# Dietary Factors and Microbial Profiles Related to Caries in Adults

Thesis

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By

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# ABSTRACT

Dietary and microbial factors were evaluated in 21 healthy subjects and 21 subjects with severe dental caries. An interview based 24-hour multiple-pass recall questionnaire to record dietary information. Supragingival plaque was collected from both healthy teeth and carious lesions. The bacteria were identified and quantified using an unbiased genetic technique. Analysis of the dietary questionnaire revealed that the exposure time of carbohydrates on the teeth was significantly different between the groups; exposure time also correlated with caries increment. There were no significant differences between percent RDI calories, total carbohydrates, or body mass index. Analysis of the bacterial profiles in plaque showed that the most significantly disease-associated bacteria are *Streptococcus mutans*, Lactobacillus species, and Veillonella species. The significantly health-associated species are *Streptococcus mitis*, *infantis*, *pneumoniae*, and *oralis*. This study shows that the eating behavior may be the most significant contributor to dental caries, which involves many different species.

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## VITA

| | |
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## FIELDS OF STUDY

Major Field: Dentistry

Minor Field: Prosthodontics

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# CHAPTER 1

# INTRODUCTION

## *Dental Caries*

Dental caries is a worldwide disease affecting every population throughout life. Some estimate that caries affects around 85% of adults. The disease is known to cause pain, infection, economic loss, and reduction in quality of life.[1] Oral health has recently become recognized as important to overall health. The report by the surgeon general in 2000 emphasized the importance of preventing oral diseases, specifically dental caries. It is known that dental caries is a preventable disease with a combination of "individual, professional, and community measures".[2]

## *Diet and Disease*

The process of dental caries has been carefully studied, from the etiology, microbiology, and epidemiology. It is well established that caries is caused by bacterial acids, which are the by-products of metabolism of dietary carbohydrates. However, in clinical disease, the role of dietary carbohydrates is not

straightforward. Although there is a clear relationship between the amount of sugar consumed and caries in developing countries, the relationship is lacking in industrialized countries.[3] This suggests that there may be additional factors that contribute to caries beyond simply the amount of dietary carbohydrate. Historic evidence from the Vipeholm study indicates that between meals snacking is a significant contributor to caries.[4] Recent published evidence does not consistently support a link between dietary carbohydrate consumption and increased caries. In a systematic review of 36 studies relating carbohydrate consumption with caries, only two studies reported a strong correlation, while most showed a weak or non-existent relationship.[5] Reports also indicate that categorization of the type of sugars may not be sufficient to explain the differences in caries experiences of children.[6]

The association between dietary carbohydrates in beverage form and caries is mostly supported by clinical observation, and only weakly supported by evidence. Recent letters published in the Journal of the American Dental Association urge dentists to make their communities aware of significant caries problems caused by carbonated beverages.[7,8] In particular, these dentists report an increase in rampant smooth surface caries occurring in patients with a pop drinking habit.[7,8] Some research supports the idea that high intakes of different beverages may indicate caries risk.[9] Children with high carbonated beverage consumption had higher risk than children with high juice, milk, or water consumption.[9] An analysis of the Third National Health and Nutrition Examination Survey (NHANES III) found that carbonated beverage consumption was associated with an increased DMFS in

persons over the age of 25.[10] Epidemiological data from adults with a high incidence of caries reports carbonated beverage consumption as a risk factor for caries.[11] Simply identifying specific food or drinks that may be indicators for high risk eating behavior would be beneficial to patients.

In addition, several studies have evaluated diet and caries with regards to obesity. Children from the Iowa fluoride cohort were examined by Marshall *et al*. The findings from this study show that caries and obesity coexist in children of low socioeconomic status. [12] Another study utilizing NHANES data reported no association between caries and obesity in children.[13] Some report that obesity and overweight status does predict more caries in adolescents and frequent snacking in early childhood can indicate a higher risk for caries later in life.[14] However, a systematic review of the literature indicated that only one study with a high level of evidence could demonstrate a relationship between obesity and caries.[15] While this research does not conclusively show whether diet may influence body weight as well as caries status, eating behaviors may be the common link in both conditions.

*Evaluating Dietary Factors: Frequency*

It would be beneficial if clinicians could have more information about which patients are most affected by dietary factors, and how much and how frequently carbohydrate consumption is significant. The World Health Organization has provided some dietary recommendations to decrease caries. They summarize

research which indicates that eating or drinking free sugars more than 4 times per day or at a level of more than 10% of the total energy intake creates higher caries risk.[16] More specific questions about dietary habits need to be developed. For example, in many studies, only the amount of food and drink is recorded, not the frequency. In addition, some record both mealtime and between meal eating and drinking and do not separate them. In some studies, food "frequency" does not consider the actual frequency of eating events, but only the frequency of choosing a particular food or drink. Even when the number of eating events per day is recorded, the amount of time that a subject takes to finish eating or drinking for that event is not evaluated. It seems appropriate to develop a way of estimating the time that substances are in contact with the teeth, since available information about the microbiology of caries supports the idea that production of acid regularly follows the consumption of carbohydrates, thus contributing to caries.

*Evaluating Dietary Factors: Type*

The type of food or drink has also been traditionally evaluated to determine cariogenicity. It is generally accepted that some sugars like lactose and starchy foods are less cariogenic than sucrose containing foods.[17] High intakes of different beverages may also indicate caries risk, as shown by Sohn *et al.*, in 2006. Children with high carbonated beverage consumption had higher risk than children with high juice, milk, or water consumption.[18] However, ingredients may be only one

explanation to support the anecdotal observation that patients with specific soda drinking habits (Mountain Dew®) have more severe caries. This beverage has more caffeine than other beverages.[19] The patients who drink these beverages may drink more or more often than patients who drink other beverages. The advertisement of these products may also be targeted towards a different population possibly at higher risk for caries. This example indicates that the type of food or drink may have an influence on the disease. This implication is not unsupported; investigators have concluded that certain foods or drinks are more damaging than others.[20] Although it may not be possible to answer all of these questions with a diet survey alone, it would be valuable in gaining some knowledge about the effect of the composition of diet on disease.

The presence of organic compounds in the diet is an area of interest. For example, phenolic compounds from the cranberry fruit are known to inhibit *Streptococcus mutans*, a major cariogenic bacterial species.[21] Other compounds found in cocoa, green tea, and grape seed are also being evaluated for their effect. Also, citric acid present in many other products is able to chemically erode enamel by its ability to cause desaturation of calcium and hydroxyapatite in saliva. This may also prevent remineralization of tooth structure following demineralization by bacterial acids. It is known that many species of *Lactobacillus* are capable of utilizing citric acid as an energy source.[22] The presence of citric acid causes increased growth of the bacteria and increased production of acid.[23] Lactobacilli have been frequently isolated from carious lesions[24], and seem to be important in the pathogenesis of

caries. Although *in vitro S. mutans* has the ability to utilize citrate, is seems to be adversely affected by its presence.[25] It is unknown if citrate metabolism occurs in the oral environment, but the effect of this and other unknown dietary factors is only clinically significant if a large difference is observed in the composition of bacteria or the severity of disease.

*Specific Aim*

It would be beneficial if clinicians could have more information about how much and how frequently carbohydrate consumption is significant. Our aim was to determine the dietary contributors to caries, and to do this we adapted a standard dietary assay, a 24-hour multiple pass recall examination, to include variables that might specifically contribute to dental disease. In this article we describe dietary information, including eating behaviors, of a group of 21 healthy participants and 21 participants with severe dental caries.

# MATERIALS AND METHODS

*Development of study instruments*

Many patient variables were recorded for this study. Patient factors and habits as well as the microbial data needed to be defined and recorded. Existing methods for recording patient factors were used. For example, demographics, caries severity, and measures of gingival health could be recorded based on standard techniques. However, some factors like dietary habits needed to be measured with consideration of factors that have not been well defined. The exposure time of the teeth to acid is the main variable that has not been standardized. A questionnaire was designed to highlight this variable.

The multiple pass technique is used to gather information about the participant's diet over the last twenty-four hour period. It begins with asking the participant to list all foods and drinks consumed. This list does not need to be in any order and is open-ended, without any suggestion or probing. Next, nine food and drink categories are listed with examples to elicit forgotten items. For this study, the example foods were selected in several instances because they are common possibly higher or lower risk foods. Also, the beverage category was expanded because

beverages are frequently implicated in caries risk. The third step is to review each item and ask when and for how long each item was consumed. The time of day was recorded exactly as the participant stated. The length of time was recorded in five minute increments that were always rounded up. If a participant answered with more than one time, like “ten or fifteen minutes”, the greater amount of time was recorded. These questions were open-ended, however, if a participant was confused about the question, a probe question was used; “From the time you started to the time you finished eating or drinking that item was is around 5 minutes, 20 minutes, or an hour or more?” When the participant indicated a time longer than 60 minutes, a follow-up question was used; “Did you have at least one bite or sip every 30 minutes during that time?” The fourth step was to gain detail about the items. Visual aids were provided to estimate portion sizes. Details about the brand, preparation, and amount of items were recorded. Several probe questions designed to obtain forgotten items were asked at this point. Finally, the participant was asked to list any other items. A few additional questions were asked unrelated to the 24-hour recall, but important to the study. The five-step multiple pass questions in this specific order provided a detailed protocol for recording the dietary habits of participants, including exposure time.

*Identification and Selection of Study Participants*

The clinical protocol for this study was reviewed and approved by the Ohio State University Institutional Review Board. Patients presenting to the Ohio State University College of Dentistry for treatment were evaluated for participation. Faculty and students in the pre-doctoral Comprehensive Care Clinics, Advanced General Dentistry, and Advanced Prosthodontics clinic were contacted by a single examiner to seek out eligible patients. Once possible participants were identified, the examiner determined their eligibility. Participants must: be 18 years or older, have at least 20 teeth, and speak English. They may not: have diabetes, have xerostomia, abuse amphetamines, have taken antibiotics within the past thirty days, or have had a prophylaxis within the past thirty days. The healthy controls also must have no smooth surface caries and have not had a restoration placed within the past 5 years. The caries group must have at least six cavitated carious lesions with at least three on accessible smooth surfaces.

*Clinical Protocol*

Once patients have been identified as eligible for the study, the clinical protocol was performed by one examiner. Written consent was obtained from all participants. A brief oral exam was performed to record the number of teeth, their

condition, gingival inflammation, and plaque. Plaque samples were collected after the oral exam.

After the oral exam and plaque samples, further information and the diet questionnaire was administered. This occurred in a private office, not in the treatment cubicle. The general information form was completed by interview by a single examiner. The diet questionnaire was administered by interview as well. Visual aids were available for this questionnaire. The participants were offered a copy of their consent forms at this time. The participants were given a choice of a parking voucher or dental supplies in return for their participation. This clinical protocol was utilized for all participants.

*Analysis of Nutritional Variables*

Participants completed a 24-hour recall of food and drinks with details regarding timing. This information needed to be systematically reduced into meaningful variables, like total calories or total exposure time. The completed recording form for the questionnaire is like a timeline of events. Both nutritional information and timing information was analyzed.

First, each food or drink item was entered into an online program called Nutrition Analysis Tool.[26] Nutritional information in this online database is from the USDA. Not every food or drink is included, so approximations had to be made for

some items. For example, if an item was recorded as “Red Bull”, the item closest to matching is “Carbonated beverage; Cola”. Next, portion sizes were entered for each item. The participant’s age and sex were used by the program to calculate the recommended value for nutrients. After the items were reviewed for accuracy, the final data was presented and recorded. On occasion, there were some generalizations made during the analysis of the data. Whenever insufficient detail was present from the recording form an average item was used consistently for all cases of insufficient detail. For example, if an item was recorded as “2% Milk”. There are several options for this type of milk in the database, but in all cases the item “Milk; Cow, Lowfat, Past & Raw, Fluid, 2% fat, w/added NFMS & Vit A” was used. Overall, the program processed the dietary data into several variables of interest.

*Analysis of Exposure Time*

Additionally, the timing data needed to be processed to obtain the variable exposure time. All of the events were first examined to determine if they caused exposure to bacterial acid. Any item that contained less than 1.5 grams of total carbohydrates for the entire serving was not included in the analysis for exposure time. This means items like water, black coffee, or sugar free gum were excluded. All other items with greater than 1.5 grams of carbohydrates were analyzed by creating a timeline beginning with the first event, or exposure. If another event occurred during that time, it would be accounted for in the timeline. This means that the total

number of minutes was not simply added up to produce the exposure time, but that the number of minutes was added once factoring in possible overlapping events. The exposure time was obtained by following this process for all participants.

*Analysis of Short-term and Long-term Beverages*

The type of beverage that was most frequently consumed was evaluated. This was accomplished by selecting the item that was consumed for the longest time period during the 24-hour recall. Only items that were consumed for 30 minutes or longer were considered. This was an attempt to determine which beverage if any could be implicated as a significant repeated exposure. Also, the subjects were asked if they had a favorite drink that they had nearly everyday. This gave the long-term, typical, or favorite beverage. On occasion the subject would give more than one response. In this case the first response was the recorded typical beverage. These beverage choices were categorized as high or low carbohydrate beverages. The low carbohydrate beverages included: none, water, diet sodas, black coffee, and flavored waters. All others were high carbohydrate beverages. The presence of caffeine was ascertained by the manufacturer's nutrition information. This categorization allowed the comparison of the type of beverage most frequently selected by the participants over a 24-hour period and the favorite or long-term beverage choice.

In addition, the participants were asked about their assessment of snacking habits. The question, "Do you consider yourself a frequent snacker?" was asked. If

clarification was needed, the statement, “Do you think that you eat or drink frequently?” was used. These questions were used to identify the self-described snackers in both groups.

*Analysis of Citric Acid Exposure*

The dietary information was used to estimate the amount of citric acid exposure. Two types of exposures were evaluated: Short-term citric acid exposure and long-term citric acid exposure. To evaluate the short-term exposure, the dietary information was reviewed for each participant. Items that were consumed for 30 minutes or more were evaluated. The item with the highest citric acid content was reported as the short-term item. The long-term item was determined by recording the answer to the question “Do you have a favorite drink that you drink nearly everyday?”. Both the short-term and long-term citrate items containing citric acid were grouped into low citrate beverage (LCB), medium citrate beverage (MCB), and high citrate beverage (HCB) groups. These categories were defined by Haleblian et al. in 2008.[27] Potassium citrate supplements are used to treat patients with hypocitraturic calcium oxalate nephrolithiasis. The authors of the study analyzed the citric acid concentration of various beverages in an attempt to determine if these beverages could be used in place of the supplement. Many of the items reported by the participants in this study exactly match the beverages examined by Haleblian et al. However, some items do not match. Using information from this research,

reasonable estimations about unanalyzed beverages that do contain citric acid can be made. In the analysis, the authors report that beverages with citric acid, sodium citrate, or potassium citrate as the primary ingredients have a higher concentration of citrate.[27] For this study, if an item has citric acid of some type listed within the top five ingredients (excluding water), the item was determined to be similar to the analyzed beverages of the LCB group. If the item has a citrate source within the top three ingredients (excluding water), the item was determined to be similar to the MCB group.

In addition, the percentage of the standard item, lemon juice, was reported by Haleblian. For this study the average of this percentage was used as a numeric factor in the analysis. The conversion of the items from this study into the categories and numeric values is shown in Table 1. Although this method is not precise, the citrate concentration of most items is unknown. This estimation provides a way to group the dietary exposure of the participants to citric acid both in the short- and long-term.

**Table 1 – Citric Acid: Comparison of beverages analyzed by Haleblian[27] and the beverages categorized in this study. The citric acid content is expressed as a percentage of lemon juice, for this study the average of this percent was used. ∗The category of none was not a category established by Haleblian, it was only used in this study for the listed beverages.**

| | Citric Acid content as percent of lemon juice | Type of beverages analyzed by Haleblian | Citric acid value for this study | Type of beverages in this study |
|---|---|---|---|---|
| High Citrate Beverage | 80-99 | Orange juice, pineapple juice, reconstituted lemonade, not from concentrate lemonade, and lemonade flavored Crystal Light | 90 | Orange juice, lemon juice, lime juice, lemon drop |
| Medium Citrate Beverage | 20-50 | Cranberry juice, lemon flavored Gatorade, homemade lemonade | 35 | Gatorade, cranberry juice, Aquafina flavored water |
| Low Citrate Beverage | 0-20 | Diet Mountain Dew, Diet 7Up | 10 | Diet 7Up, Diet Mountain Dew, Sprite, Vitamin Water Focus |
| None | * | * | 0 | Coffee, sweet tea, wine, tea, Dr. Pepper, Coke, Monster, Pepsi, Diet Pepsi |

*Analysis of Participant Information*

Information about each participant was recorded in addition to the dietary survey. The date of birth, race, ethnicity, sex, height and weight of each participant was noted. The date of birth and the date of sample was used to categorize the

participants into groups. The groups applicable are 19-30, 31-50, 51-70, 71+. These groups, along with the participant's sex are used by the USDA to determine the recommended daily allowance of certain nutrients. Height and weight was entered into an online calculator from the National Institutes of Health (NIH) to determine body mass index (BMI).[28] These items were used as variables in the statistical analysis. All comparisons in all categories were made by Chi Square or Analysis of Variance (ANOVA), using the program JMP (SAS Institute, Carey, NC). A p value of 0.05 or less was selected as a level of statistical significance.

# RESULTS

## *Comparison of Groups*

The healthy and caries groups were compared to determine any significant differences in demographic, oral factors, and dietary variables. The participants were not matched. Patient factors like age, sex, race, and ethnicity were compared. Figure 1 and Tables 2 and 3 show the comparisons for these factors. None were found to be significantly different between the healthy and caries groups. However, for the race and ethnicity factors, the total number of responses in some squares was less than five.

The oral condition of the groups was evaluated by comparing the variables: number of surfaces with caries, DMFS, gingival inflammation, and plaque. These factors were all highly significantly different between the groups (Figure 2). Participants in the caries group had more surfaces with caries, higher DMFS, more gingival inflammation, and more plaque.

Many dietary factors were compared. Total carbohydrates, body mass index, and number of servings of carbonated beverages are shown in Figure 3. There is not a significant difference between the groups for any of these factors. In general, most participants tended to consume more than 100% of the RDI for calories and protein,

and their body mass index is higher than recommended. The participants were also asked whether their reported diet for the past 24 hours was typical or not. There was not a significant difference between the groups for this question, and 70% of all participants answered “yes”.

Other dietary factors were analyzed. The exposure time was compared between the healthy and caries groups. This is shown in Figure 3. This factor was significantly different. Also, the actual exposure time was compared to whether the participant stated that they were a snacker or not (Figure 4). There was not a significant association between the participant’s assessment of snacking and the actual 24-hour exposure time (Figure 4). The citric acid consumption over a short and long-term period was compared, along with the type of beverage typically consumed. The citric acid levels between the groups were not significantly different. However, there were some differences in the type of beverage consumed. The 24-hour and typical beverage choices of the caries subjects were significantly more often high carbohydrate beverages, rather than low. These beverage choices are shown in Table 4. The caffeine content of the beverage choice was not significantly different (Table 5). The dietary factors that were significantly different between the groups are exposure time and type of beverage consumed.

*Comparisons within the Caries Group*

Several factors were compared within the caries group to evaluate any interactions. Since the exposure time was significantly different between groups, the exposure time was compared to all other clinical and dietary factors. Interestingly, in the caries group the exposure time was directly related to the number of surfaces with caries, but not with any other factors (Figure 3). The exposure time was not correlated with DMFS. The exposure time also did not indicate the total carbohydrate or calorie consumption. The number of surfaces with caries was also not related to other factors like plaque or carbohydrate consumption. It was correlated with the amount of carbonated beverage consumption (Figure 3). The caries subjects who indicated that they were snackers did not have significantly more surfaces with caries, nor a higher DMFS. They did indicate high-carbohydrate beverage choices more often, but not statistically significantly. However, a high-carbohydrate beverage choice did not indicate a higher number of surfaces with caries. Also, since slightly more males were present in the caries group, sex was used to compare other factors, like number of surfaces with caries, plaque, and typical beverage. No factors were significantly different between males and females in the caries group, however, the sample size was small. In summary, the only significant factor related to caries was exposure time.

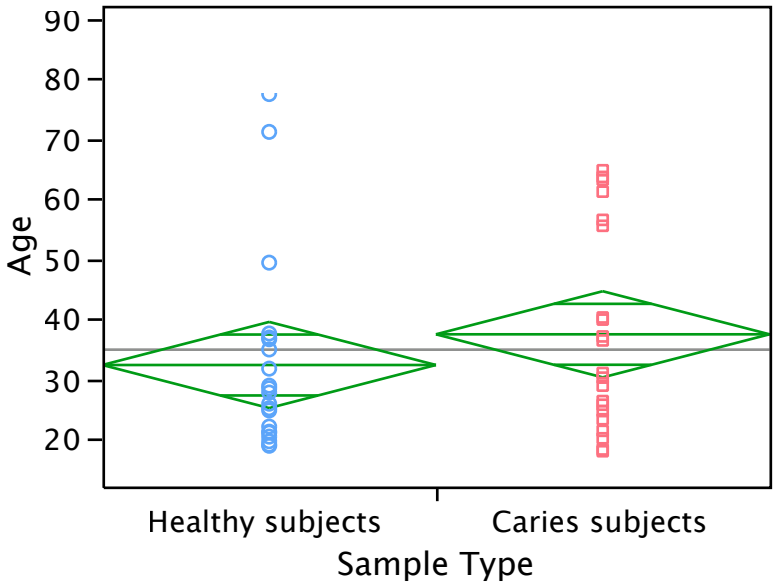


**Figure 1 – Age: Comparison of age of subjects in healthy and caries groups. Grey line represents mean, green diamonds width represents sample size, height represents 95% confidence interval.**

**Table 2 – Race: Comparison of race of participants in healthy and caries groups.**

| | American Indian | Asian | Black | More than one race | Unknown | White | Total |
|---|---|---|---|---|---|---|---|
| Healthy subjects | 0 | 1 | 3 | 0 | 1 | 14 | 19 |
| Caries subjects | 1 | 3 | 1 | 1 | 0 | 14 | 20 |
| Pearson | 0.4120 | | | | | | |

**Table 3 – Sex: Comparison of sex of participants in healthy and caries groups.**

| Sex | Healthy Group | Caries Group |
|---|---|---|
| Female | 12 | 6 |
| Male | 9 | 15 |
| Pearson | 0.0614 | |

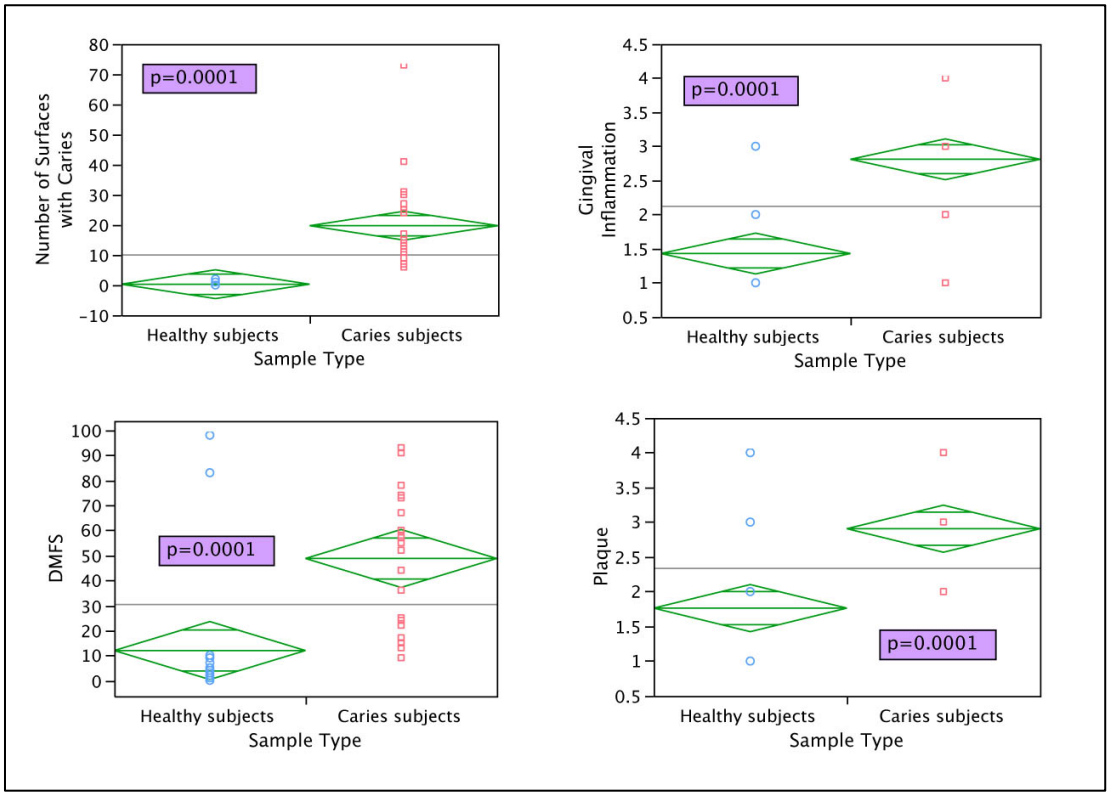


**Figure 2 – Oral factors: Comparison of number of surfaces with caries, DMFS, gingival inflammation (1 is lowest, 4 highest), and plaque (1 is lowest, 4 highest) between healthy and caries groups. Grey line represents mean, green diamonds width represents sample size, height represents 95% confidence interval.**

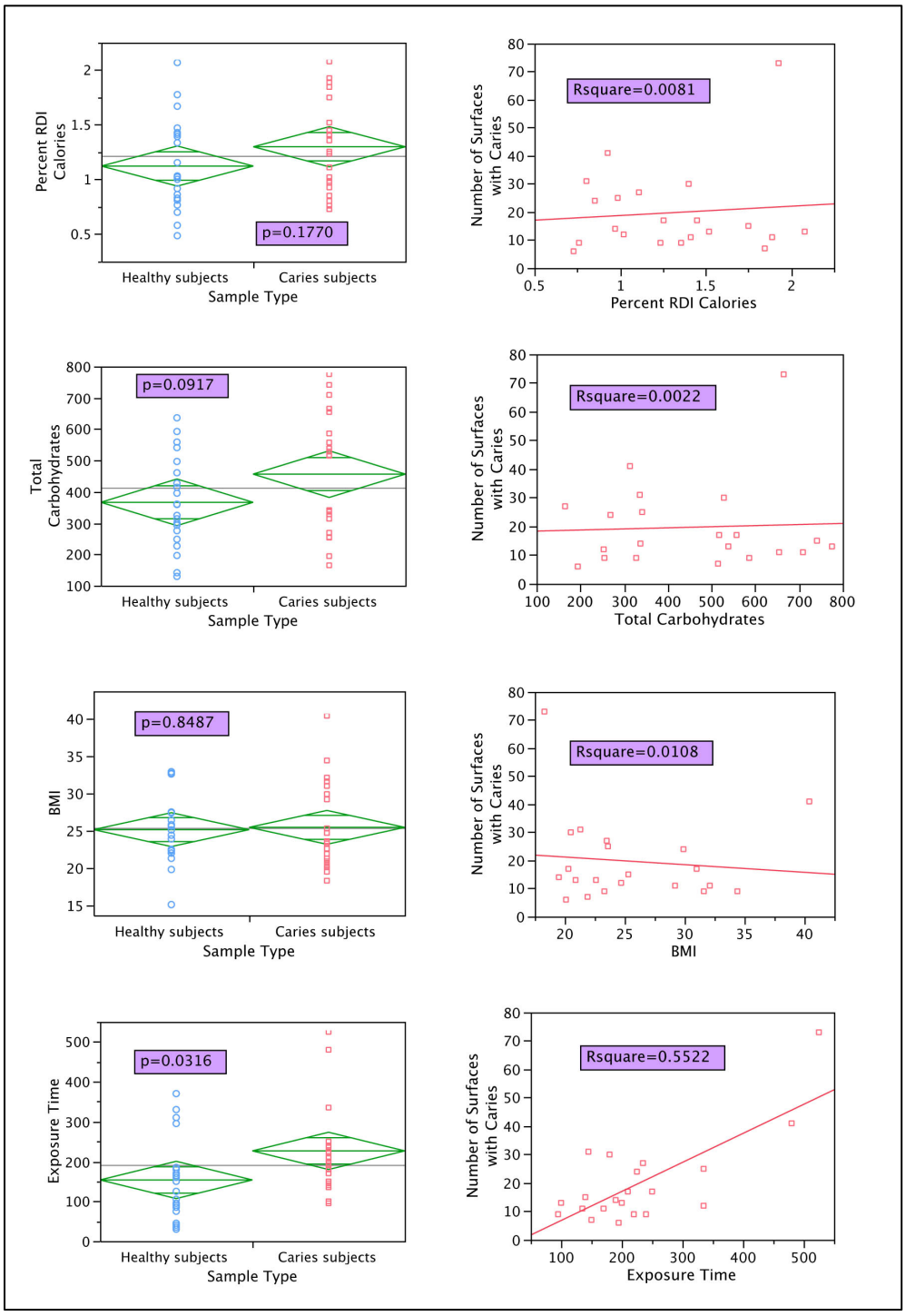


**Figure 3 – Dietary Factors: Left column: Four variables compared between the healthy and caries groups – Percent RDI calories, total carbohydrate (grams), body mass index, exposure time (minutes). Grey line represents mean, green diamonds width represents sample size, height represents 95% confidence interval. Right column: The same five variables plotted against number of surfaces with active caries in the caries group only. Red line shows the best fit.**

**Table 4 - 24-hour and Typical Beverages: Comparison of low and high carbohydrate level beverages (24-hour and typical) between healthy and caries groups.**

| | Low Carbohydrate 24-hour Beverage | High Carbohydrate 24-hour Beverage | Low Carbohydrate Typical Beverage | High Carbohydrate Typical Beverage |
|---|---|---|---|---|
| Healthy Subjects | 12 | 9 | 16 | 5 |
| Caries Subjects | 3 | 18 | 7 | 14 |
| Pearson | 0.0038 | | 0.0053 | |

**Table 5 – Caffeine: Comparison of Caffeinated Beverages (24-hour and typical) between healthy and caries groups**

| | Not Caffeinated 24-hour Beverage | Caffeinated 24-hour Beverage | Not Caffeinated Typical Beverage | Caffeinated Typical Beverage |
|---|---|---|---|---|
| Healthy Subjects | 15 | 6 | 15 | 6 |
| Caries Subjects | 12 | 9 | 11 | 10 |
| Pearson | 0.3340 | | 0.2037 | |

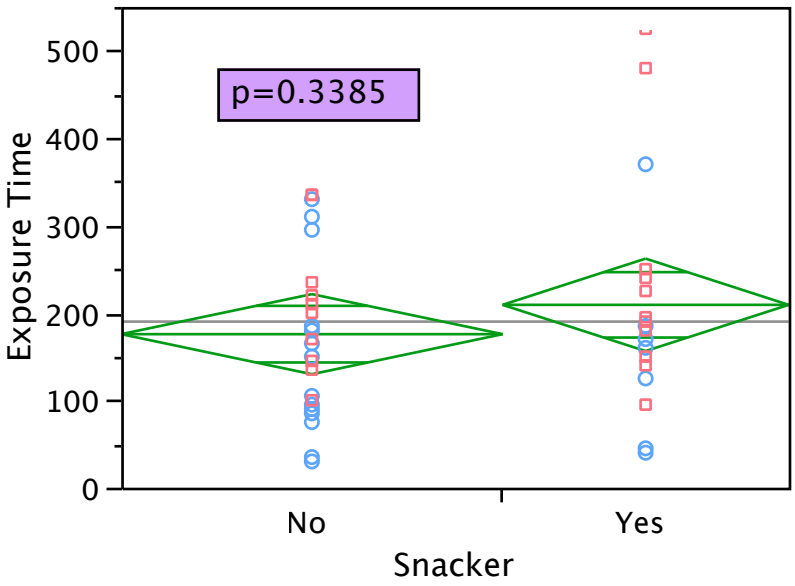


**Figure 4 – Snacking and Exposure Time: Participant evaluation of whether they are a frequent snacker or not plotted against actual exposure time in minutes. Red squares are caries subjects and blue circles are healthy subjects. Grey line represents mean, green diamonds width represents sample size, height represents 95% confidence interval.**

# DISCUSSION

The analysis of dietary factors provided several interesting and relevant results. Although the direct etiology of caries is microbial, the dietary factors are a clinically important subject. The dietary habits of patients may be easier to investigate and possibly modify through education. These results are valuable to determine which dietary habits are the most pertinent.

## *Selection of Study Population*

It is unlikely that dietary factors alone are responsible for caries in any population, however, it may be more sensible to select an adult population to somewhat isolate this variable. Significant research has been done on children and adolescents with varying results. In childhood, many other factors are important in development of caries, like time of infection and eruption, immune factors, general health, tooth development anomalies, and fluoride exposure. In addition, children's diets may be more controlled by caretakers and schools, but reporting of real dietary habits may be very inconsistent.[29] Adults who have rampant caries probably have newly developed risk factors or are experiencing the cumulative effects of a

low level risk factor. An analysis of the Third National Health and Nutrition Examination Survey (NHANES III) found that carbonated beverage consumption was associated with an increased DMFS only in persons over the age of 25.[30] This indicates that dietary factors may be more influential in adult patients. In addition, since the effect of diet seems to produce only weak correlations with caries in relatively healthy patients, selection of patients with severe disease will allow for a more clear association. Epidemiological data from adults with a high incidence of caries reports carbonated beverage consumption as a risk factor for caries.[31] The population in this study was appropriate, but frequency data for the drinks was not specific. For example, diet and regular soda drinks were not differentiated, and the time of contact of the drinks with teeth was not evaluated. A threshold level of sugar consumption may be required in this disease. Specific knowledge about this risk will improve the ability of dental professionals to counsel patients about diet. Certainly complete cessation of eating or drinking between meals is ideal, but many patients may be unwilling to follow this recommendation. Targeting the most damaging habits would be beneficial to patients.

*Dietary Questionnaire*

A modified diet questionnaire was utilized to gain more information than is available from previously used and published diet questionnaires. Measurement of macronutrients like total energy intake is best done with multiple 24-hour recalls,

but for foods with day-to-day variability a food frequency questionnaire is best.[32] Since the goal of this study is to identify the unvarying daily habitual food or drink, it is very likely that a single 24-hour recall will reveal the product of interest. The questionnaire will consist of a 24-hour recall administered by a multiple-pass method. An example of a multiple-pass method 24-hour recall is the widely used USDA survey.[33] The USDA model was used to develop the stepwise questions appropriate to the information needed in this study. Questions were designed to evaluate all foods, drinks, and snacks both at meals and between. The unique aspect of the modified questionnaire in this study is that the time that foods and drinks are in contact with the oral cavity was also evaluated. Since the questions are open-ended, trained interviewers are best to administer the questionnaire rather than participant administered.[34] Although no validity or reliability measures were undertaken in this study, 24-hour recall exams have produced acceptable validity in other studies.[35]

*Internal Validity*

The validity of the 24-hour recall questionnaire also must be examined. Investigating the daily habits of people can be difficult when direct observation is not possible. Relying on self-recall of information is most feasible, but can be unreliable for many reasons. People may not remember needed information, or may intentionally or unintentionally alter their responses. In dietary questionnaires,

these factors are inevitable, but can be somewhat controlled. The diet questionnaire was designed as an open-ended reporting of items to minimize under-reporting or generalizations. The main questions are scripted to minimize interviewer bias. In addition, bias can be introduced through many other aspects of a study. In this study, sampling of the population is a form of bias due to selection.

It may be suggested that because the participants are undergoing dental treatment, they may have altered their diet. All participants were asked if they had changed their diet significantly in the past year. Several participants did respond "yes" and were prompted to describe the changes. Some stated that they have started to eat more fruits and vegetables or have tried to eat less fast food. Most responses did not suggest that they have completely changed their diets in response to recommendations from their dentist. There is also the possibility that a 24-hour recall may not identify the dietary habits that are associated with caries because it is only a 24-hour period and people usually vary their diets from day to day. This is certainly a disadvantage of the 24-hour recall questionnaire. However, the majority of both healthy and caries groups indicated that their 24-hour recall diet was typical. On occasion, some subjects responded about why they stated that their 24-hour recall diet was not typical. Many reported that they ate more vegetables or less fast food on typical days. Some reported that the previous day was a holiday and so they felt that they ate more unhealthy items. However, the most significant variable, exposure time, is something that is not dependent on daily food choices, but may be more of a habit. The factors like total calories and carbohydrates are likely more

variable from day to day. These factors may be better examined by a week long recording, but for the scope of this study, that was not possible. Overall, the dietary questionnaire has limitations, but seems to be able to detect some significant differences between the dietary habits of the two groups.

*Comparison Between Groups*

The comparison of groups was useful to determine if any significant differences existed between groups that could be confounders. Since there were no significant differences between the age, sex, race, or ethnicity, it can be reasonable to assume that the groups are similar. Also, all participants were patients receiving treatment at the Ohio State University, so there may be some similarity of geographic area and socioeconomic status. However, these factors were not directly evaluated. Overall, these factors were not likely confounders for this study.

The comparison of the dietary factors produced several important relationships between the healthy and caries groups. Interestingly, BMI was not significantly different between the groups. This is in contrast to several other studies.[12,14] However, in one study, the relationship was very weak, and so for this study the sample size or the different population could account for the lack of difference.[36] The variables that recorded the total of what was eaten did not produce any significant relationships. This includes percent RDI calories, protein, total carbohydrate, and citric acid. This may mean that the amount of calories or even

carbohydrate may not be significant in the dietary factors responsible for caries. However, when the most frequently consumed beverage choice was evaluated in both the 24-hour period and long term, the high carbohydrate choice was seen more frequently in the subjects with caries. In addition, the amount of carbonated beverages consumed was not different between the groups but was associated with more carious surfaces in the caries group. This finding may relate to previous research which found an association between carbonated beverage consumption and caries in adults.[30] This may be a useful guide to identify patients at risk for caries. It is simple to ask what beverage was consumed the most frequently in the past day and also what is the favorite beverage. The question with the highest sensitivity was the evaluation of the 24-hour beverage, rather than asking for the favorite beverage. Asking the subject whether they eat or drink frequently or consider themselves to be a snacker may not be a good way to identify the risk. In this study the "yes" response to that question was not associated with the caries group or actual exposure time (Figure 4). Although the type of beverage choice was different between the groups the most significant finding was the exposure time.

*Exposure Time*

To examine the time that acids are in contact with the teeth, an estimation of the period of demineralization can be made. The initial research into the pH drop of dental plaque illustrated several variables in the exposure time. In 1944 Stephan

showed that the location of the plaque in the mouth and the caries activity status of the subject made a difference in the amount of pH drop and time to recovery following a carbohydrate rinse (Figure 5).[37] The most significant variable was the caries status of the subject. In general, patients with low or no caries activity experienced less of a drop of pH, while patients with high caries activity experienced a more significant drop. It is known from laboratory studies that when in a completely saturated solution, a pH of about 5.0 or lower is needed to cause enamel demineralization. [38] The pH was measured below 5.0 by Stephan in several circumstances. In general, all subjects experienced a lower pH for one hour following exposure to glucose.[37] In this study, this period of pH decrease was not factored in to our analysis. However, when 30 minutes was added to each eating event, the difference between the groups was even greater. Depending on several factors, the pH of the plaque can reach levels to demineralize the enamel for a varying period of time following exposure to dietary carbohydrate.

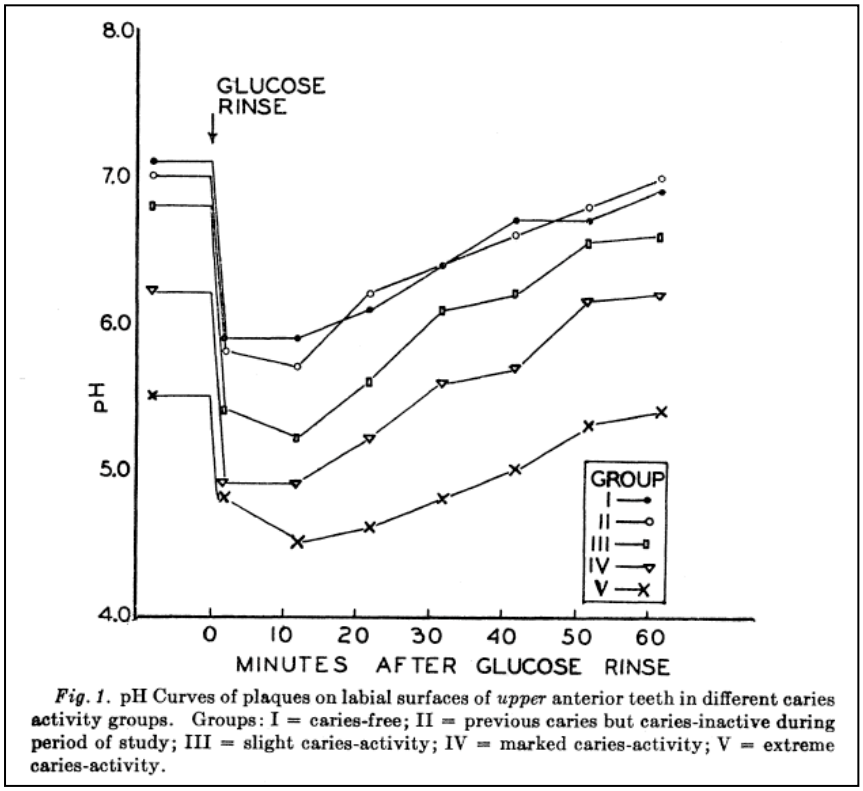


*Fig. 1.* pH Curves of plaques on labial surfaces of *upper* anterior teeth in different caries activity groups. Groups: I = caries-free; II = previous caries but caries-inactive during period of study; III = slight caries-activity; IV = marked caries-activity; V = extreme caries-activity.

**Figure 5 – pH Curves: From Stephan, 1944**[37]

The exposure time was a unique way to determine how frequently and how long the teeth are exposed to bacterial acids. This factor was very significantly associated with the caries group and the number of surfaces with caries. This may mean that this factor is most important in the dietary etiology of caries. This fits with the accepted model of caries on the bacterial level. As the subject ingests dietary carbohydrate, bacteria begin a complex set of events that results in the uptake, utilization, and storage of these carbohydrates. By-products of this sequence are acids which erode the tooth mineral. By repeating this sequence before the salivary components can replace the tooth mineral, permanent damage is done to

the tooth structure. The time periods for the demineralization to occur are not known exactly and may vary significantly. In fact, subjects without any caries may not reach pH 5.0.[37] This is when we believe the highest rate of demineralization occurs.[38] This difference in demineralization time is likely due to the amount and type of bacteria present in the plaque on the teeth. In healthy subjects, the same dietary carbohydrate exposure does not result in the same acid production because the bacteria are not specialized and selected for a frequently acidic environment. However, in this study, even when all subjects were assumed to have no bacterial response to carbohydrate, the amount of time was significantly different.

This study may indicate that the eating habits of patients with caries are more important than the actual dietary composition. The most damaging habit is the frequency and amount of time that any carbohydrate is consumed. It is associated with caries and the number of carious lesions. It is interesting to note that it was not associated with DMFS. This may be because DMFS is a long-term indicator of caries activity and that the dietary habits from a 24-hour recall are more predictive of the current caries state. This may also be a reflection of the idea that subjects with frequent eating and drinking habits may be less likely to have dental treatment performed and have more carious surfaces, but fewer restored surfaces. Also, it is possible that patients with more restorations have been educated throughout their treatment and have attempted to limit their food and beverage frequency. This association was not seen; DFMS was not related to any dietary variable. The

behavioral aspect of caries risk is a complicated subject, but some preliminary findings can be highlighted by this study.

# CONCLUSIONS

1. The exposure time was significantly higher in the caries group compared to the healthy group, while the percent RDI calories, total carbohydrates, and BMI were not significantly different.
2. The exposure time was significantly positively associated with the number of carious surfaces.
3. High carbohydrate beverages were chosen more frequently among the caries group over a 24-hour period. High carbohydrate beverages were more frequently listed as the favorite or typical beverage among the caries subjects.

# CHAPTER 2

# INTRODUCTION

Dental caries is a microbial disease with many complicating factors. The first writings on dental caries focused on various etiological agents, like nutritional, systemic, dietary, and microbial factors. While some of these are indeed implicated in dental caries, the actual cause of tooth demineralization resulting from caries is the bacteria in the form of plaque. Many researchers have attempted to better understand these bacteria. Early research focused on lactobacilli as the responsible group of bacteria and concluded that they were the only bacteria identified as having the ability to withstand the acidic environment of caries.[38] The specific plaque hypothesis proposed that certain bacteria like *Streptococcus mutans* and lactobacilli were more prevalent in some plaque which has the potential to become carious.[39] *S. mutans* has been well documented as a very pathogenic organism in caries.[40] With culturing dependent studies regarding caries, little has changed in regard to knowledge about the type of bacteria responsible for caries.

*Genetic Technique in Bacterial Identification*

Until recently microbial analysis of plaque has been limited by culturing and non-quantitative DNA techniques. With open-ended DNA techniques, a better understanding of the bacterial communities is possible. Limited evidence from pilot studies indicates that the microbial composition of carious plaque in patients is variable. The traditionally implicated organism *Streptococcus mutans* is not always present, and many other species may actually dominate. In one study, the dominant species present varied significantly between patients, and was either *S. mutans, Lactobacillus gasseri/johnsonii,* or *Lactobacillus rhamnosus*.[41] This is also reflected in pilot studies of caries in the young permanent dentition, in which *S. mutans*, lactobacillus species, or other bacterial species dominate.

The quantitative microbial analysis has been well established in previous studies. DNA-sequence-based assays can be used to identify closely related species that are difficult to differentiate using traditional, culture-based approaches. 16S rRNA sequence-based clonal analysis provides an open-ended method of molecular analysis that is not limited to previously known species, and so allows for the detection and identification of species that are refractory to detection by traditional cultivation-based methods and closed-ended DNA-based technologies such as hybridization or PCR-based assays. Our preliminary data has demonstrated that previously unsuspected species and presently uncultivated species may be important in the caries process.[42] For this reason we completed a systematic

investigation of the flora associated with rampant caries using direct DNA sequencing. We used 16S cloning and sequencing to identify species present in caries and in health. Using this molecular approach we will be able to identify species present in health and caries without the limitations imposed by reliance on cultivation.

The microbial analysis provides an unbiased representation of the bacterial species present. No selection of species is possible with the technique to be utilized. The data is objective and cannot be manipulated.

*Microbial Communities Associated with Caries*

The variability of composition of dental plaque could be caused by several proposed factors including: the age progression of the biofilm, the severity of disease, and the sampling site. Lactobacilli have been implicated as an organism that appears in caries secondarily once the lesion is well established.[40] Actinomyces species have been associated with root caries.[40] *S. mutans*, lactobacilli, *Actinomyces*, and several other species have been associated with root caries in a recent study utilitzing genetic techniques.[43] This study also indicated that the data showed significant subject-to-subject variability, meaning that the disease process is complicated.[43] The disease state of the sampling site also has a significant contribution to the type of bacteria isolated.[44] The sampling of well-established cavitated lesions reveals more acid-producing bacterial species than the sampling of

initial white spot lesions.[44] Another study did not find a difference between the carious dentin and the advancing front of the lesion.[41] The new genetic techniques have allowed for a more detailed view of the microbial communities involved with caries and the factors that influence these communities.

The additional proposed factor is the diet of the subjects. Although it is accepted that diet and microbial factors can influence clinical disease, the effect of diet on the microbial community involved in the disease has not been evaluated. Since the bacteria get a majority of their nutrition from the host's food, it seems plausible that the types of bacteria present may be a reflection of the type of nutrients available.

*Rationale and Aims*

Clinical management and therapies for treatment of caries are the most important end-point. However, although the microbial causes for caries have been known for some time, dental caries is still a nearly universal disease. Treatments are not curative, and recurrent caries is the most frequent cause for failure of fixed restorative dentistry. New approaches need to be developed to increase success. The ecological catastrophe hypothesis predicts that pathogenic organisms are selected for and propagate because of environmental factors.[45] There is a need for therapies to directly inhibit these organisms by altering the environmental factors.

The identification of the species responsible for caries is important to understand and better target them when treating disease and indicating preventive therapies.

The specific aims of this study are to identify and compare the microbial communities from both healthy and carious sites within subjects with healthy teeth and subjects with caries. Another aim is to compare the microbial information with other variables, like demographics, oral condition, and diet.

# MATERIALS AND METHODS

## *Identification and Selection of Study Participants*

The clinical protocol for this study was reviewed and approved by the Ohio State University Institutional Review Board. Patients presenting to the Ohio State University College of Dentistry for treatment were evaluated for participation. Faculty and students in the pre-doctoral Comprehensive Care Clinics, Advanced General Dentistry, and Advanced Prosthodontics clinic were contacted by a single examiner to seek out eligible patients. Once possible participants were identified, the examiner determined their eligibility. Participants must: be 18 years or older, have at least 20 teeth, and speak English. They may not: have diabetes, have xerostomia, abuse amphetamines, have taken antibiotics within the past thirty days, or have had a prophylaxis within the past thirty days. The healthy controls also must have no smooth surface caries and have not had a restoration placed within the past 5 years. The caries group must have at least six cavitated carious lesions with at least three on accessible smooth surfaces.

*Clinical Protocol*

Once patients have been identified as eligible for the study, the clinical protocol was performed by one examiner. Written consent was obtained from all participants. A brief oral exam was performed to record the number of teeth, their condition, gingival inflammation, and plaque. Plaque samples were collected after the oral exam. The plaque sampling procedures differed for the two groups.

For the healthy group, three teeth without caries were selected for plaque sampling. At least one posterior and one anterior tooth was selected. When possible, teeth were selected in different quadrants. Teeth with visible plaque were preferred. The supragingival plaque on the buccal or lingual surface of the selected teeth was collected by rubbing a microbrush on the surface for several seconds. The microbrush was placed into a sterile microtube and cut, so that the microbrush tip remained in the microtube. This collection procedure was repeated for each tooth sampled. The final sample consisted of pooled plaque from three non-carious teeth for each participant.

For the caries group, the same procedure was followed to collect plaque from three healthy teeth each in the participants with caries. In addition, three cavitated carious lesions were selected for sampling. When possible, teeth were selected to be both anterior and posterior, in several quadrants. Plaque was sampled by rubbing a microbrush around and within the cavitation for several seconds. The microbrush was stored as previously described. The final samples consisted of pooled plaque

from three healthy teeth and pooled plaque from three carious lesions from each participant in the caries group.

After the oral exam and plaque samples, further information and the diet questionnaire was administered. This occurred in a private office, not in the treatment cubicle. The general information form was completed by interview by a single examiner. The diet questionnaire was administered by interview as well. Visual aids were available for this questionnaire. The participants were offered a copy of their consent forms at this time. The participants were given a choice of a parking voucher or dental supplies in return for their participation. This clinical protocol was utilized for all participants.

*Laboratory Protocol*

Collected plaque samples were processed to produce DNA sequences of the bacteria within the sample. All samples were frozen shortly after collection and remained stored at -20. DNA was isolated and a specific segment amplified from the samples. The segments were then cloned into *Escherichia coli*. Following incubation, the inserted DNA in the *E. coli* is amplified for sequencing. This process requires many steps and takes several days.

First, the DNA is isolated with the Qiagen DNA isolation kit. The concentration of DNA is measured using a nanodrop spectrophotometer. Total DNA

for the PCR should be within 50-100ug. No more than 4ul of DNA should be used for each sample, so the DNA may need to be diluted or concentrated to fall within these limits. The primer used is from the gene for the 16S portion of the bacterial ribosome. The primers are 317 and 318 universal bacterial primers. *E. coli* is used as a positive control and water is used as a negative control. A portion of the PCR products are visualized on an agarose gel stained with ethidium bromide to confirm that a segment of appropriate length was amplified from each sample. Once confirmed the segments will be cloned.

Next, the PCR products are purified with the Qiagen DNA purification kit. Again the concentration is measured and should be between 4-7ng/ul. Cloning was performed using the TOPO TA Cloning® kit from Invitrogen™. The DNA is combined with the vector and incubated. This solution is added to the competent *E. coli* cells which are heat shocked. The cells are plated onto LB (Luria-Bertani) medium with ampicillin and X-gal. After incubation for about 18 hours, colonies that have a white appearance have the inserted DNA segment. 95 of these colonies are selected randomly and mixed with PCR reagents. The PCR products from this amplification are visualized on agarose gel stained with ethidium bromide to confirm the expected length. These segments are then sequenced.

Sequencing is performed by the Plant Microbe Genetic Facility laboratory at The Ohio State University. It is completed by the Sanger method of DNA sequencing. Completed sequences are returned electronically. These sequences are compared to

known sequences in the database. Sequences are given a score to indicate the percentage of similarity to the closest match. Each sequence represents a randomly selected bacterium from the original plaque sample. This allows for non-selective analysis of the microbial community in the plaque.

*Statistical Analysis*

The data is returned as a list of species present and percentage of the total sample from each subject. A total of 148 species were isolated collectively from all 39 subjects. Comparisons of the species were made between three levels: the difference between the healthy subjects and caries sites of the caries subjects, the difference between the healthy subjects and healthy sites of the caries subjects, and the difference between the healthy sites and caries sites of the caries subjects. In addition, all of the bacterial species were compared with the demographic variables, oral condition, and dietary variables. These comparisons were made by Analysis of Variance (ANOVA), using the program JMP (SAS Institute, Cary, NC), or PROC MIXED analysis using SAS (SAS Institute, Cary, NC). Samples within the caries group were matched, since each subject contributed two samples. A p value of 0.05 or less was selected as a level of statistical significance. This analysis provided a way to evaluate microbial differences between the sites and groups.

# RESULTS

Several generalizations about the results of this study can be made. Overall, the greatest differences were observed by comparing the healthy group with the caries sites in the caries group. For most species there was a consistent pattern of increasing or decreasing values from healthy subject, healthy site, to caries site. The type of bacteria (health or disease associated) determined whether the levels were increasing or decreasing between these groups. The other variables like demographics, oral factors, and dietary did have some significant effects on the prevalence of species within the samples.

The comparison of groups was useful to determine if any significant differences existed between groups that could be confounders. Since there were no significant differences between the age, sex, race, or ethnicity, it can be reasonable to assume that the groups are similar. Also, all participants were patients receiving treatment at the Ohio State University, so there may be some similarity of geographic area and socioeconomic status. However, these factors were not directly evaluated. Overall, demographic factors were not likely confounders for this study.

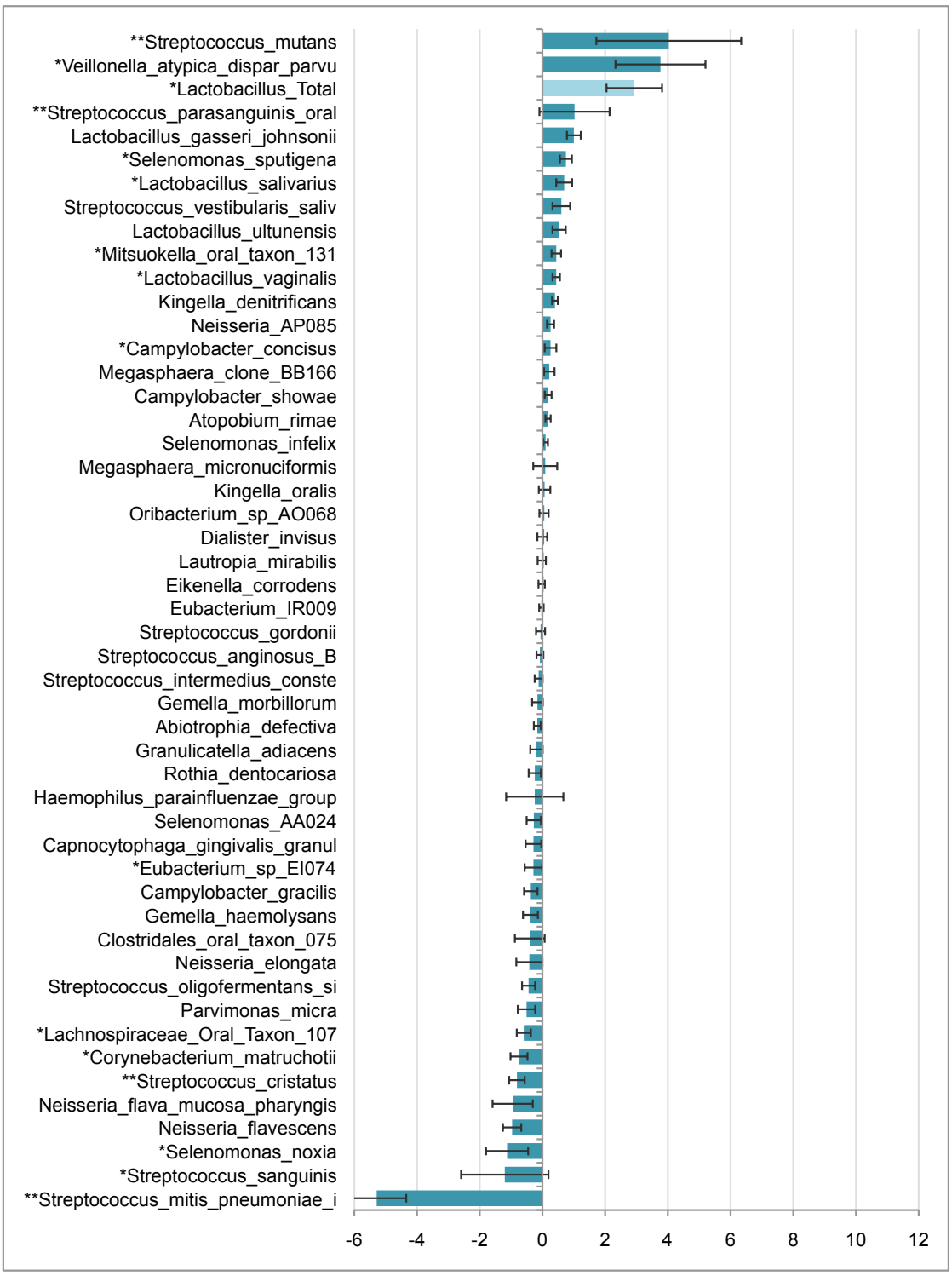


**Figure 6 – PROC MIXED: PROC MIXED analysis of top 50 most frequently isolated species. Light blue bar represents Lactobacillus Total (Total of all Lactobacillus species present in 160 species). Error bars represent 95% confidence intervals.**

*Streptococcus species, including mitis, pneumoniae, infantis-oralis*

The most frequently isolated species from all samples in this study was from this group of Streptococcus. An average of 20% of the bacteria from the samples belonged to this group. With ANOVA the statistically significant difference occurred between the healthy subjects and the caries sites from the caries subjects. There was also a statistically significant difference between the healthy site and caries sites when the subjects were matched and analysed with a paired T-Test. This species tended to present more frequently in the healthy group. In the caries subjects, the healthy sites had more of this group than the caries sites.

*Streptococcus mutans*

The third most commonly isolated species from all samples was *S. mutans*. Only two subjects in the healthy group had any *S. mutans* in their sample and the level was relatively low. The differences between the healthy subjects and the healthy sites was statistically significant as well as the difference between the healthy subjects and caries sites.

*Lactobacillus species, including gasseri, johnsonii, salivarius, ultunensis, vaginalis, fermentum, casei, paracasei, crispatus, and rhamnosus-casei*

Several species of Lactobacillus were grouped because they were relatively uncommon individually but were the sixth most commonly found among all samples. This group was not isolated from anyone in the healthy group, and was not isolated from many subjects in the caries group. When Lactobacillus species were found in the caries group, they generally made up relatively high percentage of that sample, up to nearly 40% in one case.

*Additional species more prevalent in the healthy group: Streptococcus cristatus, Streptococcus sanguinis, Selenomonas noxia, Corynebacterium matruchatii, Lachnospiracaea oral taxon 107, and Eubacterium species EI074*

These species were discovered rather infrequently, and in fewer subjects. However, subjects in the healthy group tended to have higher percentages of these species. Subjects in the caries group had higher levels of these species in their healthy sites compared to the caries sites. There were statistically significant differences between samples as shown in Figure 6.

*Additional species more prevalent in the caries group, Veillonella spp., Streptococcus parasanguinis~oralis, Mitsuokella sp. oral taxon 131, Selenomonas sputigena, and Campylobacter consisus*

These species were also infrequently isolated from the samples. In addition, when discovered within a sample, they did not occur in very high levels. For example, the highest level was less than 15% of the total isolates in one case. However, they were overall more prevalent among the subjects with caries, and within the caries sites rather than the healthy sites. There were statistically significant differences as shown in Table 6.

**Table 6 – ANOVA Comparisons of Species: Statistically significant differences between groups and sample sites for each species listed analyzed by ANOVA.**

| Species | Healthy group v. Caries site | Healthy group v. Healthy site | Healthy site v. Caries site |
|---|---|---|---|
| *Gemella haemolysans* | X | X | |
| *Streptococcus cristatus* | X | | |
| *Streptococcus sanguinis* | X | | |
| *Parvimonas micra* | | | X |
| *Neisseria elongata* | | X | |
| *Streptococcus parasanguinis* | X | | |
| *Mitsuokella sp. oral taxon 131* | X | | |
| *Selenomonas sputigena* | X | | |

*Interaction of demographic factors, oral conditions, and dietary factors*

The samples of the caries group were evaluated to determine if any demographic factors affected the population of bacteria. Although there were no significant differences between the oral conditions of males and females in this group, there were significantly higher levels of *S. mutans* within caries sites in female subjects (Figure 7). Females also had lower *S. mitis, pneumoniae, infantis-oralis* within the healthy sites. No other factors like age, race, ethnicity, number of surfaces with caries, plaque, or gingival inflammation were significantly associated with higher or lower levels of any species.

The dietary factors were also analyzed with the bacterial species from the caries group. The exposure time was correlated weakly, although statistically significantly, with three species. *S. sanguinis, Gemella morbillorum, and Kingella denitrificans* were all found to decrease with increasing exposure time. Interestingly, as total carbohydrates increased, the levels of *S. mutans* and *Selenomonas noxia* decreased statistically significantly in the caries sites. Within the healthy sites, the group *S. mitis, pneumoniae, infantis-oralis* increased with increasing carbohydrates. There were no other dietary influences on the bacterial species when all other factors like RDI calories, RDI protein, BMI, short and long-term beverages, caffeine content, carbonated beverage servings and time, and citric acid were considered. The bacterial species in the healthy group were also analyzed in the same way with no significant interactions.

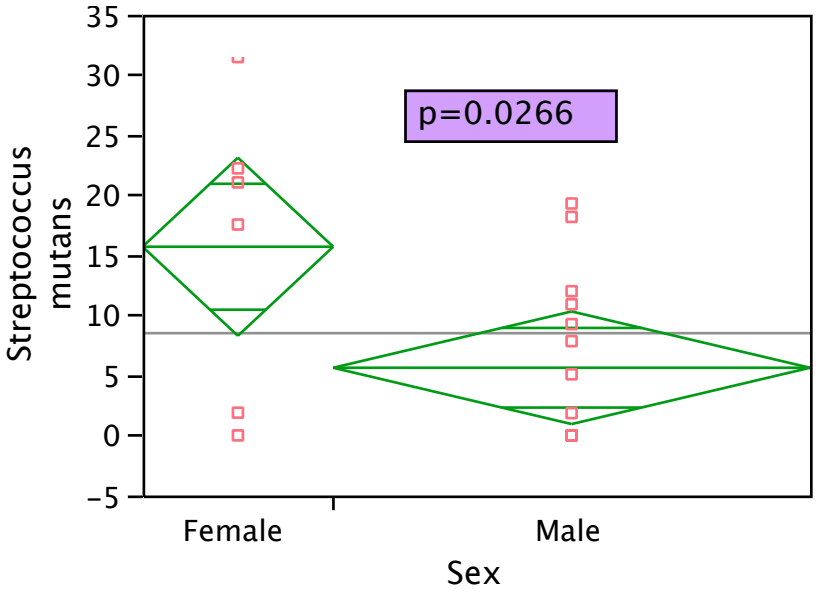


**Figure 7 – *S. mutans* and sex: Comparison of *S. mutans* levels between females and males within the caries group, caries sites. Grey line represents mean, green diamonds width represents sample size, height represents 95% confidence interval.**

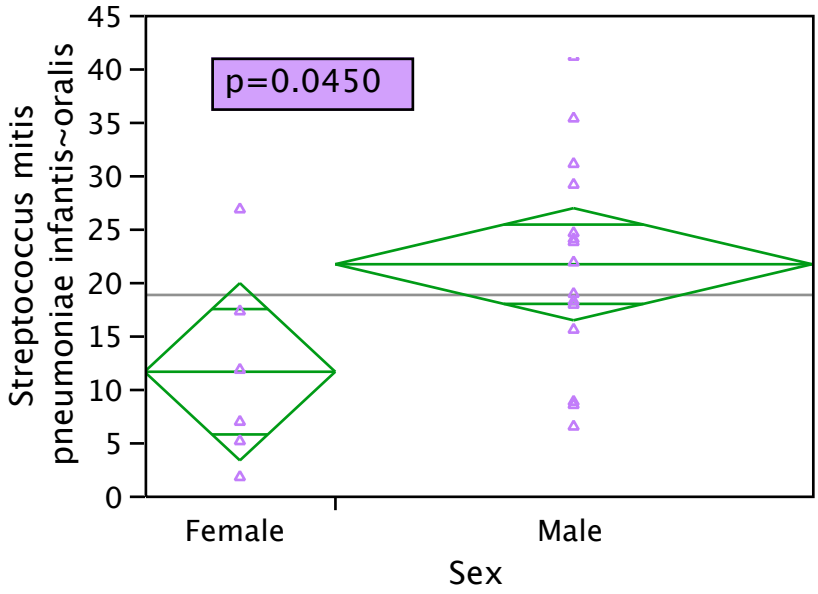


**Figure 8 – Streptococcus Species and Sex: Comparison of *S. mitis, pneumoniae, infantis~oralis* between females and males in the caries group, healthy sites. Grey line represents mean, green diamonds width represents sample size, height represents 95% confidence interval.**

*Subject-to-Subject Variability*

The most profound differences were the differences in dominant species between the samples. When viewing all of the isolates from just one sample it is evident that in general a few species tend to be much more prevalent than the others. A species that comprised more than 15% of a sample was considered to be a dominant species for that sample. A tabulation of all the dominant species found in the samples is found in Table 7.

**Table 7 – Dominant Species: Total number of samples in which the species comprises more than 15% of the isolates.**

| Species | >15% in healthy subject | >15% in healthy sites of caries subject | >15% in caries sites of caries subject |
|---|---|---|---|
| *S. mitis, pneumoniae, infantis-oralis* | 14 | 14 | 6 |
| *Veillonella atypical, dispar, parvula* | 10 | 11 | 11 |
| *S. mutans* | 0 | 2 | 6 |
| *Campylobacter gracilis* | 0 | 2 | 0 |
| *Streptococcus gordonii* | 1 | 0 | 0 |
| *Lactobacillus* total | 0 | 2 | 3 |
| *Eubacterium* sp group C | 1 | 0 | 0 |
| *Selenomonas noxia* | 1 | 0 | 0 |
| *Dialister invisus* | 0 | 1 | 0 |
| *Neisseria flavescens* | 1 | 0 | 0 |
| *Selenomonas* AA024 | 0 | 1 | 0 |
| *Neisseria* AP085 | 0 | 1 | 0 |

# DISCUSSION

With such powerful technology like the genetic techniques used for this study, a clear glimpse of the bacterial communities in plaque is possible. There are many interesting associations and interactions that are evident when reviewing the results. These results reaffirm that several species are associated, as previously suspected, with either health or disease. *S. mitis*, *pneumoniae*, and *infantis-oralis*, were most strongly associated with health. *S. mutans* and *Lactobacillus* were most strongly associated with disease. There is the possibility that other factors, like diet, may influence the microbial communities in undetermined ways. However, the most profound finding is that the communities from one subject to another are very variable. The results indicate that caries is a complex disease with many microbial interactions.

## *Streptococcus species, including mitis, pneumoniae, infantis-oralis*

This group of streptococci was the most frequently isolated group from this study. These species are grouped together because the genetic identification technique does not accurately allow for their differentiation. While this group is

found at high numbers in both healthy and caries subjects, it is significantly associated with health. This group was frequently the dominant species in the samples from healthy subjects and healthy sites. However, it was much less commonly a dominant species within the caries sites (Table 7). This may indicate that the pathogenic species, which are able to produce acid much better, can displace this group when caries occurs. This group was not found to be health or disease associated in one study on a pediatric population.[42] However, *S. mitis* has been reported as health-associated in a subsequent study.[44] Species from this group of streptococci were not frequently isolated in root caries or on healthy root surfaces in an elderly population.[43] Species from this group were also infrequently isolated from carious dentin in other studies.[41,46] These previous reports give an inconsistent view of this group. This study, however, shows that this group is very common in the plaque of adults with and without caries. Perhaps if the carious dentin was sampled, this group would have been rarely isolated from those samples. These bacteria are probably common inhabitants in health and may be simply displaced in disease by other species.

*S. mutans*

This species was isolated frequently among subjects with caries. In eight samples they comprised more than 15% of the isolates, making them a dominant species in several subjects. This species was clearly associated with carious lesions

and was also found at the healthy enamel sites of subjects with caries. This is consistent with the findings of a recent similar study on root caries. In that study, *S. mutans* was found at levels greater than 15% in two subjects in both plaque from healthy sites and carious dentin.[43] Similar findings have been reported for a pediatric population as well. *S. mutans* in this population is not present in all samples and dominates others.[44] Additional evidence indicates that *S. mutans* is not always found in cavitated carious sites, and may occur at very low levels.[46] This study overall reaffirms the role of *S. mutans* as a potent pathogen in the caries process.

*Lactobacillus species, including gasseri, johnsonii, salivarius, ultunensis, vaginalis, fermentum, casei, paracasei, crispatus, and rhamnosus-casei*

This group of bacteria represented a commonly isolated group found within the caries group only. Historically, this group was one of the first to be associated with caries. Animal studies have indicated that lactobacilli are associated with caries progression.[39] A recent study utilizing genetic techniques, evaluated the microbial composition of carious dentin. Lactobacilli comprised 50% of the species isolated from the samples.[46] This study did not observe similar frequencies of lactobacilli, they were less common. Only five of the samples in the caries group contained more than 15% lactobacilli. However, this is expected because that study sampled carious dentin, which is a site with further disease progression than the plaque over

cavitated lesions. In this study, lactobacilli were not associated with any clinical findings or dietary factors. However, since they were not frequently isolated, the sample size may be too small to eliminate possible interactions. The overall finding from this study is the clear association of lactobacilli with subjects with caries.

The possibility of interactions with the oral condition or dietary factors is interesting. There was not an increase of *S. mutans* with the number of surfaces with caries, or with plaque levels. The exposure time did not affect the levels of *S. mutans*. The only significant differences were unexpected. The levels of *S. mutans* did decrease with increasing amounts of dietary carbohydrates. The possibility that this statistically significant finding may be clinically insignificant or represent a random occurrence cannot be discounted. However, the possibility remains that this is a real result. Theoretically, the species *S. mutans*, while very acid-tolerant, is not the most acid-tolerant of all cariogenic species. Lactobacilli tend to be more aciduric, and actually a slight increase was observed for lactobacilli with increasing carbohydrates. The amount of carbohydrates may have resulted in more acid production, and could have caused this result. However, the evidence is not conclusive for this association.

*Other species associated with health or disease*

Several other species were associated with health or disease although they occurred at relatively low levels. None of these species ever comprised more than

15% of any sample. However, finding them with the techniques used means that they were present in significant numbers to be analyzed. *S. sanguinis* and *S. cristatus* have been associated with health in previous studies.[42,44] Low-pH non-mutans streptococci have been associated with initial white spot caries in children.[44] This would include the species *S. parasanguinis*, *vestibularis*, and *salvarius* which were associated with disease in this study. Chhour *et al*. described *Selenomonas* species and *Dialister* species as disease associated in advanced dentinal caries.[46] This supports the finding that *Selenomonas* species were associated with disease in this study and both families were dominant in two samples from caries subjects. The remaining species that were health or disease associated in this study have not been previously specifically associated. *Neisseria elongata* was health associated in this study and two other species in the Neisseria group were dominant species in both healthy and caries subjects. These findings may indicate that members of the same genus may be very different and have opposite clinical behaviors. This is very well known to be the case for streptococci. Several species were found to be health or disease associated in both low and high levels, indicating their possible contribution to or prevention of the caries process.

Aiding the acid producers is an important role that other bacterial species may play as well. The finding of *Mitsuokella* sp. oral taxon 131 as disease associated may indicate that this species is associated with caries, but not causing the actual demineralization. This species is proteolytic, and may simply be taking advantage of all of the tooth proteins that are being exposed during the caries process. However,

proteolytic species are very prevalent in deep caries, and may be important in the disease process as they break up the tooth structure allowing the acid producers to penetrate the tooth more.[47] This is not the only species thought to contribute to the virulence of caries pathogens. *Veillonella* species are very prevalent in both healthy and caries subjects. Species from this group are able to use lactic acid as a carbon source, which explains their high levels in dental plaque. They may aid the pathogens by providing nitrate reduction.[48] In fact, streptococci and *Veillonella* species are known to coaggregate and together are formers of the initial plaque biofilm.[49] This may explain why both of these groups were so numerous in the smooth surface plaque of healthy subjects. Since these subjects had significantly less plaque on their teeth it may be that this plaque is newly formed, and represents the initial colonizers of the tooth surfaces. Some studies have found that *Veillonella* are associated with disease, or are present significantly more in deep dentinal lesions.[44,42] Another study did not frequently isolate this group in carious dentin.[41] In this study, a disease-associated relationship was present, but was not statistically significant. It appeared that some caries subjects had very high levels of *Veillonella*, up to nearly 50% of all isolates, but the majority had levels consistent with the healthy controls. However, *Veillonella* levels were not observed to depend on any other factors, such as level of *S. mutans*, number of surfaces with caries, exposure time, or dietary carbohydrates. It appears that *Veillonella* species are present in both health and disease, but may increase dramatically in the presence of disease. The

ability to enhance the growth of pathogenic acid-producers is a key asset to several species and may provide a target for disease prevention and treatment in the future.

Above all else, the identification of bacteria responsible for the healthy state and the diseased state is important for the ultimate end-point: treatment of the patient. Vaccines for dental caries are currently being proposed. Antimicrobial therapies in the form of chlorhexidine varnishes and rinses are not yet proven effective.[50] Xylitol in the form of chewing gum is beneficial because it does not cause caries, may be anticariogenic, and does prevent the transmission of caries causing bacteria from mothers to their children.[51] Recent attention has focused on the identification of patients who have cariogenic plaque. The CAMBRA, or caries management by risk assessment, protocol publicized by Featherstone *et al*. relies on microbial testing of plaque to determine caries risk. One device measures ATP (adenosine tri phosphate) bioluminescence in an attempt to determine bacterial load. The evidence provided by the manufacturer is an unpublished abstract indicating that the ATP bioluminescence does correlate to salivary levels of streptococci.[52] The recent focus on microbial factors in caries control indicate that clinicians desire better therapies and preventive measures for their patients. This study is one small step in the process of better understanding those microbial factors. Unfortunately, the most consistent finding of this study is that caries is a complex disease that varies from one patient to another. However, several good conclusions about the relative prevalence of certain species in dental plaque can be made and are significant in the understanding of dental caries.

# CONCLUSIONS

1. *S. mitis, pneumoniae*, and *infantis-oralis* were significantly associated with health
2. *S. mutans* and *Lactobacilli* were significantly associated with disease
3. Subjects with caries had more disease associated species within their caries sites and more healthy associated species within their healthy sites
4. Additional bacterial species were observed to be correlated with disease and healthy in low and high levels
5. Subject-to-subject variability was high

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