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MOLECULAR ANALYSIS OF THE CARIES BIOFILM IN YOUNG PERMANENT TEETH

A Thesis

Presented in Partial Fulfillment of the Requirements for

the Degree Master of Science in the

Graduate School of The Ohio State University

By

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The Ohio State University
2006

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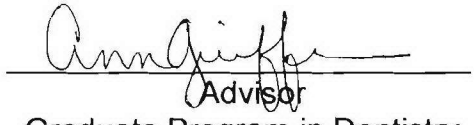

Advisor
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The views expressed in this article are those of the author and do not reflect the official policy or position of the United States Air Force, Department of Defense or the U.S. Government

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I wish to thank my advisor, Dr. Ann Griffen for her guidance and support during my two years of training in pediatric dentistry. If it were not for Dr. Griffen, and her interest in caries research, I probably would not have chosen the program at The Ohio State University. I am glad I kept her business card in my wallet for two years after meeting her for the first time at the 2002 IADR meeting in San Diego.

I would also like to thank Dr. Eugene Leys and Dr. Purnima Kumar for their support on my thesis committee.

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ABSTRACT

Dental caries is the most common infectious disease in childhood. Only a small percentage of children will go through adolescence and not develop caries, and the disease typically is concentrated in children from poor socioeconomic backgrounds with limited access to routine dental care. 16S DNA based bacterial sequencing technology is beginning to make it possible to more accurately identify the pathogens responsible. Most of the previous research has only focused on cultivatable bacteria. This has created a bias in the reported diversity of the bacterial communities in the oral cavity. The purpose of this study is to define the bacterial plaque diversity on permanent teeth in school age children by replacing the traditional culture techniques with new non-culture open-ended methods for identification. Nine caries-free subjects and 9 subjects with caries (8 to 16 years of age) were selected for identification of bacteria by cloning and sequencing 16S ribosomal DNA. A total of 43 plaque samples (34 samples collected from caries subjects and 9 samples from caries-free subjects) were analyzed. A total of 2,151clones were identified, and 112 species were detected. There was a large representation of known cariogenic taxa present. *Streptococcus* spp., *Veillonella* spp., *Lactobacillus* spp., *and Selenomonas* spp. were the most predominant genera found in samples. The bacterial flora on the tooth surfaces is quite heterogenous with a small number of taxa represented in the caries biofilm. The findings show that *S. mutans* and

Lactobacillus spp are the prominent bacteria in caries of young permanent teeth. While *S. mutans* is higher in white spot lesions, *Lactobacillus* spp clearly out number *S. mutans* in the advanced lesions past the initial demineralization of tooth structure.

ABSTRACT

Dental caries is the most common infectious disease in childhood. Only a small percentage of children will go through adolescence and not develop caries, and the disease typically is concentrated in children from poor socioeconomic backgrounds with limited access to routine dental care. 16S DNA based bacterial sequencing technology is beginning to make it possible to more accurately identify the pathogens responsible. Most of the previous research has only focused on cultivatable bacteria. This has created a bias in the reported diversity of the bacterial communities in the oral cavity. The purpose of this study is to define the bacterial plaque diversity on permanent teeth in school age children by replacing the traditional culture techniques with new non-culture open-ended methods for identification. Nine caries-free subjects and 9 subjects with caries (8 to 16 years of age) were selected for identification of bacteria by cloning and sequencing 16S ribosomal DNA. A total of 43 plaque samples (34 samples collected from caries subjects and 9 samples from caries-free subjects) were analyzed. A total of 2,151clones were identified, and 112 species were detected. There was a large representation of known cariogenic taxa present. *Streptococcus* spp., *Veillonella* spp., *Lactobacillus* spp., *and Selenomonas* spp. were the most predominant genera found in samples. The bacterial flora on the tooth surfaces is quite heterogenous with a small number of taxa represented in the caries biofilm. The findings show that *S. mutans* and

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INTRODUCTION

During the past 30 years, significant advancements have been made in the field of dentistry regarding dental caries. Focus on fluoride in municipal water systems, tooth paste, professional applications, hygiene instruction, prenatal care, and improved access to care have helped to lower the overall caries experience in this country. However, dental caries remains the most common chronic disease in childhood (1). Often labeled as "the silent epidemic"; dental caries is an infectious disease that is not evenly distributed. Those affected most are the individuals who have the most difficult access to care. Nearly 4.2 million children are unable to obtain needed dental care (2). By the age of 17, eighty percent of young people have had a cavity resulting from caries infection (3).

The estimated annual world wide financial burden is certainly in the billions, and current research has only begun to show that caries is a complex entity. The combination of host, environment, and bacteria factors comprise an accepted multifactorial model for the caries process (4). Any overlap of the three components in this model creates a situation where an individual is at an increased risk for developing dental caries. However, the dogma of SUGAR + PLAQUE = CAVITIES is being challenged. While sugar consumption in the United States has been increasing, caries rates in permanent teeth have actually dropped during the same period (5, 6). Other factors such as

socioeconomic status, ethnicity, overall nutrition, number of meals and snacks per day, oral hygiene, and exposure to fluoride must also be considered when assessing caries risk (6).

It was often thought that only a small number of bacterial organisms were responsible for the destruction to the dentition, and the role of microorganisms in the etiology of caries has been the subject of much study. Dental caries results from acid production of bacteria in dental plaque. Clarke was the first to identify streptococcus from a British child with caries in 1924, which he named *Steptococcus mutans* (7, 8). Currently, the mutans group of *Streptococcus* is thought to be the primary causative agent. *Lactobacillus* species have been known for quite some time to aid in the progression of caries (9, 10, 11). The taxonomy of the mutans group of *Streptococcus* has been well studied; however, oral lactobacilli and other microorganisms have been poorly classified (11, 12, 13).

Until the last few decades, oral bacteria have been identified on the basis of cell morphology and biochemical/physiological criteria (11). However, the specificity of these tests is questionable, because bacterial morphology is an unreliable indicator of relatedness, and many closely related bacteria have similar nutritional requirements and metabolic capabilities. In addition, many organisms were not previously identified at all due to biases in their inability to be cultivated after collection (14, 15).

There has been a shift from the "organismal" level to the molecular level. Recent studies have shown 16S ribosomal RNA gene sequences to be the most powerful technique for reliable classification of bacteria without the need for cultivation (13, 16, 17). An average 16S rRNA molecule has a length of 1,500 nucleotides. 16S ribosomal

gene sequencing has become a tool for determining phylogenetic and evolutionary relationships among bacteria (16, 17, 18). With the use of molecular genetic technology, it is now possible to identify organisms in the oral cavity with greater confidence. As a result, significant changes in nomenclature have occurred. Previously identified species of Lactobacilli, for example, have been reclassified more accurately (19).

The specific aim of this study is to improve the taxonomy of oral bacteria in caries biofilm and healthy controls. There is a potential to include novel species, isolate bacterial strains from carious lesions that may be genetically different from the probiotic counterparts, and identify specific oral bacteria distributed on various tooth surfaces. Understanding the complexity of the bacterial flora may lead to improved prevention strategies.

MATERIALS AND METHODS

Subject Selection: All subjects were recruited from Columbus Children's Hospital Dental Clinic, Columbus, Ohio. The general exclusion criteria include the following: subjects requiring SBE prophylaxis, history of oral antibiotic use in previous 30 days, professional cleaning, or significant systemic disease. The inclusion criteria for severe caries group were the presence of 2 or more permanent teeth with caries with at least one visibly extending into dentin. Deep carious lesions with necrotic pulps were not sampled. The inclusion criteria for the caries-free group was absence of dental caries. Subjects with a previous dental history of amalgams, composites, or stainless steel crowns on permanent teeth or existing primary teeth were excluded. Caries-free subjects with preventive dental sealants were allowed to participate in the group. Consent was obtained by parents and assent was obtained by subjects 9 years old and older for this IRB approved study. Thirty-eight subjects with caries and fifty caries-free control subjects were sampled and paired on the basis of age, race, and gender. Subjects ranged in age from eight to sixteen years old.

Data Collection: Demographic information including age, race, gender, and highest grade completed by either parent was collected. An open-ended survey was completed regarding possible risk factors for caries. The parents were asked the following questions:

1. Has your child been on antibiotics in the last month?
2. How many times would you estimate your child has been on antibiotics in his/her life?
3. Has your child ever been on any other medications for more than one month? If so, which medications? (what is the route of administration?)
4. What source of water do you use as your drinking water? (city or well)
5. Is this the source of water that your child has used his/her entire life?
6. Does your child eat or drink anything before bed?
7. Does your child use toothpaste?
8. How often does your child use toothpaste?
9. Does your child take fluoride vitamins, tablets, or drops?
10. Does anyone in your household smoke?
11. Is you child exposed to smoke anywhere on a regular basis? (daycare, other family)
12. What does your child prefer to drink? How often do they consume these beverages? (Kool-aid, juice, soda)
13. What does your child like to have for snack foods? (cookies, cereal, fruit, chips, candy, fruit snacks) How often do are they snacking on these foods?

Teeth Present/ Caries Scoring Form: the teeth present were recorded for both groups. Scoring for severe caries groups is as follows:

w = visible white spot lesion

c = surface cavitation with caries no extended into pulp

p = caries penetration deep enough to warrant pulp therapy

x = caries so severe that extraction is indicated

Gingival Health: The amount of gingival inflammation was scored with the following criteria:

Healthy = pink, firm, no bleeding

Mild to moderate gingivitis = some puffiness, redness, bleeds when instrumented, includes at least one sextant

Severe = red, shiny, edematous, bleeds when touched, includes at least one sextant.

Plaque Severity: The amount of visible plaque was ranked and recorded according to the following criteria:

None = not detectable by visual inspection or explorer on nearly all surfaces

Moderate = detectable on visual inspection or with explorer on many but not all surfaces

Heavy = obvious on visual inspection; present on nearly all teeth.

Biofilm Sampling: Plaque samples were collected with a sterile dental instrument and whipped onto a sterile, extra coarse endodontic paper point or placed directly in sterile 1.5-mL microfuge tubes and transported to the laboratory where they were frozen until further analysis. Biological debris from the following sites was collected.

e Intact enamel samples included pooled plaque from one tooth from each of the four quadrants, with at least one anterior tooth and one posterior tooth.

w White spot lesion samples included plaque removed from the surface of white spot lesions of at least one, and no more than four teeth.

c Cavitated lesion samples included the surface biofilm on cavitated lesions from at least two and no more than four teeth.

d Carious dentin was obtained from one tooth requiring deep caries excavation or vital pulp therapy. The surface biofilm was removed with a low speed round bur or spoon excavator and discarded prior to sampling the deep dentin.

Biologic Samples: A human DNA sample was obtained by swirling two MasterAmp swabs against the buccal mucosa for one minute. The swabs were then returned to their sterile tubes and transported to the laboratory for long-term storage and later analysis.

Molecular Analysis: The laboratory portion of this project was carried out at The Ohio State University College of Dentistry. Bacterial DNA was isolated using a bead beater. The samples were laced in 300ul of TE buffer and then beaten with 0.25g of 0.1mm glass beads for 60 seconds at 5,000 rpm in a Biospec Products bead beater and chilled on ice immediately after. The DNA was purified using glass beads as previously described (20) and frozen for later analysis.

Cloning of the PCR amplified DNA was performed using a TOPO TA Cloning Kit (Invitrogen) following the manufacturer's instructions. Transformation was done using competent *E. coli* TOP10 cells provided by the manufacturer. The transformed cells were plated onto LB agar plates supplemented with kanamycin and incubated overnight at 37°C. The size of the inserts (approximately 1500 base pairs) were determined by PCR using flanking vector primers followed by electrophoresis on 1.5% agarose gel. Prior to sequencing, the PCR amplified 16S rDNA fragments were purified

and concentrated using Microcon 100 (Amicon), followed by the QIAquick purification (Qiagen).

A data base was compiled representing 2,151 clones and preliminary statistical analysis performed using one-way analysis of variance (ANOVA) comparisons of baseline samples from healthy controls (one sampled site) and caries subjects (four sampled sites) differentiating between healthy controls and caries subjects. In addition, the percentage of species found in each sample was calculated and displayed graphically to depict trends in healthy subjects and differences between early and progressed lesions. Further statistical analysis is currently underway to examine the relationships of bacterial levels to caries severity.

RESULTS

Nine plaque samples from 9 caries-free control subjects and 34 plaque samples from 9 caries subjects were selected for cloning and sequencing of 16S ribosomal DNA. Approximately 50 clones were sequenced from each sample for a total of 2,151 clones. A total of 112 different species or phylotypes were found. Forty-one of the total species represented 90% of the total population, and 19 clones did not have matching species in GenBank. Figure 1 lists the predominant species in order of decreasing frequency. The most frequenly isolated taxa in relation to health and caries status are shown in Figure 2.

A one-way ANOVA was used as a preliminary analysis to determine if differences in number of species existed between healthy control subject sites and caries subject sites. The number of bacterial species decreased as the caries lesions progressed deeper (Figure 3). *Lactobacillus rhamnosus, Lactobacillus casei, Lactobacillus paracasei,* and *Lactobacillus gasseri/ johnsonii* were all observed to have significant differences at sites which caries developed ($P = 0.02$). Several other lactobacilli species including: *Lactobacillus delbrueckii, Lactobacillus salvarius*, and *Lactobacillus vaginallis* were found in non- statistically significant high levels on affected and effected tooth surfaces. In one individual *Lactobacillus* spp. comprised the entire bacterial community in a deep dentin sample (Figure 4). *Streptococcus mutans* was also found in high levels on white spot, cavitated, and deep carious lesions but did not demonstrate

significance. The mean values for *Lactobacillus* spp., which reflects lactobacilli population levels on teeth, were found in higher levels compared to *S. mutans* in the cavitated biofilm and deep carious dentin (Figure 5). Conversely, a few unsuspected bacteria were found primarily on healthy teeth with significant differences in regards to disease. Both *Streptococcus cristatus/ sinensis* and *Eubacterium sp.* (Figure 6) were found predominantly on intact tooth surfaces ($P = 0.002$) and began to disappear in the plaque population as caries progressed. Other bacteria that followed this trend but were not in significant levels include: *Capnocytophaga gingivalis, Capnocytophaga granulose, Gemella haemolysans/ morbillorum, Lautropia sp. clone 2.15, and Streptococcus sanguinis*. *Actinomyces* spp. appeared elevated on some white spot lesions.

DISCUSSION

Results from the 16S DNA gene sequencing indicate that the bacteria colonizing the human mouth are quite heterogenous with 112 different species identified. Even in this small study, the presence of several known cariogenic bacteria was either shown to be statistically significant or demonstrate a clear trend towards cariogenicity. In the future, the sample size will be increased to gain more power in the analysis.

The reported diversity of taxa appears to be consistent with previous studies regarding diversity of the oral bacteria on both healthy and carious teeth. A distinct shift in number and type of taxa occurred as caries progressed from health to deep cavitated lesions. It appears that the acid forming bacteria predominate in established caries and out-compete species commonly found in health. These data confirm other studies of increased levels of *S. mutans* and lactobacilli in caries lesions compared to non-carious tooth surfaces (21, 22, 23, 24).

Both *S. mutans* and lactobacilli have been known for some time to be cariogenic and have been the subject of much research. *S. mutans* has been long considered the main etiologic agent of caries (7, 25) due to its acidogenic properties and ability to form glucans from dietary substrates. While nearly all humans harbor *S. mutans*, not every individual develops caries (8). The combination of *S. mutans* and lactobacilli have been suggested to be a good predictor of future caries (24).

In the current study, *Lactobacillus* spp. were found in higher levels than *S. mutans* in patients with caries. Cariogenicity in gnotobiotic rats inoculated with *Lactobacillus acidophilus* and *Lactobacillus casei* was first shown in the late 1960's (26, 27), and a large portion of the classical literature on dental caries has described these lactobacilli as the major species of lactobacilli in caries (26, 27, 28, 29, 30). It is likely that lactobacilli are solely responsible for the cavitation past the initial enamel demineralization in some cases.

L. acidophilus was not identified in the current study. The absence of *L. acidophilus* can be best attributed to the fact that the genus has undergone significant reclassification and reorganization. Prior to 16S DNA gene sequencing, bacterial taxonomy was performed on the basis of phenotypic (morphological, biochemical, and physiological) criteria. *L. casei* and *L. acidophilus* made up two major groups of known lactobacilli and were divided mainly on heterofermentative and homofermentative characteristics, respectively (31). The two groups also contained numerous subspecies that complicated the taxonomy. It was not until the advent of DNA/DNA homology technology that classification schemes and taxonomic issues were able to be resolved (31). Currently, 16S DNA gene sequencing is the most powerful method available for phylogenetic studies.

The 16S DNA sequencing analysis in this study identified *Lactobacillus gasseri/ johnsonii* as the most predominant species of lactobacilli in the caries samples. Other recent research regarding diversity of oral lactobacilli in deep caries reported the same result (32, 33). *L. gasseri/ johnsonii* is actually two different species (*L. gasseri* and *L. johnsonii*), but they are grouped together because they are very difficult to separate even

after 16S gene sequencing. *L. gasseri* was first described in 1980 (31), and was also found to be the most dominant *Lactobacillus* sp. in the human intestine (34). This homofermentative bacterium was previously designated as *L. acidophilus*. DNA/DNA homology studies demonstrated significant homology differences between *L. gasseri* and the original type strains of *L. acidophilus* (13, 35).

A similar taxonomic reclassification occurred after a number of species in the *L. casei* group were designated with subspecies status. *L. rhamnosus* was originally identified as *L. casei* subsp. *rhamnosus*. Later, with DNA/DNA homology methods, *L. rhamnosus* was shown to only have 30-50% homology with the other species in the *L. casei* group (31). This facultative heterofermentative organism was elevated to the species status. *L. rhamnosus*. In this study, the *L. casei* complex (*L. rhamnousus, L. casei, and L. paracasei*) was present in significant levels but was not separated with confidence into individual species due the lack of sequence divergence. Previous research has shown genetic variation between *L. rhamnosus* and other closely related organisms in the *L. casei* cluster of species (36, 37), and there may be key base pair locations in the 16S gene that provide a signature for some species (38). It should be noted that these are all unique species, and further analysis, including sequence alignment, is required to establish the pylogenetic distances among the three species.

L. delbrueckii, a starter culture for dairy products, was also found only in cavitated and deep caries lesions. It is possible that the bacteria may have been introduced by a food source. *L. delbrueckii* and other lactobacilli do not appear to easily colonize sound tooth structure. Unlike their counterpart, *S. mutans*, lactobacilli are not primary tooth colonizers, and the ability of *Lactobacillus* sp. to colonize teeth is not

completely understood. It has been suggested that lactobacilli simply rely on mechanical retention in the caries lesion (39). Others have described proteinaceous adhesins in lactobacilli, allowing coaggregation with streptococci (40). Therefore, only low levels of *S. mutans* may be required in order for lactobacilli to become established on the tooth surface. This would indicate that perhaps lactobacilli counts would be a better clinical adjunct for determining caries risk than the present *S. mutans* testing kits because the presence of *S. mutans* alone has not routinely lead to dental caries.

Overall, the clonal analysis has shown that the microbial ecology on permanent teeth in the population studied is complex and altered in the diseased state. While there are slight differences in the species reported, when compared to other studies, some degree of variation is to be expected. The main taxa (*Lactobacillus* spp. and *S. mutans*) reported in this study further reinforces what is currently understood about caries. However, the details regarding the role of each individual species in different stages of the caries process remains unclear. In fact, the mere presence of a group of bacteria may be much more important than the presence of one specific bacterial species alone. It seems possible to identify a caries-inducing environment based on a small number of organisms with in the ecosystem with an open ended approach.

APPENDIX

FIGURES

Bacterial species or group	Total clones by species	% of total clones
Veillonella parvula	245	12
Streptococcus gordonii/ mitis/ oralis/ pneumonia	224	10
Streptococcus mutans	196	9
Lactobacillus gasseri/ johnsonii	120	5
Selenomonas noxia	94	4
Streptococcus sanguinis	82	4
Veillonella sp. oral clone AO50	79	4
Neisseria meningitidis	65	3
Corynebacterium matruchotii	63	3
Campylobacter gracilis	61	3
Selenomonas dianae/ infelix	61	3
Lactobacillus vaginalis	56	3
Abiotrophia defectiva	47	2
Veillonella dispar/ atypica	47	2
Streptococcus sobrinus/ downei	43	2
Lactobacillus rhamnosus/ casei/ paracasei	40	2
Lactobacillus delbrueckii	26	1
Gemella haemolysans/ morbillorum	25	1
Streptococcus salivarius/ thermophilus	22	1
Selenomonas sputigena	22	1
Lautropia sp. clone 2.15	21	1
Kingella/ Bergeriella/ Simonsiella	20	1
Selenomonas sp. oral clone CS024	20	1
Lactobacillus salivarius	20	1
Streptococcus cristatus	19	1
Streptococcus anginosus	19	1
Propionibacterium sp.	19	1
Capnocytophaga granulosa	19	1
Neisseria flava/ sicca/ meningitidis	16	1
Eubacterium sp.	15	1
Lactobacillus fermentum	14	1
Selenomonas sp.	14	1
Rothia dentocariosa	14	1
Capnocytophaga gingivalis	13	1
Granulicatella adiacens	13	1
Streptococcus parasanguis	11	1
Peptostreptococcus micros	11	1
Neisseria elongata	11	1
Lactobacillus buchneri/ parabuchneri/ kefiri	11	1
Other species (71 total)	233	11
TOTAL	2,151	

Figure 1. Predominant species (all healthy and caries sites combined).

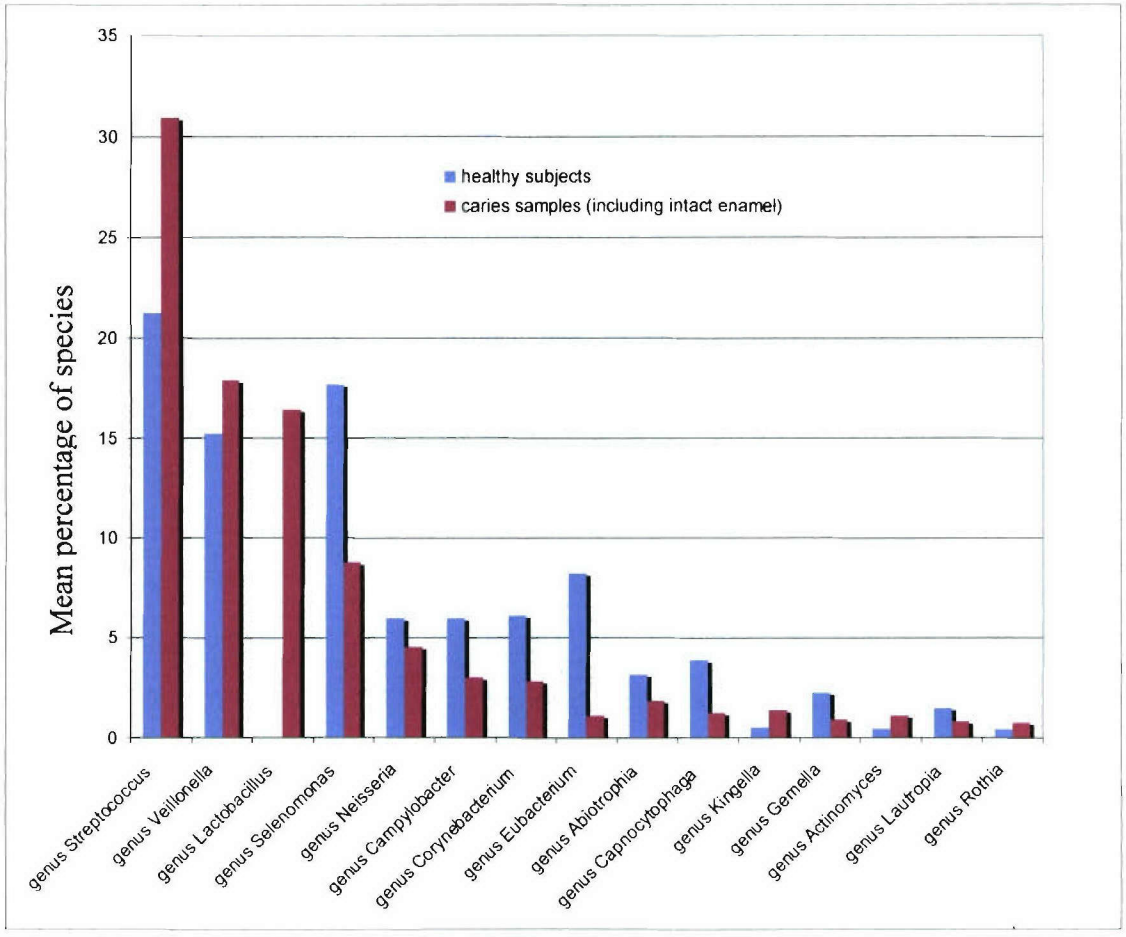

Figure 2. Mean levels of genera in healthy subjects and caries subjects.

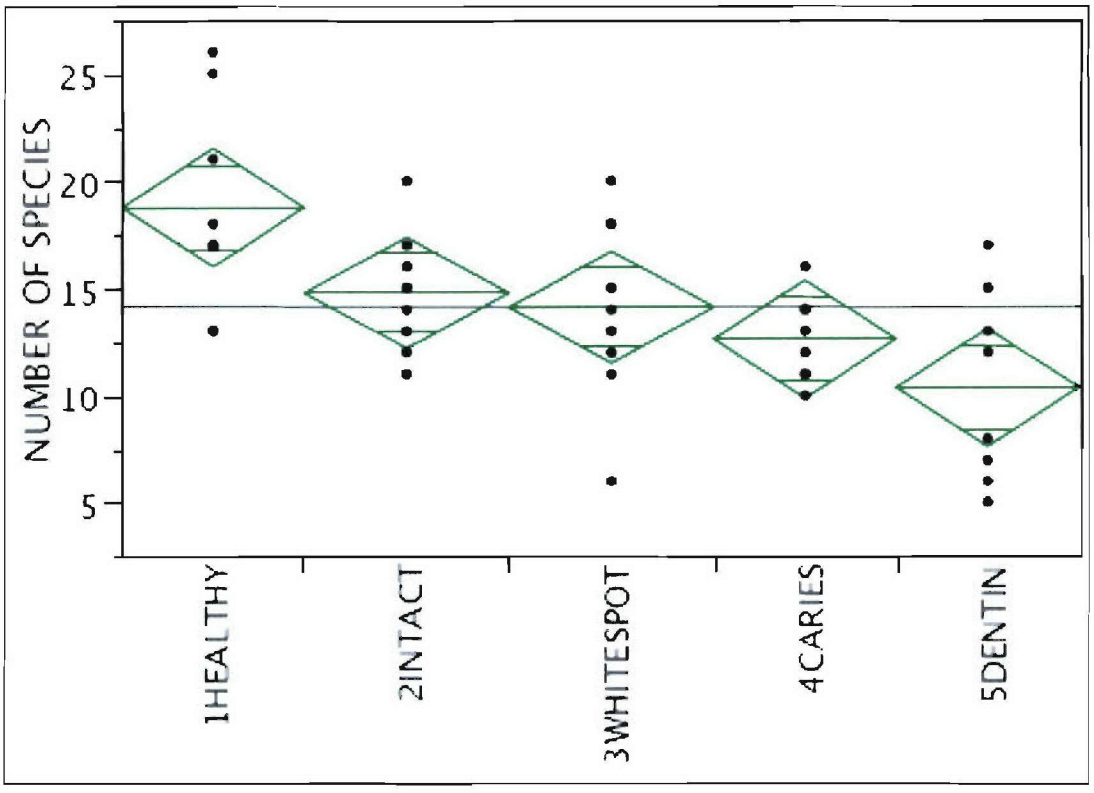

Figure 3. One-way ANOVA of bacterial species by sample type.

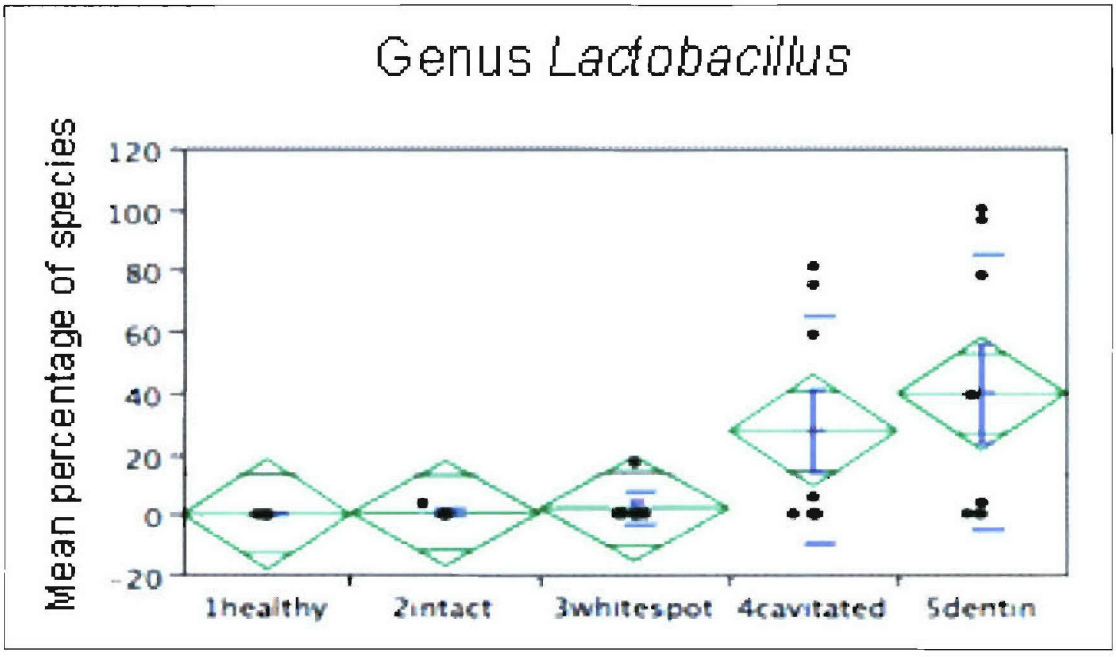

Figure 4. One-way ANOVA of healthy and caries subjects for genus *Lactoabcillus*.

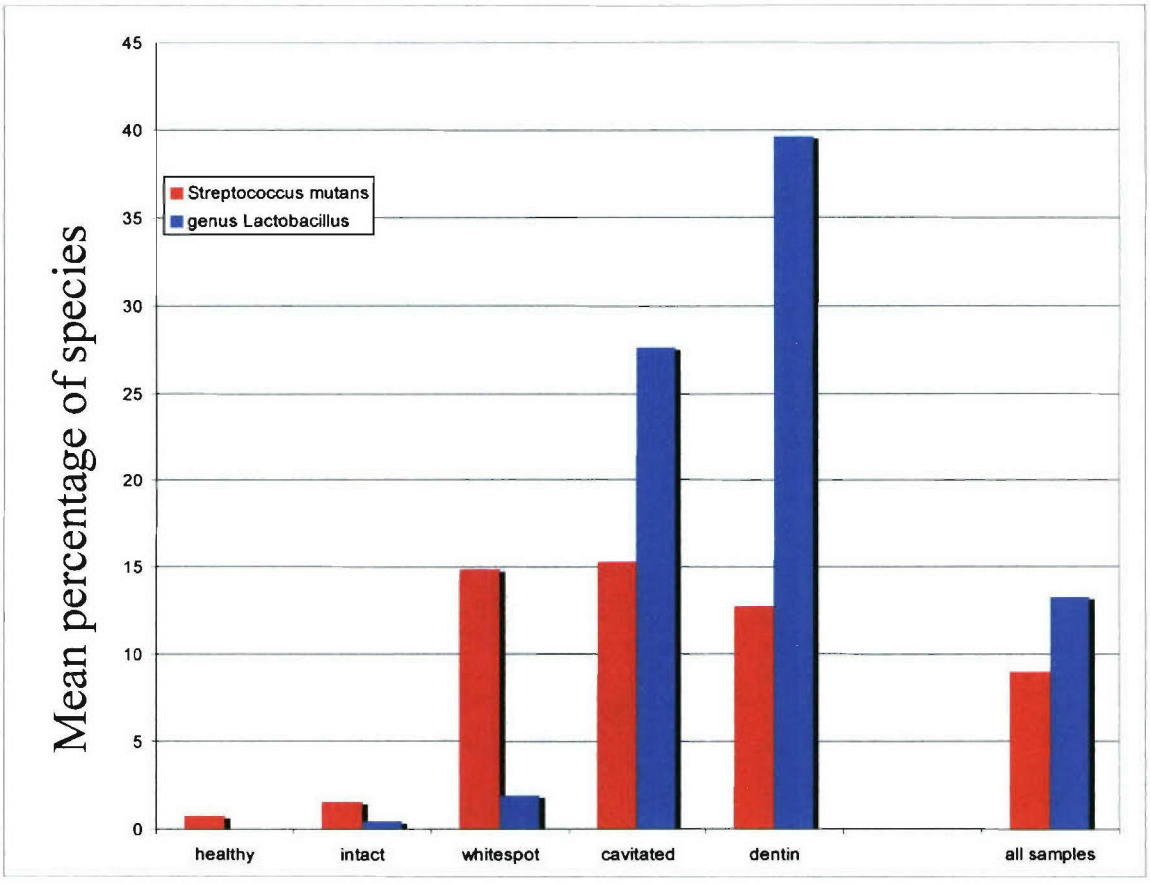

Figure 5. Comparison of *S. mutans* levels to *Lacotbacillus* spp. in health and caries.

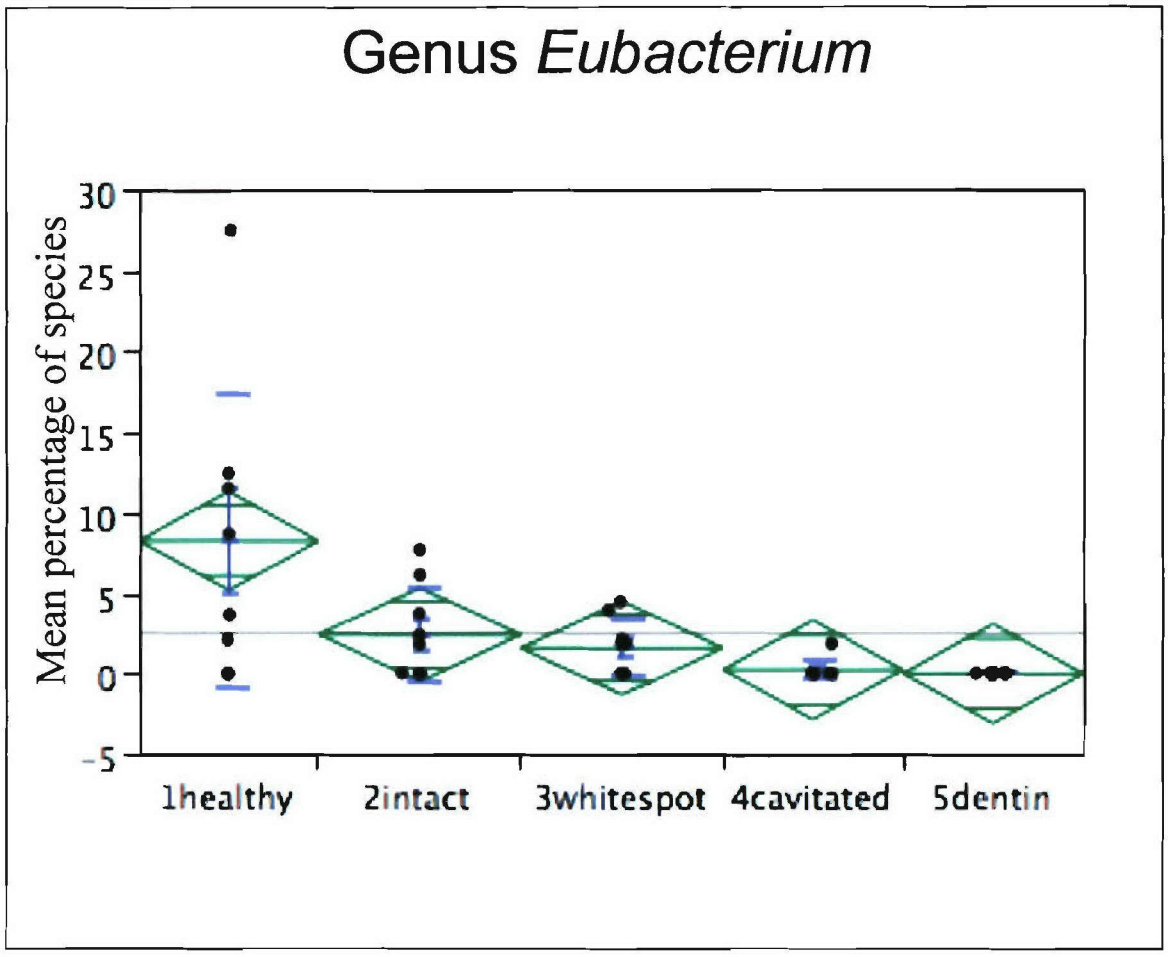

Figure 6. One-way ANOVA between healthy and caries subjects for genus *Eubacterium*.

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