium dentium* and *Gardnerella vaginalis* in the vagina (Huttenhower et al. 2012). This presence of opportunistic pathogens even

in healthy individuals shows that there is potential for a transition to a dysbiotic or disease state if conditions change to favor the opportunistic pathogen.

The Human Microbiome's Role in Health and Disease

The microbiome is inherently linked to health of the human host and can either enhance or harm health. The effects that the microbiome exerts on the human body are directly linked to the state of the microbiome itself: changes in species composition and relative abundance can be characterized on a spectrum from health to dysbiosis. Many endogenous and exogenous factors (e.g. immune function, body site, diet, antibiotic use, lifestyle) influence the environmental conditions of the niches within the human body, which in turn influence microbial community composition and thus overall host health (Gilbert et al. 2018; Figure 1). The gut microbiome is highly implicated in both gastrointestinal and overall health (Turnbaugh et al. 2007). For example, commensal microbes within the gut contribute to human health by aiding in the digestion of nutrients, metabolism of drugs, epithelial cell turnover, and immune activity. Various immune disorders are associated with dysbiosis of the gut microbiome, including asthma and inflammatory bowel diseases like Crohn's disease (Turnbaugh et al. 2007). A shift in the composition of the microbial communities within the human microbiome from favoring commensal and beneficial species to favoring opportunistic pathogens is the general etiology of microbiome-associated diseases (Turnbaugh et al. 2007; Xiao et al. 2020). For example, the loss of beneficial species such as *Bifidobacteria* and the subsequent increase in proinflammatory bacteria impacts the body on a systemic level and leads to diseases like obesity and other inflammation-driven conditions such as osteoarthritis (Schott et al. 2018; Xiao et al. 2020).

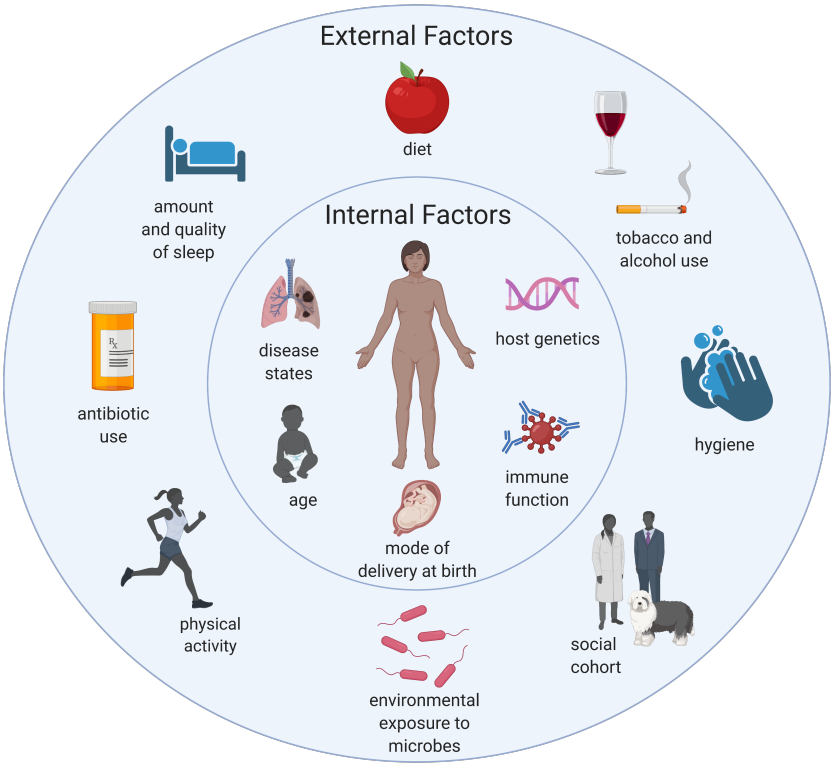

Figure 1: Internal and external factors that influence the human microbiome. Factors that shape the human microbiome can be internal (a condition of the body that is not easily changed, such as age, genetics, and mode of delivery at birth) or external (including lifestyle choices such as diet, exercise, and drug use).

External factors such as diet, antibiotic use, and various lifestyle factors impact the composition of the microbiome. For example, occupation (Ying et al. 2015), exposure to animals (Song et al. 2013), the amount of exercise (Cook et al. 2016) and sleep (Benedict et al. 2016) all affect the microbiota of the human body. The microbiome is so intimately tied with the host's bodily functions that disruptions in microbial circadian rhythms have been shown to impact the host circadian rhythms in mice (Gilbert et al. 2018). Dysbiosis, a state in which the microbial community composition is unbalanced between commensals and opportunistic pathogens, is triggered by alterations in factors such as diet, immune function, hygiene, and hormonal imbalance (Jia et al. 2017). It is well known that a dysbiotic microbiome is associated

with a multitude of disease states, such as cancers, obesity, cardiovascular disease, psoriasis, and major depressive disorder (Figure 2; Cho & Blaser 2012; Wade et al. 2013; Farrell et al. 2012; Gilbert et al. 2018). The impacts of the microbiome on health are plentiful and significant, and thus the ways in which we humans care for our microbiomes is of high importance.

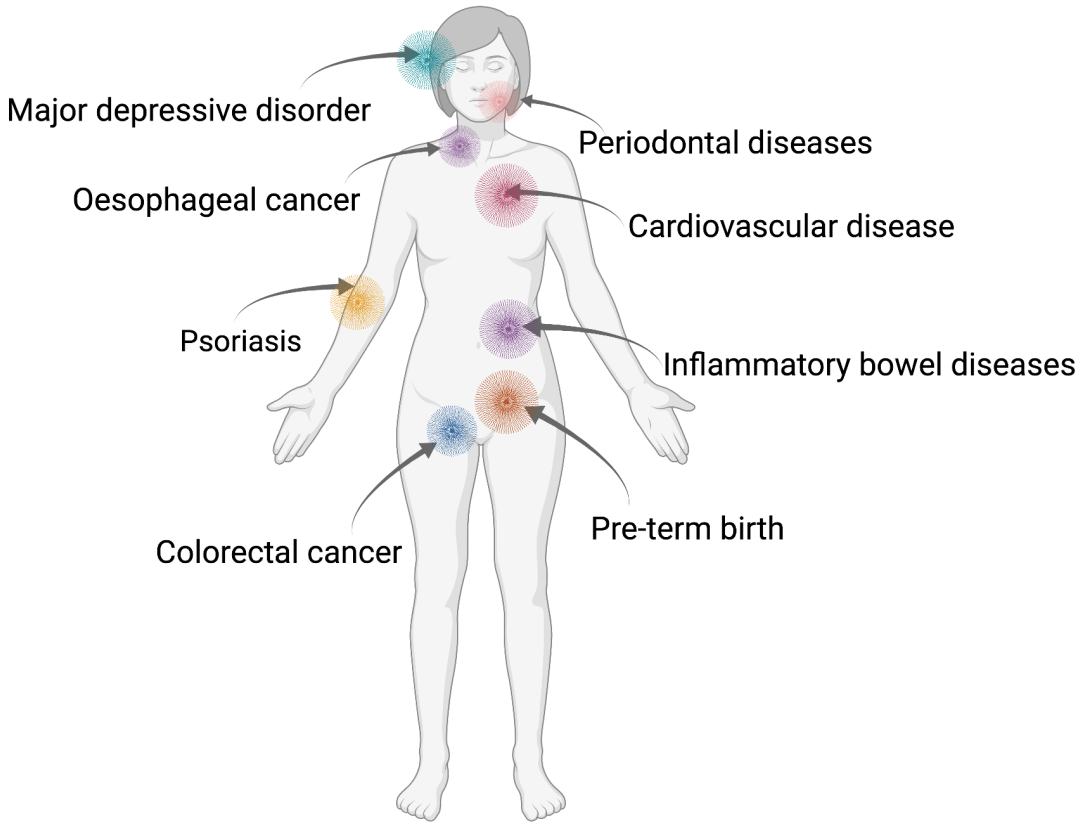

Figure 2: Some of the diseases and conditions associated with dysbiosis of the microbiome. Various localized and systemic diseases and conditions are linked to dysbiotic shifts in the human microbiome, ranging from conditions like pre-term birth to major depressive disorder.

The Oral Microbiome

The oral cavity is only second to the GI tract in both diversity and depth of study (Verma et al. 2018). This is due to both the physical continuity between the oral cavity

and the GI tract and the relative ease of access to the oral cavity, which lends itself to non-invasive scientific study (Xiao et al. 2020). Because of this physical continuity of the oral cavity with the GI tract, the community compositions are more similar than with other niches in the body (Xiao et al. 2020). The number of species that inhabit the oral microbiome is estimated to be between 700-1,200 (Dewhirst et al. 2010). Some studies suggest that each individual person harbors a unique set of these species in their oral microbiome (Jenkinson 2011), whereas others provide evidence of a core microbiome which is constituted by a number of microbial species that most individuals possess in their oral microbiomes (Zaura et al. 2009).

Within the oral cavity, there are even smaller niches with specific environmental conditions in which some microbes are more suited to inhabit than others. There are 11 described microenvironments within the human oral cavity: the hard palate, tongue dorsum, saliva, palatine tonsils, throat, buccal mucosa, keratinized gingiva, supra-gingival plaque, dentures, and lips (Jia et al. 2018; Table 1). Each of these microenvironments are exposed to variable amounts of oxygen, saliva, food particles, and gingival crevicular fluid (GCF) and have distinct community composition (Jia et al. 2018; Palmer 2014; Table 1). In addition, the hard surfaces of the teeth do not shed regularly like the epithelium of the tongue, hard and soft palate, and buccal mucosa. Because of these wide variations in environmental conditions between the different sites within the oral cavity, there are sub-communities of microbes that are specific to each of these sites with unique compositions, both in terms of species richness and relative abundance (Jenkinson et al. 2011; Palmer 2014; Table 1). While there are between 700 - 1,200 species of oral microbial colonizers, only an estimated 10% of those are

present at any sub-site within the oral cavity (Kolenbrander et al. 2010). As mentioned before, a significant change in the composition of the microbiome can indicate a state of dysbiosis or disease. The same is true at the level of sub-communities within the oral microbiome, with disease states having a generally more complex collection of microbes (Bowen et al. 2018; Jenkinson 2011).

Table 1: The 11 microenvironments within the human oral cavity. Each of these microenvironments have varying environmental conditions such as substrate type and exposure to oxygen, which drives the community composition of bacterial species found in that environment.

Site	Substrate	Description	Prominent Species
Hard palate	shedding (mucosa)	aerobic, high exposure to saliva	*Streptococcus, Pasteurellaceae, Veillonella, Prevotella, Lactobacillales*
Tongue dorsum	shedding (mucosa)	papillate surface creates anaerobic pockets, high exposure to saliva	*Streptococcus, Veillonella, Prevotella, Pasteurellaceae, Actinomyces*
Saliva	fluid	high salivary turnover prevents stable colonization	*Prevotella, Streptococcus, Veillonella, Pasteurellaceae*
Palatine tonsils	shedding (mucosa)	aerobic, high exposure to saliva	*Streptococcus, Veillonella, Prevotella, Pasteurellaceae, Fusobacterium*
Throat	shedding (mucosa)	aerobic, frequent mechanical forces prevent biofilm formation	*Streptococcus, Veillonella, Prevotella, Pasteurellaceae, Actinomyces, Fusobacterium, Lactobacillales*
Buccal mucosa	shedding (mucosa)	aerobic, high exposure to saliva, high cell turnover prevents biofilm formation	*Streptococcus, Pasteurellaceae, Gemella*
Keratinized gingiva	shedding (mucosa)	aerobic, high exposure to saliva	*Streptococcus, Pasteurellaceae*
Supragingival plaque	shedding (mucosa)	crevice between dentures creates anaerobic pockets	*Streptococcus, Capnocytophaga, Corynebacterium, Pasteurellaceae, Neisseriaceae*
Subgingival plaque	shedding (mucosa)	crevice between gingiva and dentures creates anaerobic pockets	*Streptococcus, Fusobacterium, Capnocytophaga, Prevotella, Corynebacterium*
Dentures	solid	non-shedding surface allows for the development of biofilm (dental plaque)	*Staphylococcus epidermis, Streptococcus*
Lips	shedding (mucosa)	aerobic, exposure to saliva, high amounts of interface with external environment	*Streptococcus, Candida albicans*

The microbial communities within the oral cavity exhibit polymicrobial synergy, defined as the interactions between organisms that enhance fitness, to form a biofilm on the oral surfaces (Lamont et al. 2018). The creation of biofilm is one way to maintain stability within a dynamic environment, thus enhancing fitness. The fact that oral microbes live in communities with many inter- and intraspecies interactions may account for why only about 50% of oral microbiota can be cultivated in a lab, as many of

these species may rely upon each other for survival (Jenkinson 2011). Constituent species aid in increasing other species' fitness in order to form a more robust biofilm: pioneer colonizers like mitis group *Streptococci* and other Gram-positive bacteria adhere to previously uncolonized dental surfaces and form a linking film, which is the foundational structure that allows the adhesion of subsequent species in the process of biofilm formation (Lamont et al. 2018; Jenkinson 2011). Many of these early colonizers utilize their capabilities to adhere to and metabolize a wide variety of substrates within the oral cavity. It is thought that these species are successful in colonizing surfaces due to their ability to recognize different tissue types and prevent competition from other microbes (Nobbs et al. 2009). In addition, some commensal bacteria can inhibit the growth of opportunistic pathogens, such as the reactive oxygen-producing *Streptococci* that provide the substrates for an enzymatic pathway that produces the antimicrobial hydrogen peroxide (Wescombe et al. 2011).

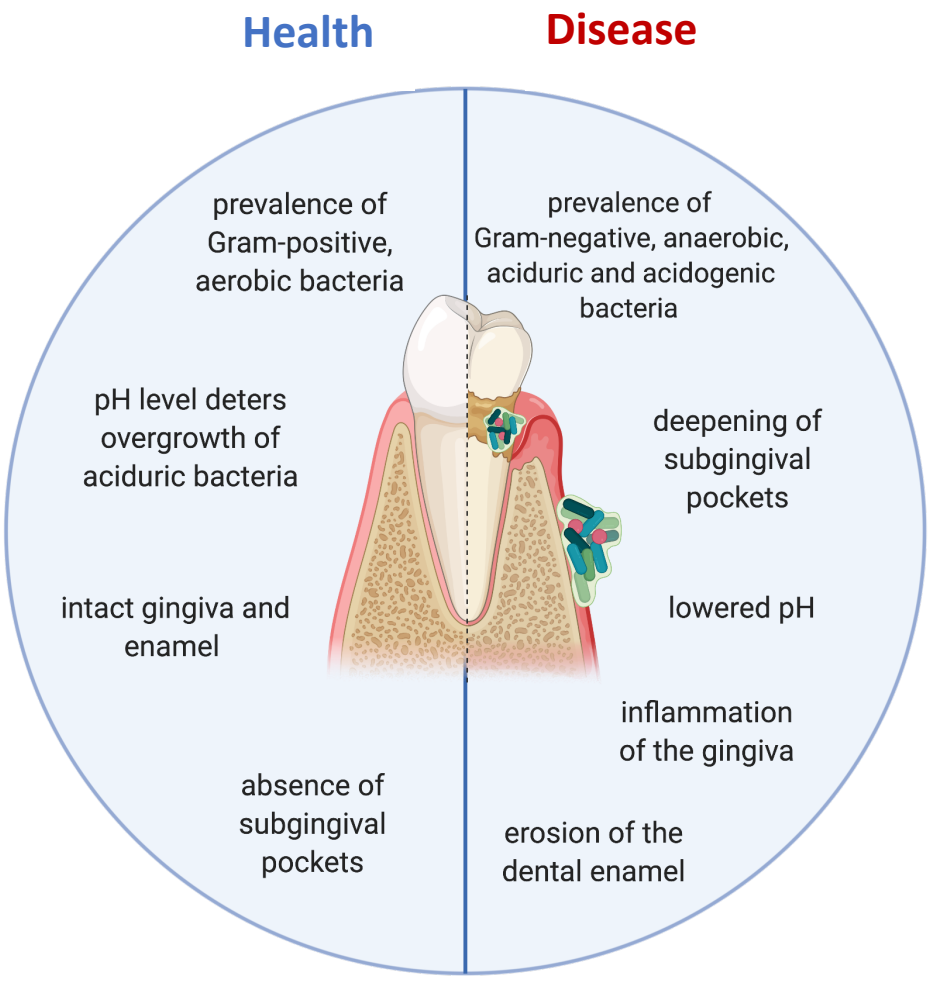

Figure 3: Healthy and disease stats of the oral cavity and microbiome. A healthy oral microbiome is characterized by an abundance of commensal bacteria and high pH, while a diseased oral microbiome is characterized by an overgrowth of pathogenic bacteria, low pH, erosion of the dental enamel and inflammation of the gingiva.

Conversely, opportunistic pathogens, such as the acidogenic (acid-producing) and aciduric (acid-tolerant) *Streptococcus mutans*, can interact synergistically with other opportunistic pathogens to form a dysbiotic biofilm by lowering the oxygen levels and producing enough acid to lower the pH of the microenvironment to a point at which commensal species cannot survive. This process is the general etiology of the most common oral diseases, such as gingivitis, dental caries, and periodontal disease (Figure 3: Lamont et al. 2018). Another example of symbiotic interaction between oral microbial

species is that of *Veillonella* species and *Streptococcus* species. *Streptococci* produce lactic acid as an end product of glycolysis, which *Veillonellae* consume. The *Veillonellae's* consumption of lactic acid may prevent end-product inhibition of glycolysis, leading to enhanced growth of the *Streptococci* (Jenkinson 2011). This shift to an increase in *Veillonellae* and acidogenic *Streptococci* species could have detrimental effects on the host's health, as the presence of these taxa is associated with dental caries (Jenkinson 2011). The balance between healthy and dysbiotic communities within the oral cavity can be shifted by a number of external factors, such as oral hygiene, diet, drug use, and immune function (Lamont et al. 2018; Figure 4).

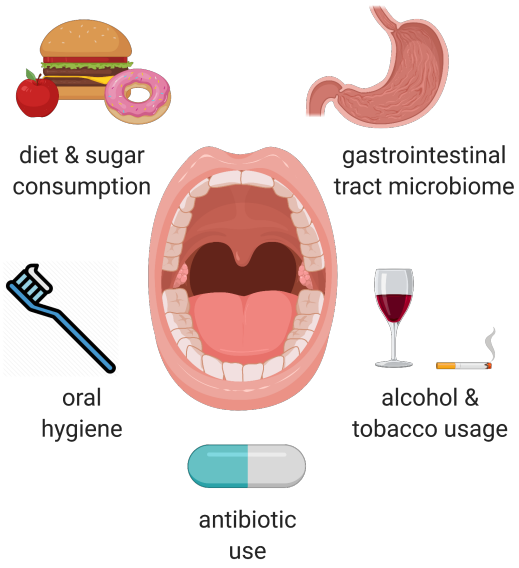

Figure 4: External and internal factors that influence the oral microbiome. Both external factors (i.e. diet, oral hygiene, alcohol and tobacco usage, and drug use) and internal factors (i.e. the gastrointestinal tract microbiome) affect the community structure of the oral microbiome.

The Early Oral Microbiome and Health

The acquisition and development of a healthy oral microbiome is important for a child's overall health, as several childhood diseases, including dental caries, periodontal

disease, and various cancers (i.e. oral, esophagus, pancreatic, and colorectal) and gastrointestinal diseases, are associated with a dysbiotic oral microbiome (Xiao et al. 2020). Early childhood caries is prevalent across the globe and is linked with overgrowth of the opportunistic pathogens *Streptococcus mutans, Streptococcus salivarius, Streptococcus sobrinus*, and others (Wade et al. 2013; Xiao et al. 2020). These species disrupt the normal balance of the oral microbiome by producing acids that lower the pH within the environment to a level that favors aciduric, pathogenic species. This triggers the overgrowth of pathogenic species such as *S. mutans* and the simultaneous inhibition of commensal species like *Streptococcus sanguinis* that are less tolerant to low pH conditions (Wade et al. 2013; Xiao et al. 2020). In fact, the composition of an infant's oral microbiome can also give insight into the future body weight of the child. Recently, Craig et al. (2018) reported that both oral microbial diversity and the *Firmicutes* to *Bacteriodetes* ratio may be able to predict weight gain during the first two years of life by demonstrating that infant growth curves were found to be negatively correlated with oral microbiome diversity and positively correlated with the *Firmicutes*-to-*Bacteroidetes* ratio (Craig et al. 2018). One of the most important findings from this study is that the oral microbiome composition was found to be a better predictor of weight gain in infants than gut microbiome composition (Craig et al. 2018). These findings demonstrate the importance of characterizing and understanding the causal links between oral microbiome composition and systemic diseases, as well as the potential for using the oral microbiome as an early diagnostic tool for various health conditions.

The state of the oral microbiome is also linked to disorders in more remote body regions, not only the oral cavity. One study illustrates how the oral microbiome is correlated with changes in the body on a systemic level: children diagnosed with obstructive sleep apnea syndrome (OSAS), a condition in which the airway is obstructed during sleep, showed significantly dissimilar oral microbiome composition compared to healthy children (Xu et al. 2018). This study also compared urinary metabolomes of both OSAS and healthy children and found that OSAS children had significantly different urinary metabolomes from healthy children (Xu et al. 2018). Another factor at play in childhood OSAS is body weight, as childhood obesity and OSAS are correlated (Narang & Mathew 2012). However, the exact causal links between oral microbiome composition, urinary metabolome, OSAS, and obesity are not yet known (Xu et al. 2018). Chronic inflammatory bowel disease (IBD) also has links to the oral microbiome. It is thought that IBD is caused by an abnormal response to the microbial symbionts by the immune system and is linked with inflammation of the oral mucosa. In a study on individuals with Crohn's disease, an IBD, children with Crohn's had a significantly less diverse oral microbiome than healthy children (Docktor et al. 2012). This study also found that shifts in the prevalence of certain taxa occurred in children with Crohn's disease, including an increase in *Spirochaetes, Synergistetes*, and *Bacteroidetes*, while a decrease in *Fusobacteria* and *Firmicutes* was observed (Docktor et al. 2012).

Aside from disease states of the body, the oral microbiome has been found to be implicated in childhood neuropsychological disorders as well. Autism spectrum disorder (ASD), in which the patient has impaired social communication and a restrictive set of

repetitive behaviors, is linked with an altered gut microbiome (Moradi et al. 2020). One study aimed to elucidate the connection between ASD and the oral microbiome by comparing the salivary microbiome of three groups of children: children with ASD, children with developmental delays, and typically developing children (Hicks et al. 2018). This study found that ASD children had significantly different oral microbiome composition compared to both children with developmental delays and typically developing children. These differences include an increase in prevalence of *Limnohabitans spp*. and *Planctomycetales sp*. and a decrease in prevalence of *Ramlibacter tataouinensis, Mucilaginibacter spp., Bacteroides vulgatus*, and *Gemmata spp.* compared to typically developing children. The current understanding of the shift in oral microbiome composition in children with ASD is that the altered gut microbiome, which may influence behavior via the microbial-gut-brain-axis, causes a change in the osopharynx and oral microbiome due to physical proximity (Hicks et al. 2018). This correlation is another example of how the ease of access to the oral microbiome could be utilized as an early indicator of various diseases and conditions in children. It is apparent that a state of dysbiosis within the oral microbiome can have effects on the physical and mental health of the human host; thus, it is of high importance to ensure the trajectory of the developing microbiome in children is towards a robust, balanced state.

Development of the Oral Microbiome over Time

Since the oral microbiome is such an integral aspect of human health, it is important to understand the process by which this community is acquired and changes over time. The process of acquiring the oral microbiome begins during gestation,

continuing through parturition and infancy, and is highly influenced by factors such as genetics, gestation length, delivery mode, and diet (Xiao et al. 2020). Because a core microbiome has not yet been established, the oral microbial community of an infant's mouth shifts rapidly in response to the aforementioned factors (Gilbert et al. 2018).

The infant's first exposure to microorganisms may be in the womb, as some recent studies have found that the placenta and amniotic fluid harbor their own microbial community (Al-Shehri et al. 2016). One study found that the placental microbiome was most similar to the oral microbiome and included *Prevotella, Streptococcus*, and *Veillonella* species (Gomez-Arango et al. 2017). These findings, however, are only a few from an understudied field and much more research is needed to provide evidence for the correlation. The mode of delivery influences the composition of the infant's microbiome, with vaginally delivered infants developing microbiomes that are more similar to their mother's vaginal microbiome which tend to be commensals, and infants delivered via cesarean section developing microbiomes more similar to that of their mothers' skin which tend to be opportunistic pathogens (Dominguez-Bello et al. 2016). The broad consensus indicates that mode of delivery has lasting impacts on the infant microbiome; however, it is important to note that, other studies have found that mode of delivery only has a limited timeframe of influence, with the effects of birth mode dissipating after the first month of life (Hurley et al. 2019).

Following birth, the infant is exposed to the multitude of microbes present within their environment. In fact, interaction with the environment is one of the primary methods of microbial inoculation (Figure 5; Xiao et al. 2020). The oral cavity is colonized in a sequential manner, first with pioneer colonizers attaching and enabling subsequent

colonizers to adhere and form a biofilm (Sulyanto et al. 2019). The most common pioneer colonizers of the oral cavity are Gram-positive bacteria including *Streptococci* species such as *S. mitis, S. epidermis,* and *S. salivarius*, because of their ability to adhere to previously uncolonized epithelial tissue and their presence in breastmilk. As time progresses, the overall amount and diversity of bacteria increases (Xiao et al. 2020). While the primary colonization of the oral surfaces is more thoroughly understood, the subsequent stages of oral microbiome development are more convoluted due to the greater number of interacting species (Jenkinson 2011). Since the later-stage microbes must interact with the primary colonizers for integration into the biofilm, the composition of the initial community plays a large role in determining the composition of the later stages of the oral microbiome (Jenkinson et al. 2011).

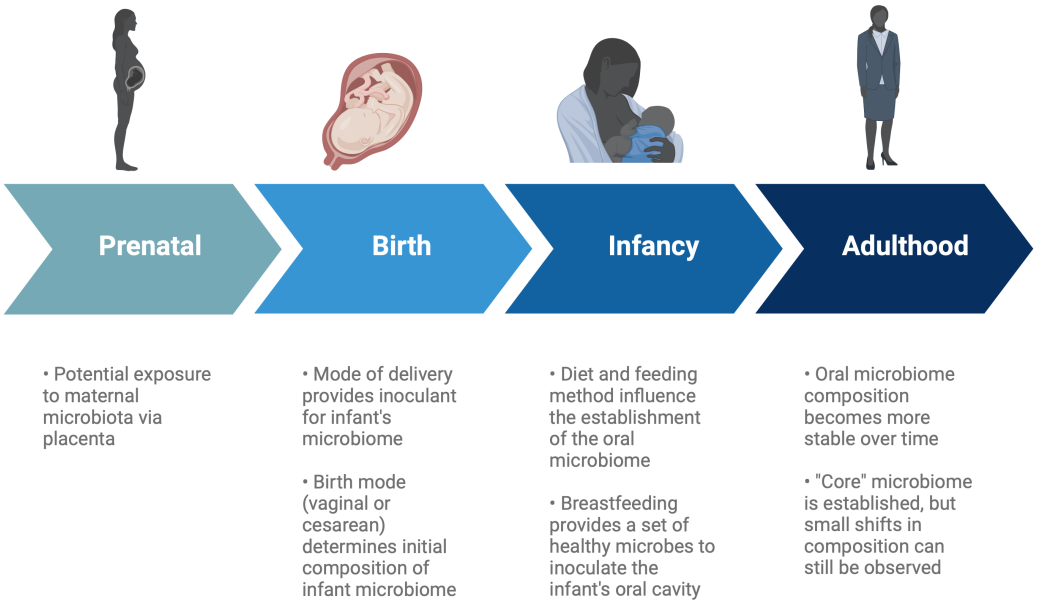

Figure 5: The development of the oral microbiome over time. The oral microbiome is established over the course of childhood and is shaped by factors such as mode of delivery at birth and feeding method.

A turning point in the development of the oral microbiome is the time of dental eruption, which both provides a new substrate for the microbial growth and coincides with the introduction of solid foods, which provides new nutrients for microbial growth. These changes create a new microenvironment for previously unintroduced bacteria to colonize (Sulyanto et al; Xiao et al. 2020). Following the eruption of the first tooth, the infant's oral microbiome shifts to hosting a higher level of Gram-negative facultative and lower level of Gram-positive microbes (Crielaard et al. 2011). Predentate oral microbiomes are dominated by *Escherichia coli, Pseudomonas spp*., and *Staphlyococcus spp*., while species such as *S. mutans* begin colonizing the dentate infant oral cavity at an increased rate, as the dental surfaces are their preferred colonization surface (Crielaard et al. 2011).

In addition to novel surfaces and nutrient availability, the development of the oral microbiome over time is affected by the specific site within the oral cavity, the health of the individual host (including the state of the immune system), and the age and sex of the individual (Jenkinson 2011). While the oral microbial community is widely variable before the first tooth eruption, community shifts become less transient with the establishment of the first set of teeth (Xiao et al. 2020; Jenkinson 2011). The wide range of factors influencing the development of the oral microbiome along with the implications of the oral microbiome and health assert the importance of early establishment of a healthy microbiome.

The Early Microbiome & Nutrition

Nutrition is an important factor that can influence the oral microbiome, and thus the health of the individual as a whole. During infancy, the primary source of nutrition,

and early microbial exposure, is either breastmilk or infant formula. Breastfeeding is widely considered to be the optimal nutrition source for infants (Al-Shehri et al. 2016; Timby et al. 2017). Formula-fed infants gain weight more rapidly than breast-fed infants and develop infections at higher rates (Al-Shehri et al. 2016). In addition, recent studies indicate that breastmilk and infant saliva interact to produce antimicrobial agents through an enzymatic pathway (Al-Shehri et al. 2015). Xanthine oxidase, an enzyme present in breastmilk, produces antimicrobial agents including hydrogen peroxide when exposed to the enzyme's substrates found in infant saliva. These antimicrobial agents inhibit the growth of opportunistic pathogens *in vitro* (Al-Shehri et al. 2015; Sweeney et al. 2018). For these reasons, organizations such as the World Health Organization and the American Academy of Pediatrics recommend breastfeeding as the primary nutrition source for the infants' first year of life (More et al. 2018).

However, there are many reasons why breastfeeding may not be a suitable dietary choice. For instance, preterm or low birth weight infants may need a more nutritionally dense food source such as infant formula to achieve a healthy weight (More et al. 2018). Similarly, infant formulae are desirable for infants suffering from malnutrition due to famine (More et al. 2018). HIV-positive mothers also rely on infant formulae to avoid transmission of the virus to the infant (More et al. 2018), and the advent of soy-based formulae in the 1920's provided a solution for lactose-intolerant infants (More et al. 2018).

Breastmilk is a complex fluid that provides not only nutrition, but immune factors and beneficial commensal bacteria, such as *Lactobacilli, Bifidobacteria*, and *Streptococci* (Holgerson et al. 2013). There is evidence that breastmilk contains factors

that can induce the proliferation of commensals such as *Lactobacillus* species while inhibiting growth of pathogens like *S. mutans* (Holgerson et al. 2013; Timby et al. 2017). This is not the case with infant formula, which is composed of a known nutritional profile and generally lacks immune factors and bacteria while having higher protein and calorie content than breast milk (Al-Shehri et al. 2016; Holgerson et al. 2013).

Infant formula utilizes either cow's milk, soy, amino acids, or goat's milk as a replacement for breastmilk. These commercially available powdered formulae are often supplemented with vitamins and minerals such as iron and vitamin D (Enfamil 2020). One study examined the effects of infant formula composition on the growth of the pathogen *S. mutans* and found that sucrose-based formulae yielded more growth of *S. mutans* when compared to formulae containing lactose (Hinds et al. 2016). Iron levels in the formulae also affected the growth of the pathogen, with lower iron levels having increased pathogen growth compared to formulae with high iron levels (Hinds et al. 2016).

Other infant formulae containing probiotics tout claims of boosting the microbiome, including *Lactobacilli* and *Bifidobacteria* in the formulations (Enfamil 2020). However, these claims have not been clinically supported, and the effects of these formulations on the composition of the oral microbiome has not yet been thoroughly investigated. Some studies have shown beneficial effects of probiotic supplementation, including an increase in oral microbiome diversity (Dassi et al. 2018), decrease in pathogenic growth and dental caries (Naase et al. 2001), and a decrease in symptoms of gastrointestinal conditions like gastroenteritis (Buyukeren at al. 2020) and

neuropsychological disorders like autism spectrum disorder (Shaaban et al. 2018) and anxiety (Bravo et al. 2011).

This study aims to elucidate the relationship between the nutritional profile of infant formulae with the composition of the oral microbiome. A commensal species, *S. mitis*, and a pathogenic species, *S. mutans*, were used to represent beneficial and detrimental *Streptococcus* species commonly found in the infant oral cavity (Figure 6). These species were grown on media containing one of two infant formulae: a cow's milk based formula without probiotic supplementation (Enfamil NeuroPro) and a cow's milk based formula with probiotics (Enfamil Nutramigen with Enflora LGG). These two infant formulae were chosen for their similarity in nutritional composition to elucidate the effect of probiotic supplementation on the growth of the commensal and pathogenic species. The overall growth of the two species was assessed by counting colony forming units (CFUs) to determine the cell density of the culture media at different time points throughout the duration of the experiment. The metabolic activity of acidogenic bacteria was measured by pH level of the culture media to determine the rate of converting sugars into lactic acid (Figure 6). Metabolic responses indicative of commensals thriving suggest that infant formula is beneficial for the establishment of a microbiome on a healthy trajectory, while the opposite is true with the opportunistic pathogens.

The potential for the composition of these infant formulae to affect the development of the oral microbial community demands further inquiry given the correlation between many childhood diseases and the oral microbiome. Data concerning these effects could aid parents seeking alternatives to breastmilk in

selecting an infant formula that will support the development of a balanced microbiome, and thus, an overall healthier child.

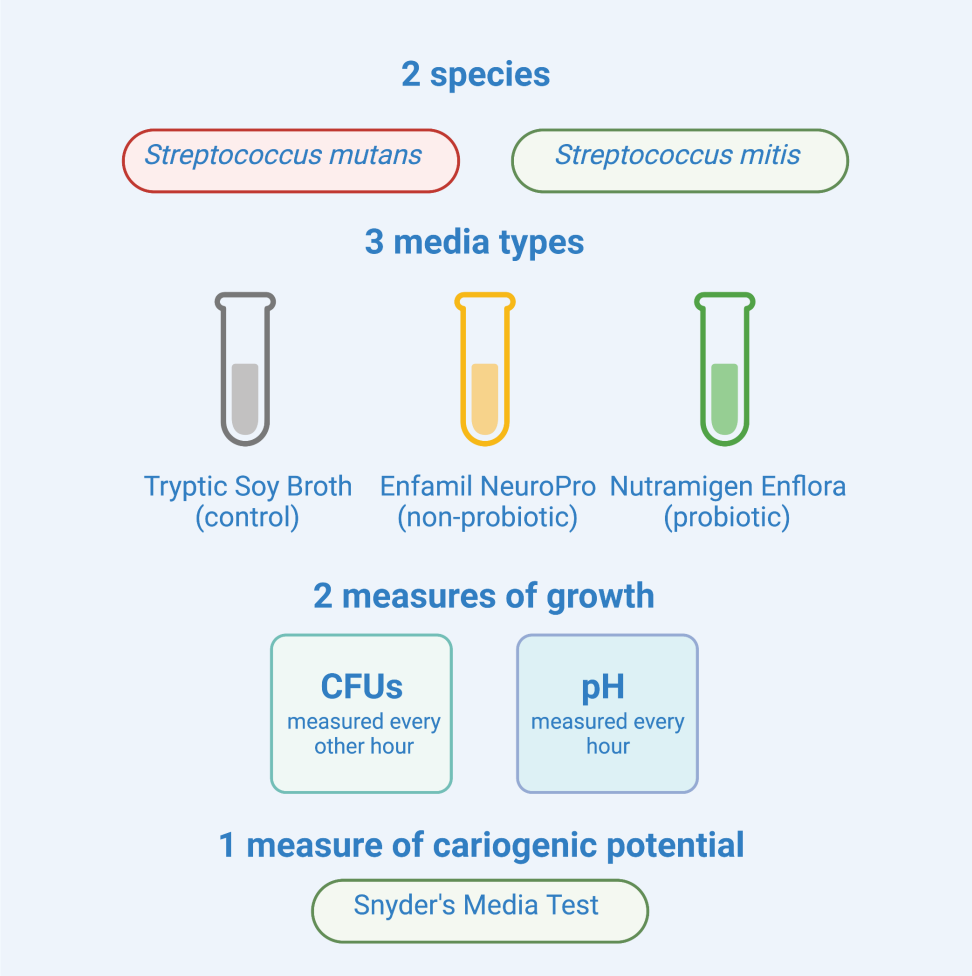

Figure 6: Experimental design of the study. One pathogen (S. mutans) and one commensal (S. mitis) were grown in a control media (Tryptic Soy Broth), a non-probiotic infant formula media (Enfamil NeuroPro), and a probiotic infant formula media (Nutramigen Enflora). Colony forming units and pH were used to assess bacterial growtn and Snyder's Media Test was used to assess cariogenic potential.

METHODS

Species Descriptions

Two species of *Streptococcus*-group bacteria were chosen for this study: one commensal (*S. mitis*) and one opportunistic pathogen (*S. mutans*) that are commonly

found within the infant oral cavity (Xiao et al. 2020). All cultures were sourced from the American Type Culture Collection (atcc.org). *S. mutans* type strain ATCC 25175 was selected for its origin from carious dentine. *S. mitis* type strain ATCC 49456 was the sole strain available from ATCC.

S. mutans (ATCC 25175) is a gram-positive facultative anaerobe that is known for its role in the development of dental caries, especially in infants and children (Hinds et al. 2016). Two characteristics of *S. mutans* that contributes to its pathogenicity are its acidogenity and acidurity: the ability to metabolize sugars (glucose, fructose, lactose, sucrose) to lactic acid and thrive in low pH conditions, respectively (Madigan et al. 2005). This increase in acidity resulting from *S. mutans'* metabolism erodes the dental enamel and leads to dental caries. Therefore, a decrease in pH is indicative of the growth of *S. mutans* and the dysbiotic state that can be associated with its growth (Madigan et al. 2005).

S. mitis (ATCC 49456) is a gram-positive commensal species of the oral cavity and is understood to be one of the primary colonizers of the oral cavity (Engen et al. 2017). Like *S. mutans*, *S. mitis* metabolizes carbohydrates such as lactose and sucrose into lactic acid (Kilian et al. 1989; Mashimo et al. 1985). *S. mitis* produces an enzyme known as neuraminidase in addition to a number of adhesins which may aid in the adherence and subsequent colonization of surfaces within the oral cavity (Kirchherr et al. 2005). The results from some studies suggest that *S. mitis* may supplement host immunity through modulating the expression of various immune markers (Zhang et al. 2008). Additionally, one study has shown that *S. mitis* induced the expression of an antimicrobial peptide that not only aids in deterring pathogenic microbes, but that *S.*

mitis itself is tolerant to (Eberhard et al. 2009; Nishimura et al. 2004). The production of antimicrobials that target pathogenic microbes in turn may boost host health by limiting the growth of pathogens.

A Snyder's media test (Figure 9) was used to determine the cariogenic potential of both species in the study. Snyder's media (Thomas Scientific, Swedesboro, NJ) contains a pH indicator that is used to qualitatively assess a decline in pH over time. A decline in pH reflects an increasingly acidic environment. A decline in pH is indicative of cariogenic potential as acid erodes dental enamel and causes dental caries. The more quickly the inoculated media changes color, the greater the cariogenic potential of the species. In this study, the color of the media for each species was compared after 24 hours of incubation. Both of the species were incubated at 37º C in Snyder's media in triplicate for 24 hours, then the color of the media was compared to uninoculated Snyder's media.

Growth Curves under Baseline Conditions

Growth curves were created for each species under baseline conditions in order to describe the general growth kinetics of both species. A growth curve outlines the growth of the culture, as measured by optical density by spectrophotometry, over time. An increase in optical density reflects the growth of bacterial cells: when the bacteria multiply, the culture becomes increasingly turbid due to higher concentration of cells. The turbidity is measured by the amount of light attenuated by the culture, which is referred to as optical density. The resulting graph shows the lag period of growth, in which growth slowly increases; the logarithmic phase of growth, in which the growth increases exponentially; and the plateau phase, in which the growth becomes steady as

the cell density reaches the carrying capacity of the media (Zweitering et al. 1990). The approximate time of the mid-log phase (i.e. the midpoint of the logarithmic phase) was needed for this study, as experimental cultures were to be inoculated with mid-log starting cultures because the mid-log phase is indicative of the fastest growth rate (i.e. maximum slope). The amount of time needed to reach the plateau phase for each species was used to determine the length of time for incubation during the experiment.

Growth curves were produced by measuring the optical density of the culture grown in the control media (Tryptic Soy Broth) in triplicate until the plateau phase of growth was reached (i.e. until growth became steady). For each species, three fresh cultures were inoculated in 10 mL TSB and allowed to reach turbidity (24 hours). Uninoculated media was placed in a cuvette to be used as a blank for the spectrophotometer (Vernier SpectroVis Plus). The batch culture was inoculated with a 1:20 dilution (Ashley Hawkins, personal communication) of turbid culture and media. The optical density of each batch culture was measured at 600 nm (Sonnleitner 2006) every 30 minutes for the first 4 hours, then every hour until a plateau in the growth curve was reached at ca. 8 hours for both species. These measurements were plotted against time to create growth curves (Figures 7 and 8) from which the mid-log and plateau phase could be determined.

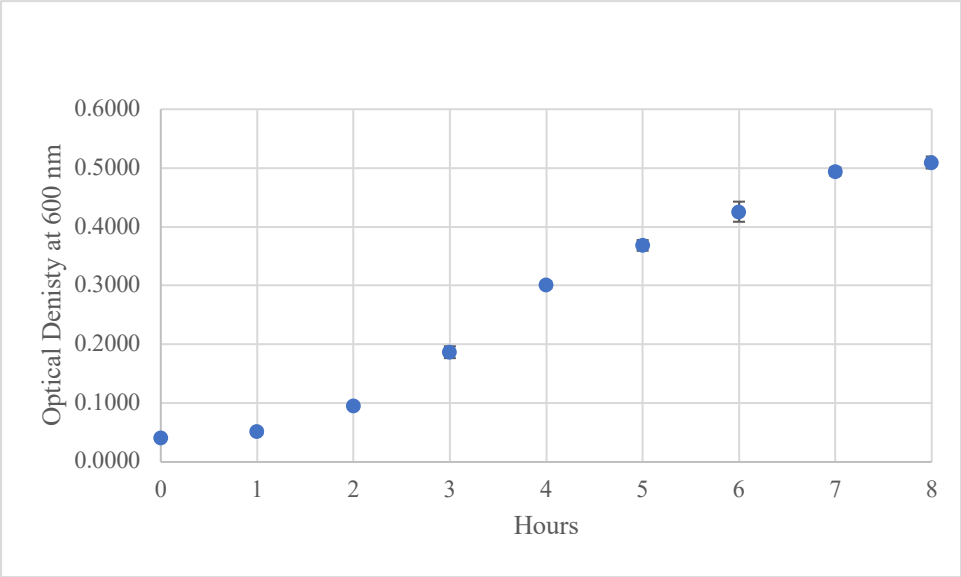

Figure 7: The growth curve of *S. mutans* under baseline conditions. Growth in TSB was quantified by optical density, measured by spectrophotometery, over 8 hours. Error bars represent standard deviation of the mean (n= 3).

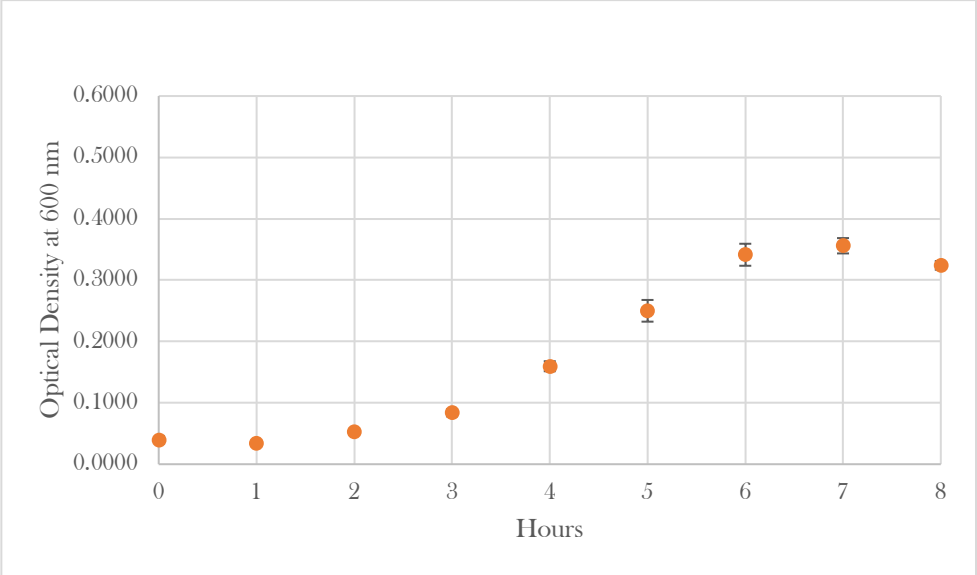

Figure 8: The growth curve of *S. mitis* under baseline conditions. Growth in TSB was quantified by optical density, measured by spectrophotometery, over 8 hours. Error bars represent standard deviation of the mean (n= 3).

Preparation of Culture Media

Once baseline growth curves were established, both species were maintained in tryptic soy broth (TSB) at 37°C. TSB was chosen as a control media because it was an

ATCC recommended growth media for each species. Two types of infant formula (Table 2) were compared in this study: a cow's milk-based formula (Enfamil NeuroPro) and a cow's milk-based formula supplemented with probiotics (Nutramigen Enflora). The Nutramigen Enflora formula is supplemented with *Lactobacillus rhamnosus*, one of the most widely used probiotic strains utilized as a dietary supplement (Isolauri et al. 2002). A 1:5 (v/v) dilution of infant formula to TSB without dextrose was prepared for each formula following Hinds et al. (2016). Infant formulae were diluted with TSB without dextrose to ensure bacterial growth without adding another carbohydrate source.

Table 2: Composition of infant formulae. The nutritional composition (per 100 kcal) of the non-probiotic (Enfamil NeuroPro) and probiotic (Nutramigen Enflora) infant formulae.

	CARBOHYDRATE	**FAT**	**PROTEIN**	**IRON**
ENFAMIL NEUROPRO	Lactose: 11.3 g	Palm olein, coconut, soy, and high oleic sunflower oils: 5.3 g	Nonfat milk, Whey protein: 2 g	1.8 mg
NUTRAMIGEN ENFLORA	Corn syrup solids: 10.3 g	Palm olein, coconut, soy, and high oleic sunflower oils: 5.3 g	Casein hydrolysate: 2.8 g	1.8 mg

Assessment of Cell Density under Experimental Conditions

For both species, cultures in TSB at the mid-log phase were prepared for the experiment in triplicate. Each of the infant formula media and the control media was inoculated with 100 µL of starting culture and vortexed to ensure homogeneity in replicates of three, for a total of 18 culture tubes including the control. Plate counts were performed by diluting the starting culture with fresh TSB to dilutions of 10^{-4}, 10^{-5}, and 10^{-6}. 100 µL of each of these dilutions were plated onto tryptic soy agar (TSA) plates in

replicates of three. The TSA plates were incubated at 37ºC for 24 hours, after which colony forming units were counted in plates that had between 30-300 colony forming units (CFUs). Only plates that had between 30-300 CFUs were counted, as plates with less than 30 CFUs are statistically unreliable and individual colonies cannot be distinguished on plates with more than 300 CFUs (Leboffe et al. 2016). The following formula was used to calculate the original cell density of the starting culture:

$$Original\ cell\ density = \frac{colony\ forming\ units}{original\ sample\ volume}$$

Assessment of Metabolic Activity under Experimental Conditions

Every hour over the course of each incubation, the pH of the culture media was measured to monitor the change in acid production using a YSI Ecosense pH 100A meter (YSI Inc., Yellow Springs, OH). The meter was calibrated according to manufacturer's instructions before use. A decrease in pH indicates an increase in acid production and the cariogenic potential within the culture media.

Statistical Analyses

Statistical analyses were conducted using JASP (Version 0.11.1, JASP Team 2019). Shapiro-Wilks tests were used to confirm a normal distribution of data. Normally distributed data were analyzed using independent t-tests; when assumptions for parametric statistics were violated, data were analyzed using the non-parametric Mann-Whitney test. Independent t-tests and Mann-Whitney tests were performed on pH data to compare the effect of each species on the pH of each media at a given time point. Analysis of Variance was used to assess the change in pH over time within the same media and species and to assess the change in pH between media types for a given species at a given point in time. Tukey's post-hoc tests were performed for all ANOVAs

with significant p-values (α = 0.05). The same tests were run on data reporting CFUs, except for time points where one of the three replicate data points were missing due to inadequate growth during plate counts. In these cases, mean values will used for data reporting, as there were not enough replicates at each time point to conduct statistical analyses. CFU and pH data were compared over time for observable trends between the two measures for each species in each media type. A linear regression was run to determine if there was a relationship between the mean CFUs and pH for both species in each media type. Data from hours 0, 4, and 8 were used as time points in the analysis because they represented the start-point, mid-log phase, and plateau phase of each incubation, respectively.

RESULTS

Snyder's Media Test

The degree of color change in the inoculated Snyder's Media was compared relative to the uninoculated test media (Figure 9b). The faster the color change of the media, the greater the acid production of the bacteria and thus higher the cariogenic potential. A species whose media turns orange after 24 hours of incubation has greater cariogenic potential than a species whose media only turns orange after 48 hours (Sherman et al. 2002). After 24 hours, a color change in the media inoculated with *S. mutans* (Figure 9a) indicated a positive result and a lack of color change in the media inoculated with *S. mitis* (Figure 9c) indicated a negative result.

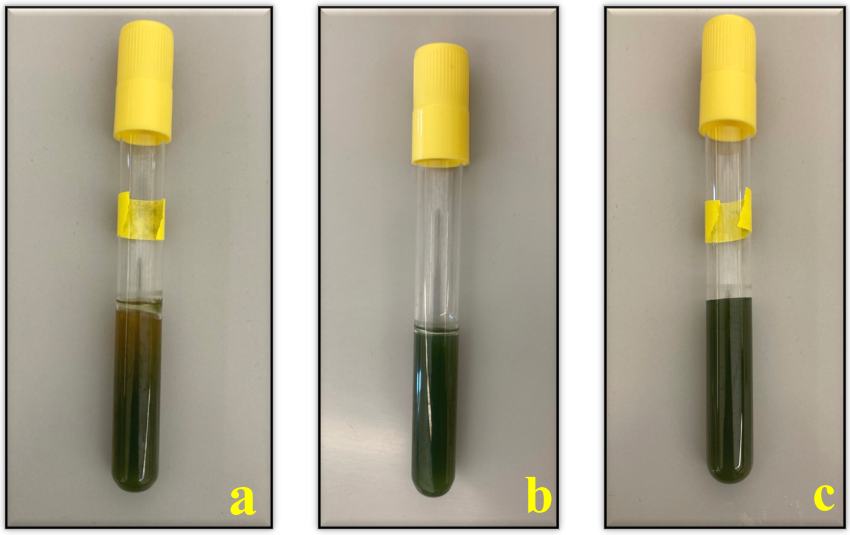

Figure 9: Results of Snyder's Media Test. A change in the color of the media from dark green to orange within 24 hours indicates a positive result. Cultures pictured are representative of all three replicates for each species: a) media inoculated with *S. mutans*, b) uninoculated media, c) media inoculated with *S. mitis.*

Colony Forming Units

The mean CFUs of *S. mutans* increased over time in TSB, with a ca. 10X increase between hour 0 and 8 (Table 3; Figure 10a). The mean CFUs of *S. mitis* decreased over time in TSB, with a ca. 16X decrease between hour 0 and 8 (Table 3, Figure 10a). The mean CFUs of *S. mutans* decreased over time in NeuroPro and Enflora, by ca. 0.18X and 6.8X, respectively. The mean CFUs of *S. mitis* increased over time in NeuroPro and Enflora, with an increase of ca. 3X and 2.1X respectively between hour 0 and 8 (Table 3, Figures 10b and 10c).

To compare the growth of each species in each media at a given timepoint, the mean values for CFUs for both species were compared within the same media types at 0, 4, and 8 hours. These three time points represent the start, midpoint, and end of the incubation, which are also significant points in the growth kinetics of these species as

shown by the growth curves (Figures 7 and 8), as they mark the start-point, mid-log phase, and end-point of the incubations.

In TSB, *S. mitis* was ca. 1.2X higher in CFUs than *S. mutans* at hour 0 and ca. 1.4X higher at hour 4 (Table 3, Figure 10a). At hour 8, *S. mutans* was ca. 141X higher in CFUs than *S. mitis*. At hour 0 in NeuroPro, *S. mitis* was ca. 108X higher in CFUs than *S. mutans*, ca. 36X higher at hour 4, and ca. 512X higher at hour 8 (Table 3, Figure 10b). In Enflora, *S. mitis* was ca. 61.5X higher in CFUs than *S. mutans* at hour 0, ca. 475X higher at hour 4, and ca. 992X higher in CFUs than *S. mutans* at hour 8 (Table 3, Figure 10c). At all time points in each media, *S. mitis* had greater CFUs than *S. mutans,* except at hour 8 in TSB.

Table 3: Mean CFU of *S. mutans* and *S. mitis* over time. Data points without standard deviation had insufficient replicates to calculate standard deviation.

Media	Hour 0	Hour 2	Hour 4	Hour 6	Hour 8
TSB	$4.31x10^6 \pm 6.58x10^6$	$2.91x10^6$	$3.50x10^6$	$1.69x10^7$	$4.23x10^7$
	$5.07x10^6 \pm 1.80x10^6$	$5.37x10^6 \pm 4.03x10^6$	$4.87 x10^6 \pm 2.51x10^6$	$1.52 x10^7$	$3.00x10^5 \pm 6.05x10^4$
NeuroPro	$2.40x10^5$	$4.02x10^5 \pm 4.37x10^5$	$1.16x10^6 \pm 1.59x10^6$	$9.15x10^4$	$2.03x10^5 \pm 1.02x10^5$
	$2.59x10^7 \pm 2.80x10^7$	$1.71x10^7 \pm 1.82x10^6$	$4.25x10^7 \pm 6.72x10^7$	$7.89x10^7 \pm 2.60x10^7$	$1.04x10^8 \pm 1.57x10^7$
Enflora	$3.90x10^5$	$1.82x10^5 \pm 1.80x10^5$	$9.30x10^4 \pm 1.31x10^4$	$6.80x10^4$	$5.03x10^4 \pm 4.81x10^4$
	$2.40x10^7 \pm 5.70x10^7$	$2.77x10^7 \pm 2.91x10^7$	$4.42x10^7 \pm 2.14x10^7$	$3.35x10^7 \pm 3.35x10^7$	$4.99x10^7 \pm 3.50x10^7$

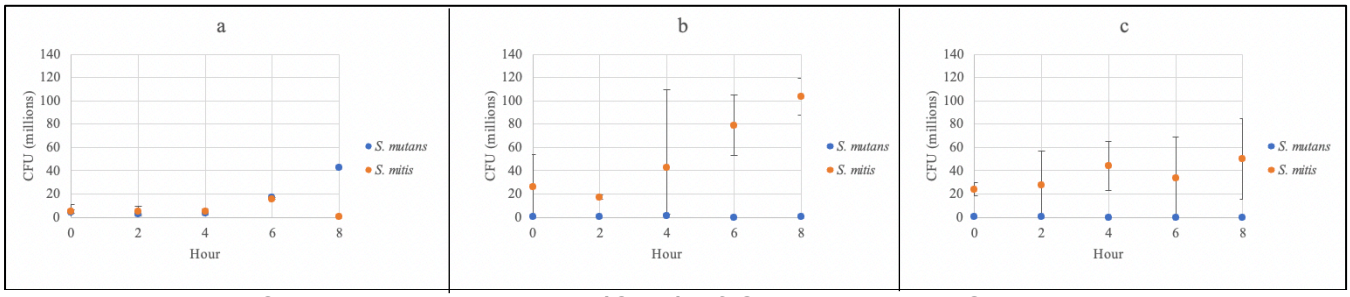

Figure 10: Mean Colony Forming Units (CFU) of *S. mutans* and *S. mitis* over time. a) in TSB, b) in NeuroPro, c) in Enflora. Error bars represent standard deviation of the mean.

To show which media yielded the highest cell density for each species at a given time, the mean values for CFUs in different media types were compared within a species (Table 3, Figure 11a). For *S. mutans* at hour 0, TSB yielded the highest CFU count of ca. 4.31 x 10^6 compared to ca. 2.40 x 10^5 in NeuroPro and ca. 3.90 x 10^5 in Enflora. For *S. mutans* at hour 4, TSB had the highest CFU count of ca. 3.50 x 10^6 compared to ca. 1.16 x 10^6 in NeuroPro and ca. 9.30 x 10^4 in Enflora. For *S. mutans* at hour 8, TSB had the highest CFU count of ca. 4.23 x 10^7 compared to ca. 2.03 x 10^5 in NeuroPro and ca. 5.03 x 10^4 in Enflora. At the end of the incubation, CFUs of *S. mutans* in TSB was 208X and 841X higher than NeuroPro and Enflora respectively. CFUs of *S. mutans* decreased ca. 15% in NeuroPro and ca. 87% in Enflora over the course of the incubation.

NeuroPro had the highest CFU count for *S. mitis* of ca. 2.59 x 10^7 compared to ca. 5.07 x 10^6 in TSB and ca. 2.40 x 10^7 in Enflora at hour 0 (Table 3, Figure 11b). At hour 4, Enflora had the highest CFU count of ca. 4.42 x 10^7 compared to ca. 4.87 x 10^6 in TSB and ca. 4.25 x 10^7 in NeuroPro. NeuroPro had the highest CFU count of ca. 1.04 x 10^8 compared to ca. 3.00 x 10^5 in TSB and ca. 4.99 x 10^7 in Enflora at hour 8. At the end of the incubation, CFUs of *S. mitis* in NeuroPro was 347X and 2X higher than TSB and Enflora respectively.

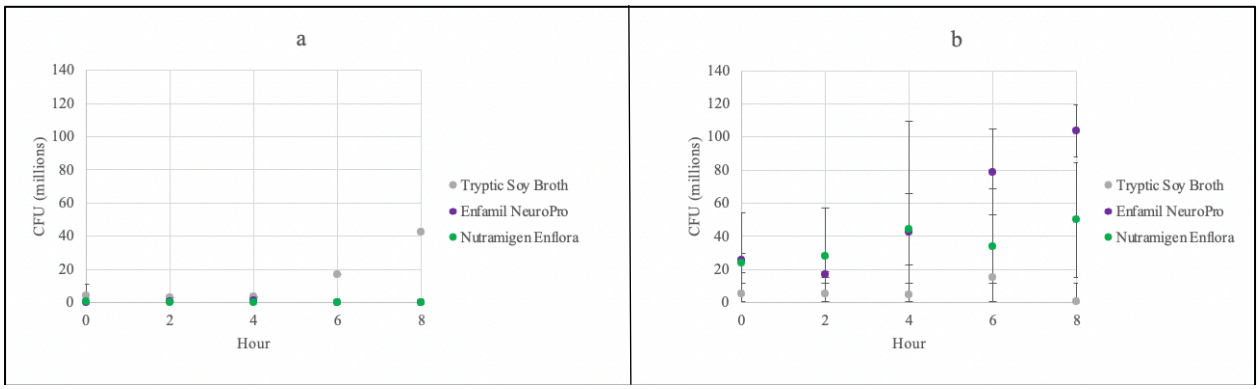

Figure 11: Mean Colony Forming Units (CFU) in of *S. mutans* (a) and *S. mitis* (b) in TSB, NeuroPro, and Enflora over time. Error bars represent standard deviation of the mean.

pH

The mean pH of *S. mitis* significantly decreased over time in all three media types (Table 4, Figure 12b). Between hour 0 and hour 8, the pH decreased by ca. 2.6% in TSB, ca. 1.9% in NeuroPro, and c.a. 1.2% in Enflora. The mean pH of *S. mutans* significantly decreased over time in TSB but increased slightly over time in NeuroPro and Enflora (Table 4, Figure 12a). Between hour 0 and hour 8, the pH decreased by ca. 13.2% in TSB and increased by ca. 0.3% and ca. 0.4% in NeuroPro and Enflora respectively.

Table 4: Mean pH values of *S. mutans* and *S. mitis* over time.

Media	Hour 0	Hour 1	Hour 2	Hour 3	Hour 4	Hour 5	Hour 6	Hour 7	Hour 8
TSB	7.28 ± 0.03	7.18 ± 0.03	7.18 ± 0.03	7.15 ± 0.01	7.11 ± 0.02	7.03 ± 0.02	6.88 ± 0.02	6.70 ± 0.04	6.32 ± 0.03
	7.15 ± 0.01	7.10 ± 0.01	7.10 ± 0.03	7.07 ± 0.01	7.08 ± 0.02	7.08 ± 0.02	7.05 ± 0.01	7.01 ± 0.01	6.96 ± 0.02
NeuroPro	6.6 ± 0.04	6.61 ± 0.01	6.60 ± 0.01	6.60 ± 0.02	6.61 ± 0.01	6.59 ± 0.01	6.62 ± 0.03	6.62 ± 0.03	6.62 ± 0.03
	6.69 ± 0.01	6.65 ± 0.02	6.63 ± 0.03	6.64 ± 0.03	6.64 ± 0.02	6.62 ± 0.02	6.63 ± 0.02	6.59 ± 0.03	6.56 ± 0.02
Enflora	6.69 ± 0.01	6.69 ± 0.01	6.68 ± 0.01	6.68 ± 0.01	6.70 ± 0.01	6.69 ± 0.01	6.72 ± 0.00	6.73 ± 0.01	6.72 ± 0.01
	6.74 ± 0.01	6.73 ± 0.00	6.73 ± 0.01	6.74 ± 0.02	6.72 ± 0.01	6.72 ± 0.00	6.72 ± 0.01	6.68 ± 0.02	6.66 ± 0.02

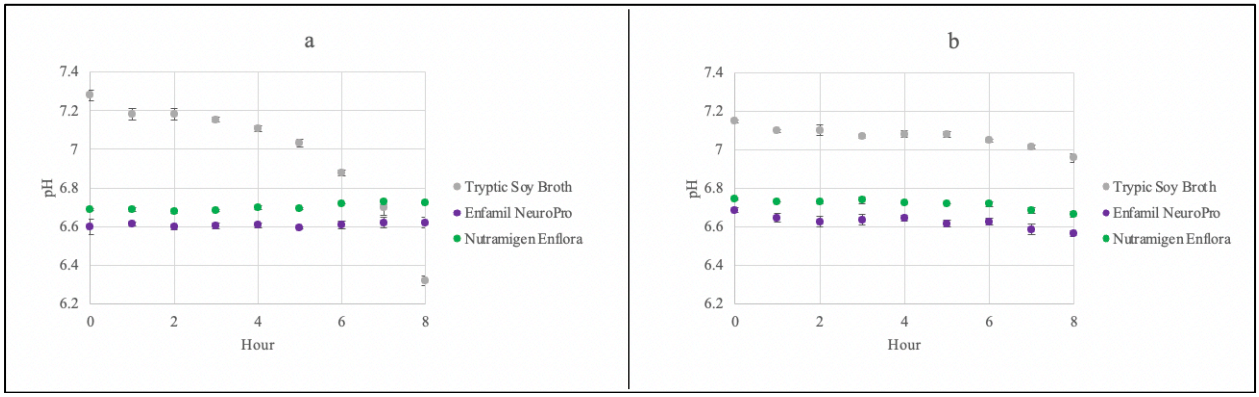

Figure 12: Mean pH values in each media type over time: a) *S. mutans,* b) *S. mitis.* Error bars represent standard deviation of the mean.

Results of the independent t-tests comparing pH levels of *S. mutans* to *S. mitis* showed a significant difference in each of the media types at least two time points (Table 5, Figure 12). None of the Mann-Whitney tests for non-normally distributed data were significant (Table 6). At hour 0 and 6, there was no significant difference in pH between the two species when cultured in all three of the media types (Table 5, 6). At hour 4, there was a significant difference in pH between the two species when cultured in NeuroPro and Enflora, but not TSB. In NeuroPro and Enflora, *S. mutans* had a lower pH than *S. mitis.* The pH levels of *S. mutans* and *S. mitis* were not significantly different from each other when cultured in TSB at hour 4. At hour 8, there was a significant difference in pH between the two species when cultured in all three of the media types. In NeuroPro and Enflora, *S. mutans* had a higher pH than *S. mitis.* In TSB, *S. mutans* had a lower pH than *S. mitis.*

Table 5: **P-values from the independent t-tests comparing the pH of media between cultures inoculated with *S. mutans* and *S. mitis* at different intervals throughout the incubation**. Non-normally distributed data were not included (denoted by nn). pH was the same for each replicate in Enflora at hour 6 (denoted by var= 0).

Media	Hour 0	Hour 2	Hour 4	Hour 6	Hour 8
TSB	nn	0.026	nn	nn	<0.001
NeuroPro	nn	nn	0.029	0.315	0.033
Enflora	nn	nn	nn	var=0	0.003

Table 6: P-values from Mann-Whitney test for non-normally distributed data comparing the pH of media between cultures inoculated with *S. mutans* and *S. mitis* at different intervals throughout the incubation. Normally distributed data was not included (denoted by nd).

Media	Hour 0	Hour 2	Hour 4	Hour 6	Hour 8
TSB	0.077	nd	0.164	0.077	nd
NeuroPro	0.077	0.184	nd	nd	nd
Enflora	0.072	0.077	0.077	nd	nd

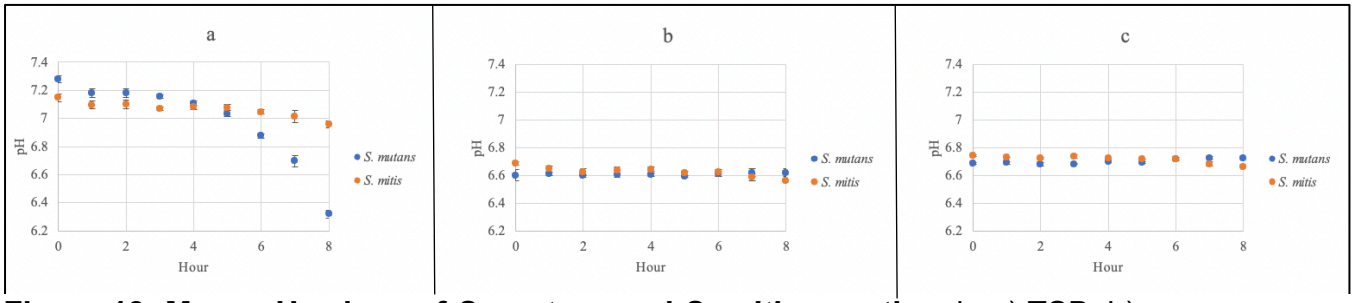

Figure 13: Mean pH values of *S. mutans* and *S. mitis* over time in a) TSB, b) NeuroPro, and c) Enflora. Error bars represent standard deviation of the mean.

The results of the ANOVAs comparing the pH levels over the course of the incubation were significant ($p < 0.001$) for both species in all three media (Tables 6, 7, and 8; Figure 13), except for *S. mutans* in NeuroPro ($p = 0.753$; Table 7). For *S. mutans* and *S. mitis,* there was a significant decline in the pH levels at 0, 4, and 8 hours when cultured in TSB. In NeuroPro, the pH levels at hours 0 and 8 significantly declined for *S. mitis.* In Enflora, the pH levels at hours 0 and 8 were significantly different in *S. mutans* and *S. mitis,* with an increase in pH for *S. mutans* and a decrease in pH for *S. mitis.* For *S. mutans,* the pH increased by ca. 0.3% and 0.4% in NeuroPro and Enflora, respectively, and dropped by ca. 15% in TSB. For *S. mitis,* the pH decreased by ca. 2.7%, 2%, and 1% in TSB, NeuroPro, and Enflora, respectively.

Table 6: Post-hoc p-values of an ANOVA comparing the pH of TSB between time points throughout the incubation. Reported post-hoc p-values from an ANOVA comparing the pH of *S. mutans* and *S. mitis* in TSB over time indicate if the difference in pH between time points is significant for a given species.

Hour	0	1	2	3	4	5	6	7	8
0		0.015	0.026	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
1	0.004		1	0.45	0.895	0.771	0.015	<0.001	<0.001
2	0.004	1		0.31	0.771	0.612	0.008	<0.001	<0.001
3	<0.001	0.925	0.925		0.995	1	0.612	0.005	<0.001
4	<0.001	0.049	0.049	0.423		1	0.202	<0.001	<0.001
5	<0.001	<0.001	<0.001	<0.001	0.049		0.31	0.002	<0.001
6	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		0.202	<0.001
7	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		0.005
8	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	

Table 7: Post-hoc p-values of an ANOVA comparing the pH of NeuroPro between time points throughout the incubation. Reported post-hoc p-values from an ANOVA comparing the pH of *S. mutans* and *S. mitis* in NeuroPro over time indicate if the difference in pH between time points is significant for a given species

Hour	0	1	2	3	4	5	6	7	8
0		0.285	0.029	0.098	0.204	0.008	0.029	<0.001	<0.001
1	0.996		0.931	0.999	1	0.628	0.931	0.29	0.001
2	1	0.985		0.999	0.975	0.999	1	0.285	0.019
3	1	1	1		1	0.931	0.999	0.098	0.005
4	1	1	1	1		0.75	0.975	0.044	0.002
5	1	0.957	1	1	0.996		0.999	0.628	0.066
6	1	1	0.996	1	1	0.985		0.285	0.019
7	0.957	1	0.904	0.985	0.996	0.825	1		0.855
8	0.957	1	0.904	0.985	0.996	0.825	1	1	

Table 8: Post-hoc p-values of an ANOVA comparing the pH of Enfamil between time points throughout the incubation. Reported post-hoc p-values from an ANOVA comparing the pH of *S. mutans* and *S. mitis* in Enflora over time indicate if the difference in pH between time points is significant for a given species.

Hour	0	1	2	3	4	5	6	7	8
0		0.791	0.559	0.995	0.337	0.18	0.088	<0.001	<0.001
1	1		1	0.995	0.995	0.946	0.791	<0.001	<0.001
2	0.977	0.821		0.946	1	0.995	0.946	0.002	<0.001
3	1	0.977	1		0.791	0.559	0.337	<0.001	<0.001
4	0.527	0.821	0.11	0.263		1	0.995	0.003	<0.001
5	0.977	1	0.527	0.821	0.977		1	0.008	<0.001
6	0.002	0.005	<0.001	<0.001	0.11	0.015		0.018	<0.001
7	<0.001	<0.001	<0.001	<0.001	0.015	0.002	0.977		0.337
8	<0.001	0.002	<0.001	<0.001	0.041	0.005	1	1	

Relationship between CFUs and pH

For the species and media that resulted in increased bacterial growth over time, there was a trend between CFUs and pH over time. For *S. mutans* grown in TSB

(Figure 14), *S. mitis* grown in NeuroPro (Figure 15), and *S. mitis* grown in Enflora (Figure 16), the general trend is as the CFUs increase, the pH also decreases. The strength of this relationship differed between conditions, with a stronger relationship between CFUs and pH in optimal growing conditions (Figure 14 & 15) compared to less optimal growing conditions (Figure 16) as visualized in the following figures. For the two conditions with the highest growth, *S. mutans* in TSB (Figure 14) and *S. mitis* in NeuroPro (Figure 15), the inflection point where CFUs increase sharply and pH decreases sharply was around the 4.5 hour mark (ca. mid-log phase). For all other conditions, a similar inflection point in the slopes of the CFU and pH graphs was not observed (Figure 16). All other conditions (where bacterial growth was suboptimal) showed a weak relationship between CFUs and pH, so those graphs were not included.

The linear regression between the mean CFUs and pH for both species in each media showed that there was a significant relationship between CFUs and pH for *S. mutans* in TSB (Figure 14) with an R^2 value of 0.968, but not any other species or media type. The other two conditions that showed a similar trend were *S. mitis* grown in NeuroPro and Enflora. For *S. mitis* grown in NeuroPro, $p= 0.122$ and $R^2= 0.605$ (Figure 15) , while *S. mitis* grown in Enflora had a $p= 0.070$ and $R^2= 0.623$ (Figure 16). For the remaining conditions, $p> 0.300$ and $R^2< 0.500$. *S. mutans* grown in NeuroPro had a $p= 0.983$ and $R^2= 0.000$ and in Enflora had a $p= 0.213$ and $R^2= 0.453$. For *S. mitis* grown in TSB, $p= 0.721$ and $R^2= 0.049$. The results of the linear regressions reflect the trends outlined above, with the high-growth conditions (*S. mutans* in TSB, *S. mitis* in NeuroPro and Enflora) having a stronger relationship between CFUs and pH than the low-growth conditions (*S. mutans* in NeuroPro and Enflora, *S. mitis* in TSB).

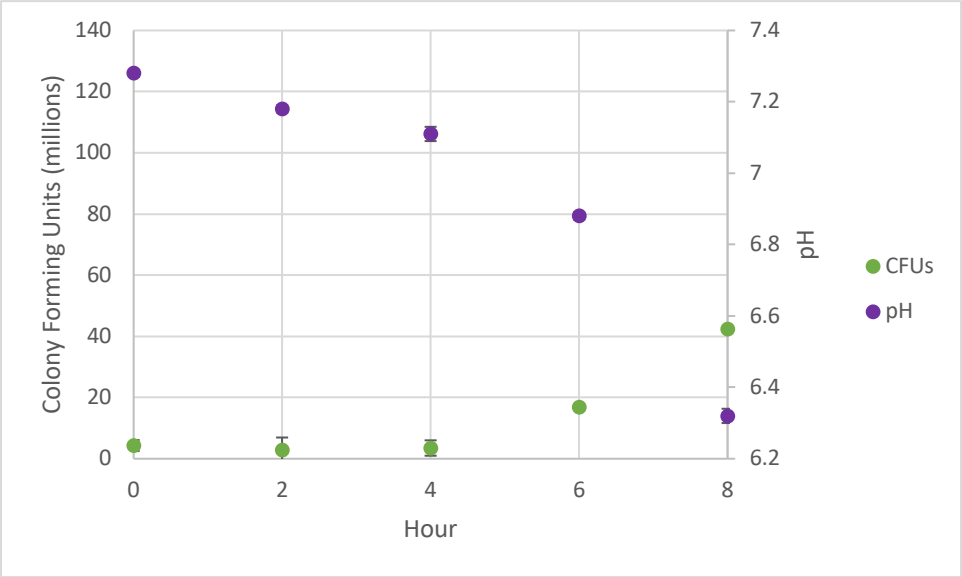

Figure 14: Relationship between CFUs and pH for *S. mutans* grown in Tryptic Soy Broth. Error bars represent standard deviation.

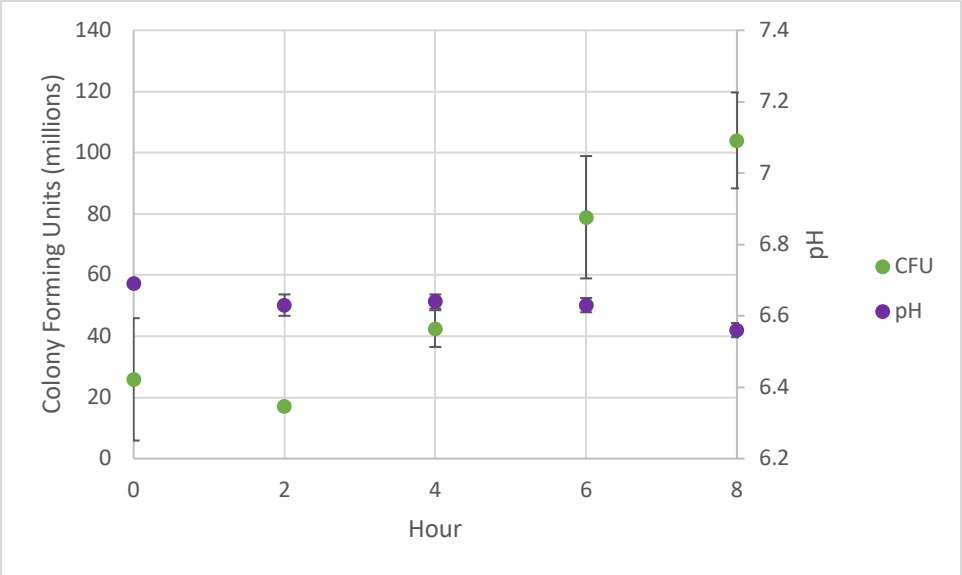

Figure 15: Relationship between CFUs and pH of *S. mitis* grown in NeuroPro. Error bars represent standard deviation.

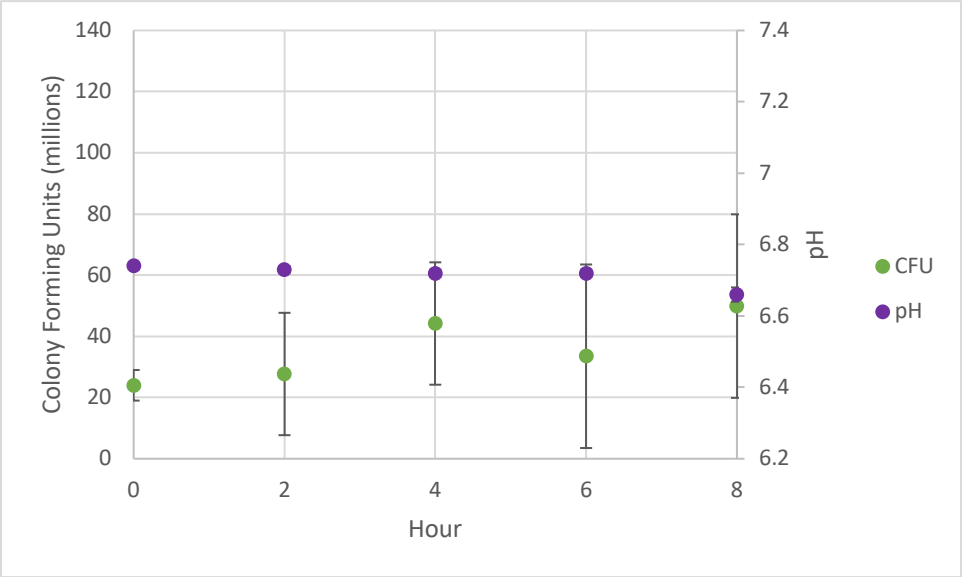

Figure 16: Relationship between CFUs and pH of *S. mitis* grown in Enflora. Error bars represent standard deviation.

DISCUSSION

The results of this study suggest that both the non-probiotic NeuroPro infant formula and the probiotic Enflora infant formula preferentially select for the commensal *S. mitis* over the pathogenic *S. mutans.* Additionally, the presence of a probiotic such as *L. rhamnosus* may help inhibit the growth of other oral bacteria, especially pathogenic species such as *S. mutans,* since the CFUs of the pathogen decreased by ca. 87% over time in the probiotic formula compared to ca. 15% in the non-probiotic formula. The probiotic's inhibition of potential pathogenic growth may influence the community of the infant oral microbiome in a beneficial manner by shifting the community structure away from acidogenic pathogens such as *S. mutans* and towards commensals such as *S. mitis* and *L. rhamnosus.* Although the probiotic formula did control the growth of *S. mitis,* this may actually benefit the overall development of the oral microbiome, since high species diversity is a hallmark of a healthy oral microbiome (Craig et al. 2018). By competing with *S. mitis* and preventing it from dominating the oral microbiome, *L.*

rhamnosus may aid in maintaining a more diverse species composition of the community.

Snyder's Media Test

The results of the Snyder's media test showed that *S. mutans* has a greater cariogenic potential than *S. mitis*. This reaffirms that *S. mutans* is an acidogenic and aciduric species. *S. mutans* produces lactic acid (Madigan et al. 2005) which lowers the pH of the surrounding microenvironment (i.e. culture media or oral cavity), limits the growth of non-aciduric species, which are commonly commensal species (Jenkinson 2011), and produces conditions that favor the growth of other aciduric pathogens (Lamont et al. 2018). In addition, the results of the Snyder's test showed that *S. mitis* does not have a high cariogenic potential. Although *S. mitis* is known to metabolize carbohydrates to lactic acid like the pathogenic *S. mutans* (Kilian et al. 1989), *S. mitis* is generally considered to act as a commensal species when its growth is kept in check by competing commensals (Mitchell 2011). To ensure the establishment of a beneficial microbial community early in life, it is important for infant formulae to have a nutritional composition that both inhibits the growth of pathogens like the cariogenic *S. mutans* and enhances the growth of commensals such as *S. mitis.*

Cell Density under Experimental Conditions

Because *S. mutans* did not show as robust increase in CFUs in the infant formulae as *S. mitis*, it appears that the infant formulae did not provide as optimal growing conditions for *S. mutans* as they did for *S. mitis*. *S. mutans* had ca. 141X more CFUs in TSB than *S. mitis* at the end of the 8 hour incubation, therefore TSB favored the growth of the pathogenic *S. mutans* over *S. mitis*. The decrease in CFU counts of *S.*

mutans over time when cultured in infant formulae suggests that the infant formulae have an inhibitory effect on the growth of this pathogen. This may be in part due to the different types and concentrations of carbohydrates found in the infant formulae and TSB. TSB contains 0.025 g/ml of dextrose while NeuroPro contains 0.015 g/ml of lactose and Enflora contains 0.014 g/ml of dextrose. The higher concentration of dextrose in TSB may have allowed for greater proliferation of *S. mutans* compared to the infant formulae.

The small difference between the CFU count of *S. mutans* in NeuroPro versus Enflora at the end of the incubation (CFUs for NeuroPro was 4X that of Enflora compared to CFUs for TSB being 208X and 841X higher than NeuroPro and Enflora, respectively) is likely not due to the different carbohydrates found in each formula, since the dextrose in Enflora is preferred to the lactose in Neuropro (Move et al. 2014). The magnitude of decrease in CFUs of *S. mutans* was greater in Enflora (ca. 6.8X decrease) than in NeuroPro (ca. 0.18X decrease) over the course of the incubation; therefore, Enflora had a greater inhibitory effect on the growth of *S. mutans* than NeuroPro. Since the concentrations of carbohydrates are similar between NeuroPro and Enflora and previous research shows that *S. mutans* does not prefer lactose over dextrose, it may be the presence of the probiotic *L. rhamnosus* that caused this differential magnitude of decrease in CFUs between the two infant formulae.

The CFU count for *S. mitis* was higher when grown in the infant formulae than when grown in TSB, indicating that the infant formulae have better conditions for supporting growth of *S. mitis* than TSB. Since *S. mutans* is known for its rapid consumption of carbohydrates (Hinds et al. 2016; Lemos et al. 2019), the lower

concentration of carbohydrates in the infant formulae, as compared to TSB, may have limited its growth. The growth of the commensal *S. mitis*, on the other hand, was not as severely limited by the infant formulae and fared better in the infant formulae than in TSB. The reasons for why *S. mitis* grew better in the infant formulae than *S. mutans* may be tied to *S. mitis'* identity as a commensal species.

While some of *S. mitis'* attributes that make it a commensal are well understood, (i.e. the secretion of proteins that aid in adherence to the dental surface, antimicrobial substances that target pathogenic bacteria, and proteins that protect itself from destruction from the human immune system), the entire story of how *S. mitis* remains a commensal species within the human oral microbiome is not fully known (Engen et al. 2017). However, the results of this study suggest that there are factors independent of adherence to surfaces, competition with other microbes, and interaction with the human host that confer the ability to thrive in an environment that inhibits the growth of pathogens such as *S. mutans*. These innate factors of *S. mitis* may be qualities of metabolism or resistance to environmental pressures, like the observed ability to thrive in lower concentrations of carbohydrates than *S. mutans,* that makes the species better suited to grow in the infant formula media. *S. mitis'* ability to outcompete *S. mutans* was not directly tested in this study; therefore, additional co-culture studies investigating the interactions between *S. mutans* and *S. mitis* are needed to elucidate these factors and their mechanisms.

One hypothesis for why the probiotic formula controlled the growth of both species is that the probiotic *L. rhamnosus* prevented uncontrolled growth by competing with the other species for resources as previous research has shown that

supplementation with *L. rhamnosus* resulted in a decrease in dental caries and amount of *S. mutans* in children (ages 1-6 years), indicating that the probiotic may have the ability to outcompete the pathogen and significantly inhibit its growth and acid production (Naase et al. 2001). This same degree of interspecies competition has not been shown between *L. rhamnosus* and *S. mitis*, perhaps because commensal species both prefer similar environmental conditions (i.e. neutral pH as opposed to the low pH that *S. mutans* produces and thrives in) and do not threaten each other by altering the environment to a state that would inhibit the other species' growth. This is good news in terms of how the infant formula impacts the community structure of the oral microbiome, because it indicates that the probiotic infant formula can potentially control both the overall growth of microbes (i.e. it controlled the growth of both the commensal and pathogen) and differentially (selectively) control the growth of the commensal and pathogen, with the commensal being favored and the pathogen being more strongly inhibited.

The decreased amount of *Streptococcus* growth may also have been influenced by the antimicrobial substances produced by *L. rhamnosus*. Existing literature shows that *L. rhamnosus* has multiple avenues of controlling microbial growth in co-culture, including the production of various antimicrobial substances along with the aforementioned competitive exclusion. *L. rhamnosus* produces microcine, a small antimicrobial peptide along with another 7 different antibacterial peptides (Lu et al. 2009). *L. rhamnosus* also produces two kinds of lectin proteins (antimicrobial molecules that target pathogenic microbes) which have been shown to successfully inhibit the growth and biofilm formation of *Salmonella* species and *E. coli* (Petrova et al. 2016).

These lectin proteins have strong carbohydrate-recognition capabilities, which allows them to distinguish pathogenic microorganisms from nonpathogens based on the types and configurations of polysaccharides on the cell surfaces (Petrova et al. 2016). The specificity of these probiotic lectin proteins for pathogenic microbes may explain why *L. rhamnosus* differentially affected the growth of *S. mutans* and *S. mitis*. These pathogen-specific antimicrobial proteins may have targeted the pathogenic *S. mutans* and exhibited a greater inhibitory effect on the pathogen compared to the commensal *S. mitis*. Competitive exclusion and the production of antimicrobial compounds present potential explanations for how the probiotic suppressed growth of the two *Streptococcus* species in this study.

L. rhamnosus' competition with *S. mitis* may also enhance host health by preventing the overgrowth of *S. mitis.* While *S. mitis* is generally considered a commensal in the oral microbiome, some studies have shown that an overabundance may cause the species to act as an opportunistic pathogen (Mitchell 2011). As previously mentioned, *S. mitis* can produce lactic acid like *S. mutans*, which in excess may lead to the formation of dental caries, giving it the potential to act as an opportunistic pathogen (Abranches et al. 2019). In a study comparing the species composition of oral microbiomes of individuals with and without dental fluorosis, a dental disease associated with dental caries, individuals with dental fluorosis had a higher abundance of *S. mitis* than those without dental fluorosis (Wang et al. 2021). Therefore, it is understood that while *S. mitis* typically acts as a commensal when kept in check by the coinhabitants of the oral microbial community, the overgrowth of *S. mitis* may lead to decreased health of the oral microbiome. By competing with *S. mitis* and preventing the

domination of the oral microbiome, *L. rhamnosus* may bolster host health by maintaining balance within the oral microbiome.

Low species diversity of the oral microbiome is associated with adverse health outcomes including dental caries (Simon-Soro et al. 2013; Xiao et al. 2020). Therefore, it is important to maintain a balanced community in which the growth of each constituent species is kept in check by interspecies competition. Despite their lower prevalence in the oral microbiome, the presence of non-cariogenic probiotics (Naase et al.2001), such as the *Lactobacilli* included in this study, are viewed as an indicator of health (Dassi et al. 2018). For example, healthy individuals tend to have a greater abundance of *Lactobacilli* than obese individuals (Yang et al. 2019), and *L. rhamnosus* supplementation specifically has been shown to prevent antibiotic-associated diarrhea; lessen symptoms of acute gastroenteritis; and prevent gastrointestinal colonization of vancomycin-resistant enterococci (Buyukeren et al. 2020; Szajewska & Hosjak 2020). *L. rhamnosus* supplementation has also shown potential for mitigating neuropsychiatric disorders including anxiety and autism spectrum disorder (Sherwin et al. 2019). One study found that mice supplemented with the probiotic experienced a decrease in stress hormones and anxiety (Bravo et al. 2011), and another study found that supplementation of a mixture of probiotics including *L. rhamnosus* improved symptoms of autism spectrum disorder in children (Shaaban et al. 2018).

Studies on the effects of probiotic consumption on the composition of the oral microbiome show promise for boosting host health. In a study comparing the oral microbiome composition of adults administered dietary *Lactobacillus* and *Streptococcus* probiotics and a control group, it was found that there was a short-term increase of

overall diversity of the oral microbiome (Dassi et al. 2018). However, the species diversity reverted to baseline (pre-treatment) levels after discontinuing probiotic intake. Since the infant oral microbiome is not as firmly established as that in adults (Xiao et al. 2020) and infants would be consuming the probiotic at every meal for presumable the entirety of infancy, the effects of consuming probiotic infant formula may have longer lasting effects than what was shown in this study.

In addition, it was shown that the *Lactobacillus* probiotic had greater effect on the diversity of the oral microbiome than the *Streptococcus* probiotic (Dassi et al. 2018). The authors of this study believe this may be due to the lower prevalence of *Lactobacilli* in the oral microbiome compared to *Streptococci,* with the relative rarity of *Lactobacilli* imparting a stronger influence on the composition of the oral microbial community (Dassi et al. 2018). Species that are rarer in a community have a greater impact on that community, as those rare species may confer functions that the more prevalent species lack (Jousset et al. 2017). Since *Lactobacilli,* like *L. rhamnosus,* are naturally less prevalent in the oral microbiome, probiotic supplementation with these organisms has longer lasting beneficial effects on the oral microbiome than supplementation with *Streptococci*, suggesting that the probiotic infant formula (Nutramigen Enflora) may bolster species diversity and thus overall health of the infant oral microbiome and be preferrable to the non-probiotic infant formula (Enfamil NeuroPro).

Metabolic Activity under Experimental Conditions

The significant decline in pH of *S. mutans* over time in TSB compared to in the infant formula media suggests that TSB provided better growing conditions for *S. mutans*, allowing the organism to metabolize sugars into lactic acid at a higher rate.

When *S. mutans* was cultured in both infant formula media, the pH actually increased slightly over time, which indicates *S. mutans'* lack of growth in the media. This lack of growth observed in the infant formulae suggests that these two infant formulae limit the growth of *S. mutans*, perhaps in part due to lower carbohydrate concentration in the infant formulae compared to TSB.

One explanation for the increasing pH of the infant formulae is *S. mutans'* ability to produce alkali under stress (Sheng et al. 2010). *S. mutans* has been shown to produce alkali by converting arginine into ammonia, CO_2, putrescine, and ATP (Lemos et al. 2019). The production of ATP through this agmatine deiminase system is a protective response against starvation, among other stressors (Lemos et al. 2019; Burne & Marquis 2000). This stress-induced ATP production provides energy for the starving cell. Arginine is found in cow's milk at 0.21 mg per gram of milk (USDA 2019), providing a substrate for the agmatine deiminase system in both cow's milk based infant formulae. It is plausible that starvation caused by low carbohydrate levels in the infant formulae induced alkali production as a protective measure in *S. mutans.* This alkali production may have in turn caused the small increase in pH over the course of the incubation. When comparing between the two infant formula media*, S. mutans* had a lower pH in the non-probiotic formula, indicating that *S. mutans* fared better in the non-probiotic formula than in the probiotic formula. The lower pH of the non-probiotic formula suggests that *S. mutans*' growth was not as strictly inhibited as in the probiotic formula, indicating that the non-probiotic formula is less effective at preventing pathogenic growth.

S. mitis' relatively high pH in comparison to *S. mutans* when grown in TSB is another indication of the commensal's low cariogenic potential, despite its shared ability to produce lactic acid as a metabolite like *S. mutans*. The small magnitude of the drop in pH over the course of the incubation in TSB compared to the pH drop of *S. mutans* shows that TSB is better suited for the growth of *S. mutans* than *S.* mitis. The fact that *S. mitis* had a greater pH drop over the course of the incubation when grown in non-probiotic formula compared to the probiotic formula may indicate that *S. mitis* also competed with *L. rhamnosus* for resources. The presence of the probiotic *L. rhamnosus* controlled the growth of both the pathogen *S. mutans* and the commensal *S. mitis*, although to differing degrees. The probiotic inhibited the growth of the pathogen more strictly and allowed for more growth of the commensal. The manner in which the probiotic differentially affected the growth of the pathogen and commensal shows that *L. rhamnosus* may support the establishment of a healthy oral microbial community by controlling the overall level of microbial growth while simultaneously favoring the growth of commensals over pathogens.

Relationship between CFUs and pH

Over the course of the incubation, an increase in CFUs is generally accompanied by a decrease in pH. This simultaneous increase in cell density and acid production relates to the overall growth of each species. For instance, when *S. mutans* experienced an increase in CFUs between 4-6 hr and 6-8 hr over the course of the incubation in TSB, this was accompanied by a sharp decline in pH at both intervals. This shows the relationship between the increase in cell number and the increase in acid production, a byproduct of the organism's metabolism. Therefore, an increase in

CFUs and decrease in pH can be interpreted as an overall increase in growth rate and cellular metabolism. This pattern that connects CFUs to pH is mirrored in the growth of *S. mitis* in both NeuroPro and Enflora.

Similarly, the decrease in CFUs of *S. mutans* grown in both of the infant formula media was accompanied by a slight increase in pH. The slow increase in pH shows that *S. mutans* was struggling to survive in these media, as there was no lactic acid production occurring to decrease the pH, and it is possible that the aforementioned agmatine deiminase system induced the production of alkali and ATP to cope with stress. This indicates that these media did not provide optimal growing conditions for this species to enable it to be a cariogenic threat. The suboptimal conditions of the infant formulae did not allow *S. mutans* to reach its full cariogenic potential, indicating that the infant formulae may help reduce the risk of developing dental caries over time. This pattern was more prominent in the probiotic formula, which provided the least optimal conditions for *S. mutans* out of all three media types, indicating that the probiotic formula had the best potential for both inhibiting the growth of the pathogen and therefore preventing it from producing enough lactic acid to induce tooth decay (enamel demineralization begins around a pH of 5.5) (Marsh 2009).

Conclusions

The community structure of the oral microbiome is constructed over time, beginning during gestation and continuing into adulthood (Al-Shehri et al. 2016; Xiao et al. 2020). The colonization of the oral cavity occurs sequentially: newly introduced species are dependent on the species already present in the oral cavity (Sulyanto et al. 2019). Since the established microbial community determines the species that are

subsequently acquired, the state of the oral microbiome earlier in life shapes the trajectory of the microbiome later in life (Xiao et a. 2020), especially since commensals and pathogens tend to exclude each other via competition (Lamont et al. 2018). For instance, if a child was fed a high-sugar diet that encouraged the growth of *S. mutans* during the time leading to primary dental eruption, *S. mutans* would likely dominate the microbial community and exclude non-aciduric commensals (Xiao et al. 2020). Eventually, this could lead to subsequent colonization of more acidogenic and aciduric pathogens like *S. mutans*, shifting the oral microbiome towards a state of dysbiosis. Conversely, if the infant is fed with a probiotic-supplemented diet such as Nutramigen Enflora preceding primary dental eruption, the presence of *L. rhamnosus* may help inhibit the growth of pathogens like *S. mutans* and thus shift the community structure towards a state that supports human health.

This study showed that a probiotic-supplemented infant formula (Nutramigen Enflora) was more successful at inhibiting the growth of the pathogen (*S. mutans*) than the non-probiotic formula (Enfamil NeuroPro). Although the growth of *S. mitis* was greater in the non-probiotic formula, the additional health benefits to the gut (i.e. preventing infection and diseases and aiding in maintaining a healthy weight) conferred by the probiotic may make Nutramigen Enflora the preferred infant formula for overall health. This information may aid parents in choosing an infant formula for their child if breastfeeding is not a viable option or choice. Since the nutrition during the first year of life is a key factor in determining the structure of the oral microbiome for the rest of the child's life, it is of parents' great concern that their child is consuming the best possible nutrition source for supporting the healthy development of the oral microbiome.

Limitations of the Study and Directions for Further Research

The health of the oral microbiome is affected by a large variety of influences, including internal factors such as host genetics (Goodrich et al. 2014), immune function (Karczewski et al. 2014), mode of delivery at birth (Dominguez-Bello et al. 2010), and external factors such as diet (Kato et al. 2016), drug use (Ferrer et al. 2017; Thomas et a. 2014), exercise (Cook et al. 2016), and environmental exposure to microorganisms (Ying et al. 2015; Song et al. 2013). The confluence of these many factors works together to mold each individual's unique oral microbiome community (Jia et al. 2017). No single factor can determine the trajectory of the development of the oral microbiome over time. This study is limited in its ability to predict the behavior of oral microbiota *in vivo*. Between 700-1,200 different species of bacteria cohabitate within the oral cavity of humans, alongside numerous fungi, archaea, and viruses that all interact with one another to shape the oral microbial community (Dewhirst et al. 2010; Wade et al. 2013). The complex environment of the oral cavity itself creates more variables that alter the oral microbiome, including the 11 microenvironments within the oral cavity that give rise to unique ecological subniches, the presence of complex biological fluids such as saliva and gingival crevicular fluid, and the constant influx of new microbial material via interface with the external environment (Jia et al. 2018; Jenkinson 2011).

In addition, this study investigated the interaction between a food source (infant formula) and one commensal and one pathogenic species. The commensal and pathogenic species selected for this study are merely representative of the hundreds of different species that colonize the oral cavity. These two species only represent the *Streptococcus* group of oral microorganisms, whereas around 185 genera are found in

the oral microbiome (Deo & Deshmukh 2019). The interactions between species and between the microbiota and the environment were not investigated in this study. As previously mentioned, there are a variety of ways in which the 700 - 1,200 species of oral microbiota interact with each other, including both competitive and cooperative interactions which influence the overall structure of the oral microbial community (Lamont et al. 2018; Jenkinson et al. 2011). Instead, the results of this study serve as an impetus for further inquiry into how nutrition may impact the acquisition of the oral microbiome through infancy. Future research on other prominent species, such as the commensals *Lactobacillus plantarum and Streptococcus sanguinis* and other pathogens including *Fusobacterium nucleatum* and *Porphyromonas gingivalis* (Jia et al. 2017) are needed to expand the understanding of the interspecific interactions that shape the oral microbiome.

In addition to the variety of internal and external factors that influence the oral microbiome that were not investigated in this study, there are naturally a large number of different kinds of infant formulae on the market that may differentially affect the growth of oral microorganisms. While this study sought to understand if and how one probiotic infant formula would differentially affect the growth of *Streptococcus* species compared to one non-probiotic infant formula of similar nutritional composition, there are many more brands and formulations of infant formula that could be studied. The two infant formulae used in this study were cow's milk based, but many other infant formulae use alternatives such as soy or goat milk and have varying nutritional composition that may affect the growth of oral microorganisms. A variety of carbohydrates (i.e. dextrose, sucrose, lactose), lipids (i.e. milk fats, palm, coconut, &

sunflower oils), and proteins (i.e. casein, whey, soy protein isolate) are present in different infant formulae (Enfamil 2020; Nutramigen 2020; Similac 2021). Additionally, comparing how breastmilk affects the growth of oral microorganisms versus infant formulae is of interest, as breastmilk is recognized as being superior in supporting a healthy microbiome over infant formulae (More et al. 2018). Therefore, comparing how different kinds of infant formulae compare to breastmilk in terms of supporting microbiome health may help parents choose an infant formula that is most comparable to breastmilk in that aspect.

While this study examined the growth of oral microorganisms over a period of 8 hours, it is understood that the oral microbiome is acquired sequentially over the years of childhood. This study focused on the growth parameters that were easily observable through the course of their logarithmic (fastest) growth phase, which was useful for understanding how those species reacted to different environments in the short term. However, more study is needed on how infant formula may influence the oral microbiota over longer periods of time. For instance, the repeated exposure *of L. rhamnosus* to the oral cavity may over time help shape the oral microbial community by outcompeting pathogens and supporting the growth of fellow commensals not only over the span of 8 hours, but over the span of weeks or months as the oral microbiome is acquired. Small shifts such as these may set a trajectory towards a healthy, mature, and stable oral microbiome in motion, where commensals dominate by outcompeting pathogens and maintaining environmental conditions that exclude pathogens (i.e. neutral pH).

An additional important area of study is how the nutrition source during pre-dental infancy may impact the colonization of the dental surfaces after primary dental

eruption (ca. 6 months of age) (Xiao et al. 2020). Since the dentures provide the only non-shedding substrate within the oral cavity, a new set of bacteria differing from the pioneer colonizers begin to colonize the novel substrate, utilizing the pioneer colonizers' ability to adhere to previously uncolonized surfaces by adhering to them (Jenkinson et al. 2011). As previously mentioned, the primary dental eruption is followed by an increase in gram-negative bacteria, notably, the cariogenic *S. mutans* (Crielaard et al. 2011). Since the dental surface is *S. mutans'* preferred colonization surface and the species is known for producing lactic acid that erodes the enamel and causes dental caries, the time of primary dental eruption is a critical point in the development of the oral microbiome (Crielaard et al. 2011). If the environmental conditions of the oral cavity allow *S. mutans* to proliferate, it may lead to the subsequent spiral of the community into a state of dysbiosis, where the child is at greater risk for aforementioned dental and systemic diseases (Xiao et al. 2020). Therefore, further study into the predentate and primary dental eruption stages of the infant oral microbiome are of great interest.

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