



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

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Abstract

The oral microbiome is a complex community of microorganisms that influences the health of the human host. A number of diseases are associated with dysbiotic oral microflora in infants and children, including dental and gastrointestinal diseases. A variety of factors can influence the composition of the oral microbial community in infants, including mode of delivery, feeding method, and diet. This study focuses on the effect of nutritional differences in infant formulae on the growth of a commensal species (*Streptococcus mitis*) and a pathogenic species (*Streptococcus mutans*) that are commonly found in the infant oral cavity. A culture-dependent model was utilized to test the effects of one infant formula (Nutramigen Enflora) supplemented with a probiotic (*Lactobacillus rhamnosus*) and a similar infant formula without probiotic supplementation (Enfamil NeuroPro) on the growth of each species. A Snyder's media test was used to assess acidogenic potential of each species. Bacterial growth in each formula was assessed by measuring colony forming units (CFUs) and by measuring the pH of the culture media over an 8 hour incubation. Results indicate that the probiotic formula may selectively inhibit the growth of the pathogen and aid in producing more favorable conditions for the commensal. These findings 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.

Introduction

The human microbiome is defined as the totality of microorganisms which inhabit the human body (25). Estimates indicate that there are about as many bacterial cells as there are human cells in the body (46). The gut microbiome alone harbors about 1,000 species of bacteria with a combined total of 2 million genes, which is 100× the number of genes in the human genome (55). Our microbial symbionts are intimately intertwined with both systemic and specific bodily function (22), and each niche in the human body nurtures a unique community of microbes.

The microbiome is inherently linked to the health of the human host. 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 (Figure 1), which in turn influence microbial community composition and thus overall host health (22).

Dysbiosis, a state in which the microbial community composition becomes unbalanced, is triggered by alterations in factors such as diet, immune function, hygiene, and hormone levels (27). It is well known that a dysbiotic microbiome is associated with a multitude of disease states of the body and the mind, such as cancers, obesity, cardiovascular disease, psoriasis, and major depressive disorder (57, 20, 22). 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.

The oral cavity is a distinct niche within the overall human microbiome that is of interest due to its association with diseases in adults and children such as dental caries, periodontal disease, various cancers (i.e. oral, esophageal, pancreatic, and colorectal) and gastrointestinal diseases (56).

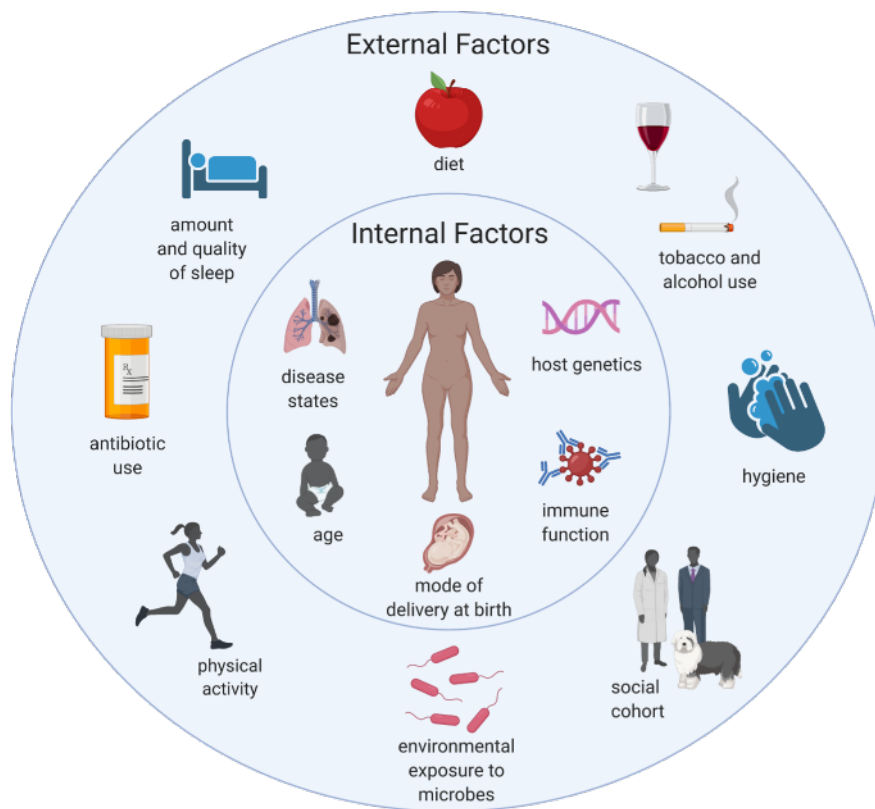


Figure 1: Internal and external factors that influence the human microbiome. Factors that shape the human microbiome can be internal (a condition of the body that is not easily changed, such as age, genetics, and mode of delivery at birth) or external (including lifestyle choices such as diet, exercise,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

The process of acquiring the oral microbiome begins during gestation, continues through parturition and infancy, and is highly influenced by factors such as genetics, gestation length, delivery mode, and diet (59). Following birth, the infant is exposed to the multitude of microbes present within their environment (27). The oral cavity is colonized in a sequential manner, first with pioneer colonizers attaching and enabling subsequent colonizers to adhere and form a biofilm (52). The most common pioneer colonizers of the oral cavity are Gram-positive bacteria including Streptococci species such as *S. mitis* and *S. salivarius*, because of their ability to adhere to previously uncolonized epithelial tissue and their presence in breastmilk. Pathogenic species like *S. mutans* are less abundant than commensal species, but their overgrowth is linked with a dysbiotic oral microbiome and diseases of the oral cavity like dental caries (57). Typically, pathogenic species such as *S. mutans* 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 the pathogenic species and the simultaneous inhibition of commensal species like *Streptococcus sanguinis* that are less tolerant to low pH conditions (57, 59). The composition of the oral microbiome fluctuates preceding the first tooth eruption, after which it begins to stabilize. Because of this, more research is needed to determine if the composition of the pre-eruptive oral microbiome influences the more permanent oral microbiome later in life (59). The acquisition and development of a healthy oral microbiome is important for a child's overall health as several childhood diseases, as mentioned above, are associated with a dysbiotic oral microbiome (59).

Nutrition is an important factor that can influence the trajectory of the development of the early oral microbiome (59) 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 (3; 54). 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 (40). Similarly, infant formulae are desirable for infants suffering from malnutrition due to famine (40). HIV-positive mothers also rely on infant formulae to avoid transmission of the virus to the infant (40), and the advent of soy-based formulae in the 1920's provided a solution for lactose-intolerant infants (40). Infant formula utilizes either cow's milk, soy, amino acids, or goat's milk as a replacement for breastmilk. Some infant formulae containing probiotics tout claims of boosting the microbiome, including Lactobacilli and Bifidobacteria in the formulations (18). However, these claims have not been clinically supported, and the effects of these formulations on the composition of the oral microbiome have not yet been thoroughly investigated

This study aims to elucidate the relationship between the nutritional profile of infant formulae with the growth of one commensal and one pathogenic constituent of the infant oral microbiome. A commensal species, *S. mitis*, and a pathogenic species, *S. mutans*, were used to represent beneficial and pathogenic *Streptococcus* species commonly found in the infant oral cavity.

S. mitis is a Gram-positive commensal species of the oral cavity and is understood to be one of the primary colonizers of the oral cavity (19). Like *S. mutans*, *S. mitis* metabolizes carbohydrates such as lactose and sucrose into lactic acid (30, 38). *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 (31). The results from some studies suggest that *S. mitis* may supplement host immunity through modulating the expression of various immune markers (61). 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 (17, 42). The production of antimicrobials that target pathogenic microbes in turn may boost host health by limiting the growth of pathogens.

S. mutans is a Gram-positive facultative anaerobe that is known for its role in the development of dental caries, especially in infants and children (24). Two characteristics of *S. mutans* that contribute to its pathogenicity are its acidogenicity and acidity: the ability to metabolize sugars

(glucose, fructose, lactose, sucrose) to lactic acid and thrive in low pH conditions, respectively (36). 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 (36).

These species were grown on media containing one of two infant formulae that were chosen for their similarity in nutritional composition to elucidate the effect of probiotic supplementation on the growth of the commensal and pathogenic species: 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). The overall growth of the two species was assessed by counting CFUs to determine the cell density of the culture media every hour 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. 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.

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 (59). Both cultures were sourced from the American Type Culture Collection (atcc.org, Manassas, VA). *S. mitis* type strain ATCC 49456 was the sole strain available from ATCC. *S. mutans* type strain ATCC 25175 was selected for its origin from dental caries.

Snyder's Media Test

A Snyder's media test 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,

which is indicative of cariogenic potential as acid erodes dental enamel and causes dental caries. The faster the inoculated media changes color, the greater the cariogenic potential of a species (48). A change in media color from blue-green to yellow within 24 hours indicates high cariogenic potential, while a change in color to orange indicates moderate cariogenic potential and no color change indicates low cariogenic potential. In this study, the color of the media for each species was compared after 24 hours of incubation. Both species were incubated at 37°C in Snyder's media in triplicate for 24 hours followed by comparisons of the media color to the uninoculated control.

Growth Curves under Baseline Conditions

Growth curves were created for each species under baseline conditions to describe the general growth kinetics of both species. The approximate time of the mid-log phase (i.e. the midpoint of the logarithmic phase) was needed for this study. The experimental cultures were 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, Thermo Fisher Scientific, Waltham, MA) 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, Vernier, Beaverton, OR). 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 (51) every 30 minutes for the first 4 hours, then every hour until a plateau in the growth curve was reached. These measurements were plotted against time to create growth curves (Figures 2 and 3) from which the mid-log and plateau phase could be determined.

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 1) were compared in this study: a cow's milk-based formula (Enfamil NeuroPro, Mead Johnson, Chicago, IL) and a cow's milk-based formula supplemented with probiotics (Nutramigen Enflora, Mead Johnson, Chicago, IL). The Nutramigen Enflora formula is supplemented with *Lactobacillus rhamnosus*, one of the most widely used probiotic strains utilized as a dietary supplement (26). 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 (Thermo Fisher Scientific, Waltham, MA) to ensure bacterial growth without adding another carbohydrate source.

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 37°C for 24 hours, after which colony forming units were counted in plates that had between 30-300 colony forming units (CFUs) (33). The following formula was used to calculate the original cell density of the starting culture:

Original cell density=(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 Incorporated, Yellow Springs, OH). The meter was calibrated according to manufacturer's instructions before use. A decrease in pH indicates acid production, which is related to the cariogenic potential of a culture.

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 (ANOVA) 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 ($\alpha = 0.05$).

The same tests were utilized 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 be 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 measurements for each species in each media type. Data from hours 0 and 8 were used as time points in the analysis because they represented the start-point and plateau phase of each incubation, respectively.

Results

Growth Curves under Baseline Conditions

The growth curves produced for both *S. mitis* (Figure 2) and *S. mutans* (Figure 3) showed a logarithmic curve with the lag phase from hours ca. 0-3 hours, a log phase from ca. 3-6 hours, a plateau phase from ca.6-8 hours, and the mid-log point ca. hour 4.5.

Snyder's Media Test

The degree of color change in the inoculated Snyder's Media was compared relative to the uninoculated test media (Figure 4a). After 24 hours, a color change in the media inoculated with *S. mutans* (Figure 4b) indicated a positive result and a lack of color change in the media inoculated with *S. mitis* (Figure 4c) indicated a negative result.

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 2; Figure 5a), whereas the mean CFUs of *S. mitis* decreased over time in TSB, with a ca. 16X decrease between hour 0 and 8 (Table 2, Figure 5a). The mean CFUs of *S. mutans* decreased over time in NeuroPro and Enflora, by ca. 0.18X and 6.8X,

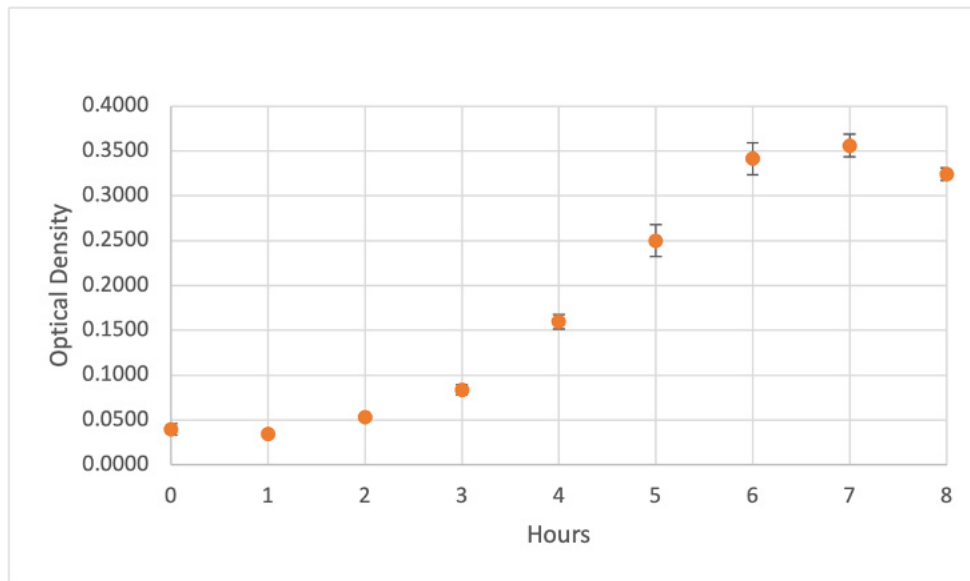


Figure 2: The growth curve of *S. mitis* under baseline conditions. Growth in TSB was quantified by optical density, measured by spectrophotometry, over 8 hours. Error bars represent standard deviation of the mean (n= 3).

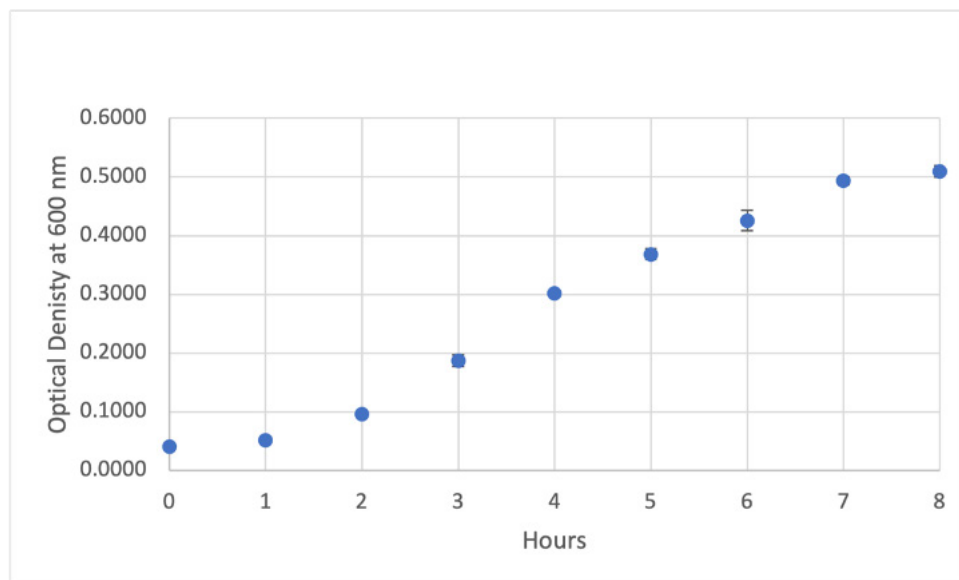


Figure 3: The growth curve of *S. mutans* under baseline conditions. Growth in TSB was quantified by optical density, measured by spectrophotometry, over 8 hours. Error bars represent standard deviation of the mean (n= 3).

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 2, Figures 5b and 5c). At all time points in each media, *S. mitis* had greater CFUs than *S. mutans*, except at hour 8 in TSB (Table 2, Figure 5).

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 2, Figure 6b). NeuroPro had the highest CFU count for *S. mitis* of ca. 2.59×10^7 compared to ca. 5.07×10^6 in TSB and ca. 2.40×10^7 in Enflora at hour 0 (Table 2, Figure 6a). At the end of the incubation, CFUs of *S. mitis* in NeuroPro was 347X and 2X higher than TSB and Enflora respectively. For *S. mutans* at hour 0, TSB yielded the highest CFU count of ca. 4.31×10^6 compared to ca. 2.40×10^5 in NeuroPro and ca. 3.90×10^5 in Enflora (Table 2, Figure 6b). At the end of the incubation, CFUs of *S. mutans* in TSB were 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.

pH

The mean pH of *S. mitis* significantly decreased over time in all three media types (Table 4, Figure 7b). Between hour 0 and hour 8, the pH decreased by ca. 2.6% in TSB, ca. 1.9% in NeuroPro, and ca. 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 3, Figure 7a). 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.

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 two time points (Table 4, Figure 7). None of the Mann-Whitney tests were significant (Table S1). 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 4, Table S1). 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*.

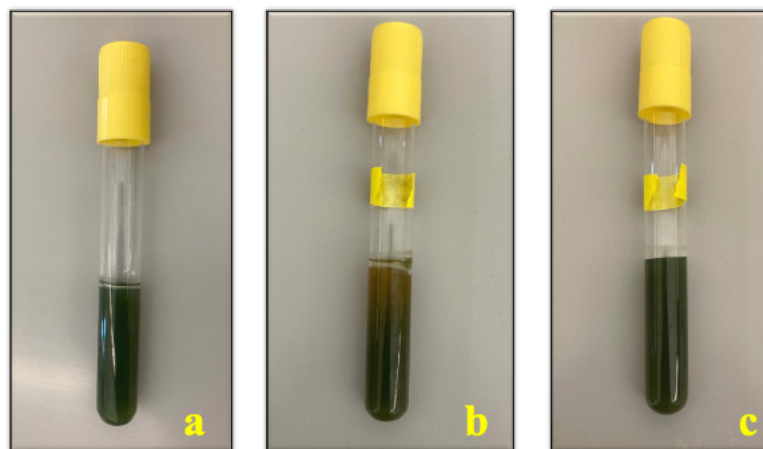


Figure 4: Results of Snyder's Media Test. A change in the color of the media from dark green to orange within 24 hours indicates a positive result. Cultures pictured are representative of all three replicates for each species: a) uninoculated media, b) media inoculated with *S. mutans*, c) media inoculated with *S. mitis*.

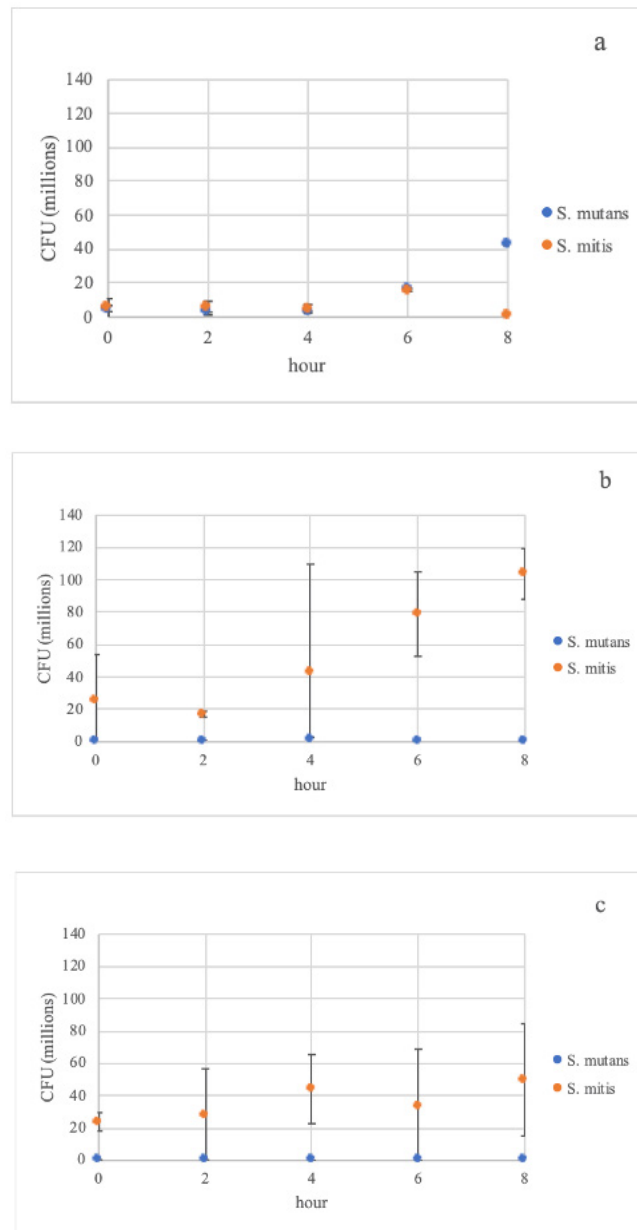


Figure 5: Mean Colony Forming Units (CFU) of *S. mutans* and *S. mitis* over time. a) in TSB, b) in NeuroPro, c) in Enflora. Error bars representing standard deviation of the mean are included when n = 3.

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 (Figure 8, Tables S2, S3, and S4), except for *S. mutans* in NeuroPro ($p = 0.753$). 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.

The Relationship between CFUs and pH

For the species and media that resulted in increased bacterial growth over time, there were notable trends between CFUs and pH over time. For *S. mutans* grown in TSB (Figure 9), *S. mitis* grown in NeuroPro (Figure 10), and *S. mitis* grown in

Enflora (Figure 11), the general trend is as the CFUs increase, the pH also decreases. In optimal growth conditions (Figure 6 & 7), there appears to be a steeper slope of the trendline relating CFUs to pH as compared to less optimal growth conditions (Figure 11). For the two conditions with the highest growth, *S. mutans* in TSB (Figure 9) and *S. mitis* in NeuroPro (Figure 10), the inflection point where CFUs increase sharply and pH decreases sharply was around the 4.5 hour mark (ca. mid-log phase). Figure 11 is representative of the remaining conditions: no inflection point in the slopes of the CFU and pH graphs was observed. The trends outlined above indicate that the high-growth conditions (*S. mutans* in TSB, *S. mitis* in NeuroPro and Enflora) had a stronger relationship between an increase in CFUs and decrease in pH than the low-growth conditions (*S. mutans* in NeuroPro and Enflora, *S. mitis* in TSB).

Discussion

Snyder's Media Test Confirms the Cariogenic Potential of S. mutans

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 (36) which lowers the pH of the surrounding microenvironment, limits the growth of non-aciduric species (which are commonly commensal species) and produces conditions that favor the growth of other aciduric pathogens (32). 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* (30), *S. mitis* is generally considered to act as a commensal species when its growth is kept in check

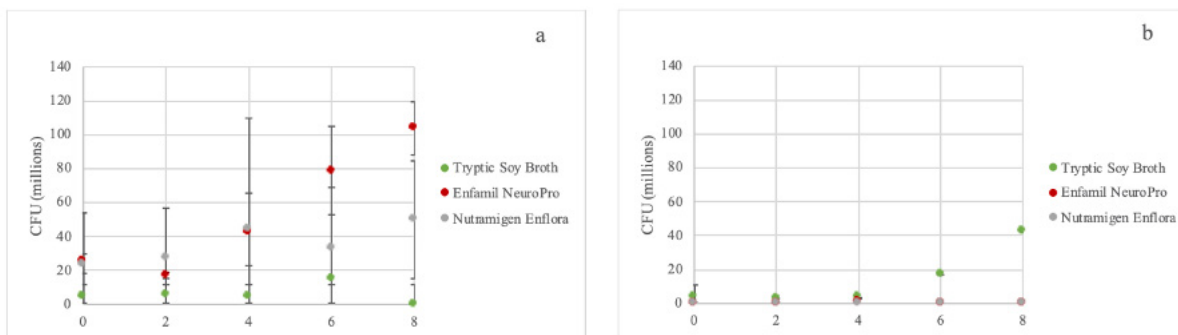


Figure 6: Mean Colony Forming Units (CFU) of (a) *S. mitis* and (b) *S. mutans* in TSB, NeuroPro, and Enflora over time. Error bars representing standard deviation of the mean are included when n = 3.

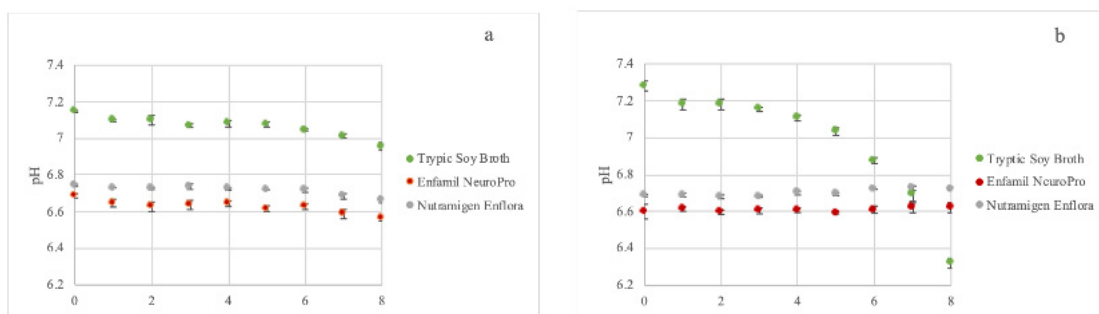


Figure 7: Mean pH values in each media type over time: a) *S. mitis*, b) *S. mutans*. Error bars represent standard deviation of the mean.

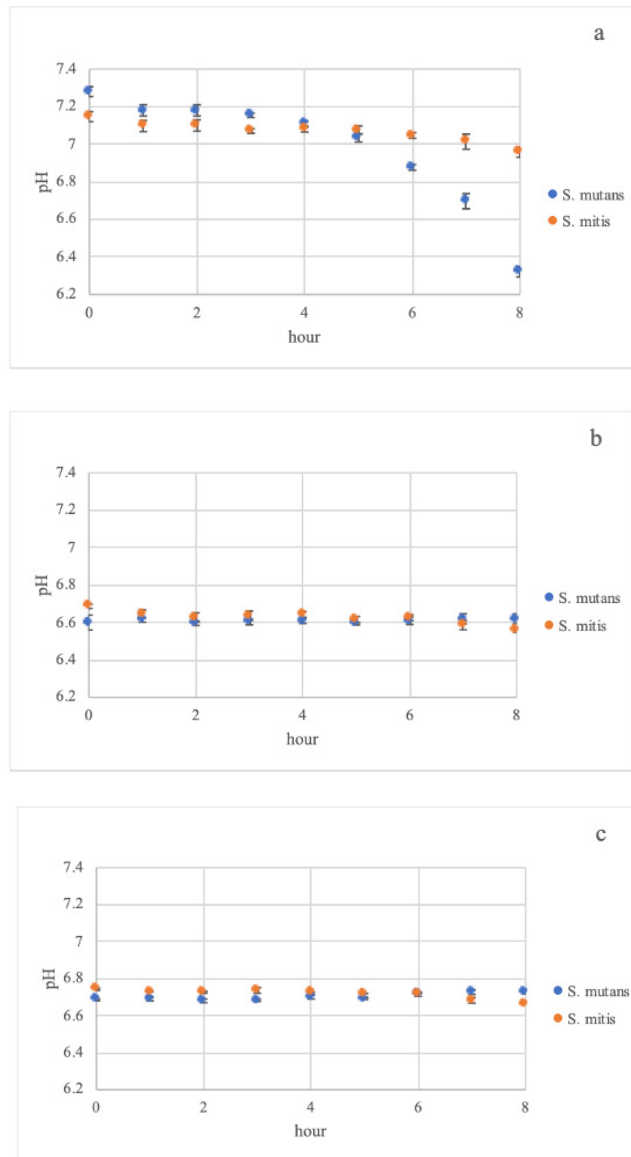


Figure 8: Mean pH values of *S. mutans* and *S. mitis* over time in a) TSB, b) NeuroPro, and c) Enfura. Error bars represent standard deviation of the mean (n = 3).

by competing commensals. 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 Reveals Lactose-based Formula Favors the Growth of *S. mitis* over *S. mutans*

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*.

When cultured in infant formula, there was a decrease in CFU counts of *S. mutans* over time, suggesting that the infant formulae did not provide optimal growing conditions for *S. mutans* compared to *S. mitis* and that the infant formulae may have an inhibitory effect on the growth of this opportunistic 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 Enfura contains 0.014 g/ml of dextrose. Previous research examined the effects of infant formula composition on the growth of the pathogen *S. mutans* and found that

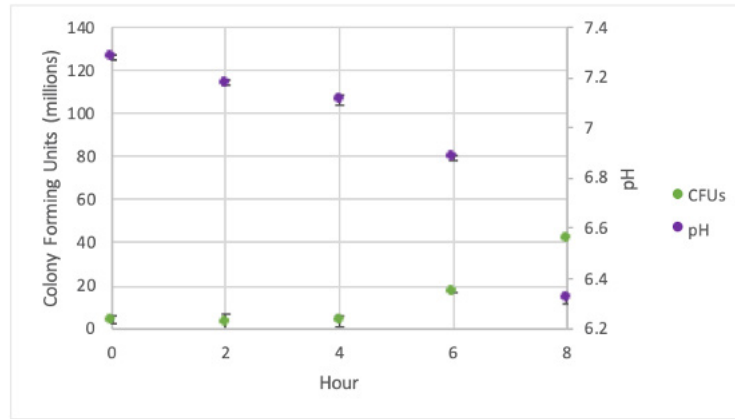


Figure 9: The relationship between CFUs and pH for *S. mutans* grown in Tryptic Soy Broth. Error bars representing standard deviation of the mean when $n = 3$.

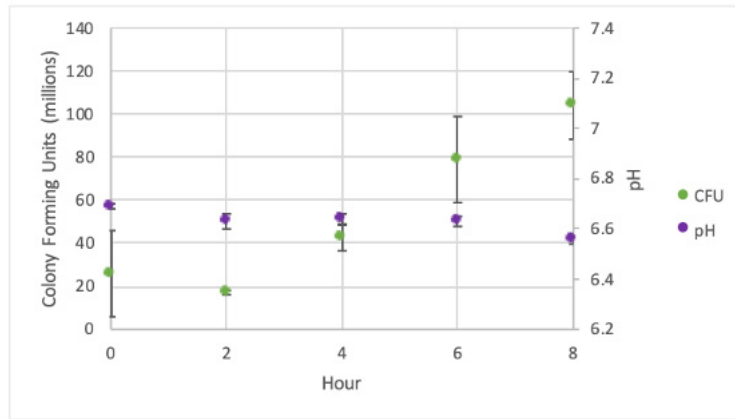


Figure 10: The relationship between CFUs and pH of *S. mitis* grown in NeuroPro. Error bars representing standard deviation of the mean are included when $n = 3$.

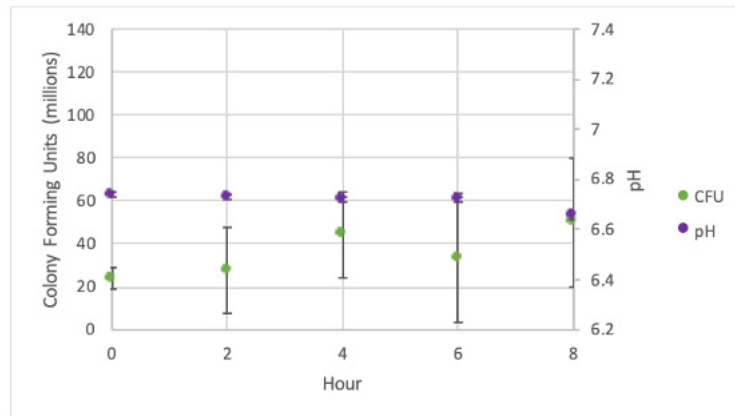


Figure 11: The relationship between CFUs and pH of *S. mitis* grown in Enflora. Error bars representing standard deviation of the mean are included when $n = 3$.

sucrose-based formulae yielded more growth of *S. mutans* when compared to formulae containing lactose (24). This is possibly due to sucrose being more readily fermentable, leading to a lowering of pH and sucrose being a substrate for the production of polysaccharides that allow bacterial adhesion and biofilm formation (45). Because *S. mutans* is known for its rapid consumption of carbohydrates (24, 34), the higher concentration of dextrose in TSB may have allowed for greater proliferation of *S. mutans* compared to the infant formulae due to sucrose being more readily fermentable than lactose and rapidly lowering the pH of the formula, creating a low pH environment in which *S. mutans* thrives.

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 (40). 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.

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. 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 (41). 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. *L. rhamnosus* produces microcine, a small antimicrobial peptide along with another 7 different antibacterial peptides (35). *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* (44). 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 (44). 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*, thus exerting 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.

In addition, *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 (39). 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 (1). 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 (58). Therefore, it is understood that while *S. mitis* typically acts as a commensal when kept in check by the cohabitants 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. In fact, 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 (13). However, the species diversity reverted to baseline (pre-treatment) levels after discontinuing probiotic intake.

S. mutans Thrives in Sucrose-dense TSB, S. mitis Thrives in Lactose-based Formulae

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 (47). *S. mutans* has been shown to produce alkali by converting

arginine into ammonia, CO₂, putrescine, and ATP (34). The production of ATP through this agmatine deiminase system is a protective response against starvation, among other stressors (7, 34). This stress-induced ATP production provides energy for the starving cell. 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 nonprobiotic 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 (ca. 2.7%) compared to the ca. 15% 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 nonprobiotic 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 demonstrates 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) (37).

Conclusions and Directions for Future Research

The community structure of the oral microbiome is constructed over time, beginning during gestation and continuing into adulthood (1, 59). The colonization of the oral cavity occurs sequentially: newly introduced species are dependent on the species already present in the oral cavity

(52). 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 (59), especially since commensals and pathogens tend to exclude each other via competition (32).

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). The growth of *S. mitis* was greater in the non-probiotic formula, this along with 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 infancy is a key factor in determining the structure of the oral microbiome for the rest of the child's life (59), 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.

Although this study provides preliminary data to help parents in choosing an infant formula for their child if breastfeeding is not a viable option, it is limited in its ability to predict the behavior of oral microbiota *in vivo*. The health of the oral microbiome is affected by a large variety of influences, including internal factors such as host genetics (23) and external factors such as diet (29). The confluence of these factors works together to mold each individual's unique oral microbiome community (27). 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 (15, 57). The commensal and pathogenic species selected for this study are merely representative of the hundreds of different species that colonize the oral cavity. Future research on other prominent species, such as the commensals *Lactobacillus plantarum* and *Streptococcus sanguinis* and other pathogens including *Fusobacterium nucleatum* and *Porphyromonas gingivalis* (27) is needed to expand the understanding of the full range of interspecific interactions that shape the oral microbiome.

In addition, there are many 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 (18, 43, 49). 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 (40). 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.

Finally, while this study examined the growth of oral microorganisms over a period of 8 hours, the oral microbiome is acquired sequentially over the years of childhood (52). Therefore, additional longitudinal studies are needed to capture how the community changes over time. One example of an important time point in the establishment of the oral microbiome is the eruption of the first tooth at ca. 6 months of age (59). The dental surface provides a new substrate for the colonization of the oral microflora and also happens to be *S. mutans*' preferred colonization niche within the mouth. Therefore, the time of primary dental eruption may be a critical inflection point in the trajectory of the development of the oral microbiome (12). Therefore, a deeper understanding of factors that influence the oral microflora during the preeruptive and primary dental eruption stages of the infant oral microbiome are of great importance.

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References

1. Abranches, J., Zeng, L., Kajfasz, J.K., Palmer, S.R., Chakroborty, B., Wen, Z.T., Richards, V.P., Brady, L.J. & Lemos, J.A. 2019. Biology of oral streptococci. *Microbiol Spectr.* 6:426-434. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6287261/>
2. Al-Shehri, S., Knox, C., Liley, H., Cowley, D., Wright, J., Henman, M., Hewavitharana, A., Charles, B., Shaw, P., Sweeney, E. & Duley, J. 2015. Breastmilk-saliva interactions boost innate immunity by regulating the oral microbiome in early infancy. *PLoS ONE* 10:e0135047. <https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0135047>
3. Al-Shehri, S., Sweeney, E., Cowley, D., Liley, H., Ranasinghe, P., Charles, B., Shaw, P., Vagenas, D., Duley, J. & Knox, C. 2016. Deep sequencing of the 16S ribosomal RNA of the neonatal oral microbiome: a comparison of breast-fed and formula-fed infants. *Sci Rep.* 6:38309. <https://www.nature.com/articles/srep38309>
4. Benedict, C., Vogel, H., Jonas, W., Woting, A., Blaut, M., Schurmann, A. & Cedernaes, J. 2016. Gut microbiota and glucometabolic alterations in response to recurrent partial sleep deprivation in normal-weight young individuals. *Mol Metab.* 5:11751186. <https://pubmed.ncbi.nlm.nih.gov/27900260/>
5. Bowen, W.H., Burne, R.A., Wu, H., & Koo, H. 2018. Oral Biofilms: Pathogens, Matrix, and Polymicrobial Interactions in Microenvironments. *Trends in Microbiol.* 26:229-242. <https://pubmed.ncbi.nlm.nih.gov/29097091/>
6. Bravo J., Forsythe, P., Chew, M., Escaravage, E., Savignac, H., Dinan, T., Bienenstock, J. & Cryan, J. 2011. Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proc Natl Acad Sci.* 108:16050-16055. <https://www.pnas.org/content/108/38/16050>
7. Burne, R. & Marquis, R. 2000. Alkali production by oral bacteria and protection against dental caries. *FEMS Microbiol let.* 193:1-6. <https://pubmed.ncbi.nlm.nih.gov/11094270/>
8. Buyukeren, M., Yigit, S., Buyukcam, A., Kara, A., Tolga, H. & Murat Yurdakok M. 2020. A new use of *Lactobacillus rhamnosus* GG administration in the NICU: colonized vancomycin-resistant enterococcus eradication in the gastrointestinal system. *J Mat-Fetal & Neonat Med.* <https://www.tandfonline.com/doi/abs/10.1080/14767058.2020.1743671>
9. Cho, I. & Blaser, M.J. 2012. The human microbiome: at the interface of health and disease. *Nature*, 13:260-270. <https://www.nature.com/articles/nrg3182>
10. Cook, M., Allen, J., Pence, B., Wallig, M., Gaskins, H., White, B. & Woods, J. 2016. Exercise and gut immune function: evidence of alterations in colon immune cell homeostasis and microbiome characteristics with exercise training. *Immunol Cell Biol.* 94:158-163. <https://onlinelibrary.wiley.com/doi/10.1038/icb.2015.108>
11. Craig, S., Blankenberg, D., Parodi, A., Paul, I., Birch, L., Savage, J., Marini, M., Stokes, J., Nekrutenko, A., Riemherr, M., Chiaromonte, F. & Makova, K. 2018. Child weight gain trajectories linked to oral microbiota composition. *Sci Rep.* 8:14030. <https://www.nature.com/articles/s41598-018-31866-9>

12. Crielaard, W. *et al.* 2011. Exploring the oral microbiota of children at various developmental stages of their dentition in the relation to their oral health. *BMC Med Genomics* 4:22. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3058002/>
13. Dassi, E., Ferretti, P., Covello, G., Bertorelli, R., Denti, M. A., De Sanctis, V., *et al.* 2018. The short-term impact of probiotic consumption on the oral cavity microbiome. *Sci Rep.* 8:1-8. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6041349/>
14. Deo, P. N., & Deshmukh, R. 2019. Oral microbiome: Unveiling the fundamentals. *JOMFP.* 23:122-128. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6503789/>
15. Dewhirst, F.E., Chen, T., Izard, J., Paster, B.J., Tanner, A.C.R., Yu, W.H., *et al.* 2010. The human oral microbiome. *J Bacteriol.* 192:2002-5017. <https://pubmed.ncbi.nlm.nih.gov/20656903/>
16. Dominguez-Bello, M.G., De Jesus-Laboy, K.M., Shen, N., Cox, L.M., Amir, A., Gonzalez, A., Bokulich, N., Song, S., Hoashi, M., Rivera-Vinas, J., Mendez, K., Knight, R. & Clemente, J. 2016. Partial restoration of the microbiota of cesarean-born infants via vaginal microbial transfer. *Nat med.* 22:250. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5062956/>
17. Eberhard, J., Pietschmann, R., Falk, W., Jepsen, S. & Dommisch, H. 2009. The immune response of oral epithelial cells induced by single-species and complex naturally formed biofilms. *Oral Microbiol Immunol.* 24:325-330. <https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1399-302X.2009.00518.x>
18. Enfamil NeuroPro infant formula. 2020. Retrieved April 30, 2021, from <https://www.enfamil.com/products/enfamil-neuropro-infant-formula/>
19. Engen, S., Rørvik, G., Schreurs, O. *et al.* (2017). The oral commensal *Streptococcus mitis* activates the aryl hydrocarbon receptor in human oral epithelial cells. *Int J Oral Sci.* 9:145-150. <https://www.nature.com/articles/ijos201717>
20. Farrell, J.J., Zhang, L., Zhou, H., Chia, D., Elashoff, D., & Akin, D. 2012. Variations of oral microbiota are associated with pancreatic diseases including pancreatic cancer. *Gut.* 61:582-588. <https://pubmed.ncbi.nlm.nih.gov/21994333/>
21. Ferrer M, Méndezgarcía C, Rojo D, Barbas C, & Moya A. 2017. Antibiotic use and microbiome function. *Biochem Pharmacol.* 134:114-126. <https://pubmed.ncbi.nlm.nih.gov/27641814/>
22. Gilbert, J., Blaser, M.J. Caporaso, J.G. Jansson, J. Lynch, & S.V. Knight, R. 2018. Current understanding of the human microbiome. *Nat Med.* 24:392-400. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7043356/>
23. Goodrich, J., Waters, J., Poole, A., Sutter, J., Koren, O., Blekhman, R., Beaumont, M., Van Treuren, W., Knight, R., Bell, J., Spector, T., Clark, A. & Ley, Ruth. 2014. Human genetics shape the gut microbiome. *Cell.* 159:789-799. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4255478/>
24. Hinds, L.M., Moser, E.A.S., Eckert, G., & Gregory, R.L. 2016. Effect of Infant Formula on *Streptococcus mutans* Biofilm Formation. *J Clin Pediatr Dent.* 40:401-408. <https://pubmed.ncbi.nlm.nih.gov/27472563/>
25. Huttenhower, C. & Gevers, D. 2012. Structure, function and diversity of the healthy human microbiome. *Nature.* 486:207-214. <https://www.nature.com/articles/nature11234>

26. Isolauri E., Kirjavainen P.V., & Salminen S. 2002. Probiotics: a role in the treatment of intestinal infection and inflammation? *Gut*. 50:iii54-iii59. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1867676/>
27. Jia, G., Zhi, A., Lai, P.F.H., Wang, G., Xia, Y., Xiong, Z., Zhang, H., Che, N., & Ai, L. 2017. The oral microbiota—a mechanistic role for systemic diseases. *Brit Dent J*. 224:447-455. <https://pubmed.ncbi.nlm.nih.gov/29569607/>
28. Karczewski, J., Poniedziałek, B., Adamski, Z. & Rzymiski, P. 2014. The effects of the microbiota on the host immune system. *Autoimmun*. 47:494:504. <https://pubmed.ncbi.nlm.nih.gov/25019177/>
29. Kato, I., Vasquez, A., Moyerbrailean, G., Land, S., Djuric, Z., Sun, J., Lin, H., & Ram, J. 2016. Nutritional Correlates of Human Oral Microbiome. *J Am Coll Nutr*. 36:88-98. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5477991/>
30. Kilian, M., Mikkelsen, L., & Henrichsen, J. 1989. Taxonomic study of viridans streptococci: description of *Streptococcus gordonii* sp. nov. and emended descriptions of *Streptococcus sanguis* (White and Niven 1946), *Streptococcus oralis* (Bridge and Sneath 1982), and *Streptococcus mitis* (Andrewes and Horder 1906). *Int J Syst and Evolut Microbiol*. 39:471-484. https://www.researchgate.net/publication/242219755_Taxonomic_Study_of_Viridans_Streptococci_Description_of_Streptococcus_gordonii_sp_nov_and_Emended_Descriptions_of_Streptococcus_sanguis_White_and_Niven_1946_Streptococcus_oralis_Bridge_and_Sneath_1982
31. Kirchherr, J.L., Bowden, G.H., Richmond, D.A., Sheridan, M.J., Wirth, K.A., & Cole, M.F. 2005. Clonal diversity and turnover of *Streptococcus mitis* by shedding and nonshedding oral surfaces of human infants during the first year of life. *Clin Diagn Lab Immunol*. 10:1184-1190. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1247832/>
32. Lamont, R.J., Koo, H., & Hajishengallis, G. 2018. The oral microbiota: dynamic communities and host interactions. *Nat Rev: Microbiol*. 16:745-759. <https://pubmed.ncbi.nlm.nih.gov/30301974/>
33. Leboffe, M. & Pierce, B. 2016. *Microbiology: Laboratory Theory and Application, Brief*. 3rd ed. Morton Publishing. Englewood, CO. pg. 412.
34. Lemos, J. A., Palmer, S. R., Zeng, L., Wen, Z. T., Kajfasz, J. K., Freires, I. A., Abranches, J., & Brady, L. J. 2019. The Biology of *Streptococcus mutans*. *Microbiol Spectr*. 7: 10.1128. <https://pubmed.ncbi.nlm.nih.gov/30657107/>
35. Lu R., Fasano S., Madayiputhiya N., *et al.* 2009. Isolation, identification, and characterization of small bioactive peptides from *Lactobacillus* GG conditional media that exert both anti- Gram-negative and Gram-positive bactericidal activity. *J Pediatr Gastroenterol Nutr*. 49:23-30. <https://pubmed.ncbi.nlm.nih.gov/19465870/>
36. Madigan M., Martinko J., eds. 2005. *Brock Biology of Microorganisms* (11th ed.). Prentice Hall. ISBN 978-0-13-144329-7
37. Marsh P.D. 2009. Dental plaque as a biofilm: the significance of pH in health and caries. *Compend Contin Educ Dent*. 30:76-78. <https://pubmed.ncbi.nlm.nih.gov/19301526/>

38. Mashimo P., Yamamoto Y., Nakamura M., Reynolds H., & Genco R. 1985. Lactic acid production by oral *Streptococcus mitis* inhibits the growth of oral Capnocytophaga. J Periodontol. 56:548-52.
39. Mitchell, J. 2011. *Streptococcus mitis*: walking the line between commensalism and pathogenesis. Mol. Oral Microbiol. 26:89-98. <https://pubmed.ncbi.nlm.nih.gov/21375700/>
40. More, S., Sankeshwari, R., Patil, P.A., Jalihal, S.S., & Ankola, A.V. 2018. Infant formula and early childhood caries. J Dent Res Rev. 5:7-11. <https://www.jdrr.org/article.asp?issn=2348-2915;year=2018;volume=5;issue=1;spage=7;epage=11;auiast=More>
41. Naase L., Hatakka K., Savilahti E., *et al.* 2001. Effect of long-term consumption of a probiotic bacterium, *Lactobacillus rhamnosus* GG, in milk on dental caries and caries risk in children. Caries Res. 35:412–20. <https://pubmed.ncbi.nlm.nih.gov/11799281/>
42. Nishimura, E., Eto, A. & Kato, M. 2004. Oral streptococci exhibit diverse susceptibility to human beta-defensin-2: antimicrobial effects of hBD-2 on oral Streptococci. Curr Microbiol. 48:85-87. <https://pubmed.ncbi.nlm.nih.gov/15057473/>
43. Nutramigen with Enflora LGG infant formula. 2020. Retrieved May 4, 2021 from <https://www.enfamil.com/products/nutramigen-powder-infant-formula/>
44. Petrova M.I., Imholz N.C., Verhoeven T.L., Balzarini, J., Van Damme, E., Schols, D., Vanderleyden, J. & Lebeer, S. 2016. Lectin-like molecules of *Lactobacillus rhamnosus* GG inhibit pathogenic *Escherichia coli* and Salmonella biofilm formation. PLoS One. 11:0161337. <https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0161337>
45. Sadan, H., Shanthala, BM., Zareena, MA., Babu, G., Vijayan, V. 2020. In vitro evaluation of milk-based, soy-based, and amino acid-based infant formulas on *Streptococcus mutans* biofilm formation.
46. Sender, R., Fuchs, S., & Milo, R. 2016. Are we really vastly outnumbered? Revisiting the ratio of bacterial to host cells in humans. Cell. 164:337-340. <https://pubmed.ncbi.nlm.nih.gov/26824647/>
47. Sheng, J., Baldeck, J. D., Nguyen, P. T., Quivey, R. G., Jr, & Marquis, R. E. 2010. Alkali production associated with malolactic fermentation by oral streptococci and protection against acid, oxidative, or starvation damage. Can J Microbiol. 56:539-547. <https://pubmed.ncbi.nlm.nih.gov/20651853/>
48. Sherman, N. & Cappuccino J. 2002. Microbiology: a laboratory manual. Pearson Education, Inc. as Benjamin Cummings.
49. Similac Soy Isomil infant formula. 2021. Retrieved May 4, 2021 from <https://www.similac.com/products/baby-formula/soy-isomil-powder/30-8oz-can-4pack.html>
50. Song, S., Lauber, C., Costello, E., Lozupone, C., Humphrey, G., Berg-Lyons, D., Caporaso, J., Knights, D., Clemente, J., Nakielny, S., Gordon, J., Fierer, N. & Knight, R. 2013. Cohabiting family members share microbiota with one another and with their dogs. eLife. 2:e00458. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3628085/>
51. Sonnleitner, B. 2006. Basic Biotechnology. Third edition. Cambridge Uni. Press. Pg 254.

52. Sulyanto, R.M., Thompson, Z.A., Beall, C.J., Leys, E.J. & Griffen, A.L. 2019. The predominant oral microbiota is acquired early in an organized pattern. *Sci Rep.* 9:10550. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6646312/>
53. Thomas A.M., Gleber-Netto F.O., Fernandes G.R., *et al.* 2014. Alcohol and tobacco consumption affects bacterial richness in oral cavity mucosa biofilms. *BMC Microbiol.* 14:1-12. <https://pubmed.ncbi.nlm.nih.gov/25278091/>
54. Timby, N., Domellof, M., Holgerson, P.L., West, C.E., Lonnerdal, B., Hernell, O., & Johansson, I. 2017. Oral microbiota in infants fed a formula supplemented with bovine milk fat globule membranes- a randomized controlled trial. *PLoS ONE.* 12:e0169831. <https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0169831>
55. Turnbaugh P., Ley, R., Hamady, M., Fraser-Liggett, C., Knight, R. & Gordon, J. 2007. The human microbiome project. *Nature*, 449, 804-810. <https://www.nature.com/articles/nature06244>
56. Verma, D., Garg, P.K., & Dubey, A.K. 2018. Insights into the human oral microbiome. *Arch. Microbiol.* 200:525-540. <https://pubmed.ncbi.nlm.nih.gov/29572583/>
57. Wade, W.G. 2013. The oral microbiome in health and disease. *Pharm Res.* 69:137-143. <https://pubmed.ncbi.nlm.nih.gov/23201354>
58. Wang, Q., Chen, X., Hu, H., Wei, X., Wang, X., Peng, Z., Ma, R., Zhao, Q., Zhao, J., Liu, J. & Deng, F. 2021. Structural changes in the oral microbiome of the adolescent patients with moderate or severe dental fluorosis. *Sci Rep.* 11:2897. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7859183/>
59. Xiao, J., Fiscella, K.A., & Gill, S.R. 2020. Oral microbiome: possible harbinger for children's health. *Internat J Sci.* 12:12. <https://www.nature.com/articles/s41368-020-0082-x>
60. Ying, S., Zeng, D., Chi, L., Tan, Y., Galzote, C., Cardona, C., Lax, S., Gilbert, J. & Quan, Z. 2015. The influence of age and gender on skin-associated microbial communities in urban and rural human populations. *PLoS One.* 10:e0141842. <https://pubmed.ncbi.nlm.nih.gov/26510185/>
61. Zhang, G., Chen, R. & Rudney, J.D. 2008. *Streptococcus cristatus* attenuates *Fusobacterium nucleatum*-induced interleukin-8 expression in oral epithelial cells. *J Periodontal Res.* 43:408416. <https://pubmed.ncbi.nlm.nih.gov/18942189/>

Tables

Table 1: 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

Table 2: Mean CFUs 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 ± 6.58x10 ⁶	2.91x10 ⁶	3.50x10 ⁶	1.69x10 ⁷	4.23x10 ⁷
	5.07x10 ⁶ ±	5.37x10 ⁶ ±	4.87 x10 ⁶ ±	1.52 x10 ⁷	3.00x10 ⁵ ±
	1.80x10 ⁶	4.03x10 ⁶	2.51x10 ⁶		6.05x10 ⁴
NeuroPro	2.40x10 ⁵	4.02x10 ⁵ ±	1.16x10 ⁶ ±	9.15x10 ⁴	2.03x10 ⁵ ±
		4.37x10 ⁵	1.59x10 ⁶		1.02x10 ⁵
	2.59x10 ⁷ ±	1.71x10 ⁷ ±	4.25x10 ⁷ ±	7.89x10 ⁷ ±	1.04x10 ⁸ ±
Enflora	2.80x10 ⁷	1.82x10 ⁶	6.72x10 ⁷	2.60x10 ⁷	1.57x10 ⁷
	3.90x10 ⁵	1.82x10 ⁵ ±	9.30x10 ⁴ ±	6.80x10 ⁴	5.03x10 ⁴ ±
		1.80x10 ⁵	1.31x10 ⁴		4.81x10 ⁴
	2.40x10 ⁷ ±	2.77x10 ⁷ ±	4.42x10 ⁷ ±	3.35x10 ⁷ ±	4.99x10 ⁷ ±
	5.70x10 ⁷	2.91x10 ⁷	2.14x10 ⁷	3.35x10 ⁷	3.50x10 ⁷

Table 3: Mean pH values of *S. mutans* and *S. mitis* over time. Reported error is standard deviation of the mean (n = 3).

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

Table 4: 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 nn	0.026 nn	nn 0.029	nn 0.315	<0.001 0.033
NeuroPro	nn	nn	nn	var=0	0.003
Enflora					

Supplemental Tables

Table S1: 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 were 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

Table S2: 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 S3: 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	

S4: 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	