

Cariogenic Virulence Characteristics of Mutans Streptococci Isolated From Caries-active and Caries-free Adults

Gloria Khoo; Ling Zhan; Charles Hoover, PhD; and John D.B. Featherstone, MSc, PhD

ABSTRACT

The objective of the study was to compare aciduricity (ability to live in acid), acidogenicity (ability to produce acid), and intracellular polysaccharide production of mutans streptococci (MS) strains isolated from caries-active (CA, with one or more cavitated lesions) and caries-free (CF, with no clinically observable new caries in the last five years) adults. Forty-three MS strains from 17 of 17 CA adults, and 14 strains from eight of 12 CF adults were investigated. MS isolates' growth, survival, and pH reduction in pH 3.5-7.0 broths were evaluated to compare their acidogenicity and aciduricity. Extracellular water-soluble polysaccharide (WSP) and water-insoluble polysaccharide (WISP) was extracted from MS culture in BHI broth with 5 percent sucrose and assessed by a colorimetric anthrone-sulfuric acid microassay. No significant differences in mean aciduricity were found between CA and CF MS isolates ($P>0.05$, t test). However, significantly more CA subjects (29 percent) were colonized by MS strains with aciduricity above the average than CF subjects (13 percent, Fisher's exact

test, $P<0.05$). Furthermore, CA MS strains produced significantly more acid at $pH<5$ (Mann-Whitney, $P<0.05$) and significantly more CA subjects were colonized with more acidogenic MS at $pH<4.5$ (Fisher's exact test, $P<0.01$). Similarly, CA MS isolates produced significantly more WISP than CF (Mann-Whitney test, $P<0.01$) while no statistical difference was found in WSP between the two groups. More CA subjects were colonized by multiple strains with aciduricity, acidogenicity, and polysaccharide synthesis ability above average. The study indicated that differences in acidogenicity, aciduricity, and polysaccharide synthesis in strains of MS may partially contribute to increased caries activity.

Authors / Gloria Khoo is a predoctoral student, and Ling Zhan is a postdoctoral fellow at the Department of Preventive and Restorative Dental Science at the School of Dentistry at the University of California, San Francisco. Charles Hoover, PhD, is a faculty member in the Department of Cell and Tissue Biology at the School of Dentistry at the University of California, San Francisco. John D.B. Featherstone, MSc, PhD, is professor in the Department of Preventive and Restorative Dental Science at the School of Dentistry at the University of California, San Francisco.

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Dental caries is one of the most common infectious diseases found in all human populations. The mutans streptococci (MS) group of oral bacteria (particularly, *Streptococcus mutans* and *Streptococci sobrinus*) is one of the major causative bacterial groups in human dental decay. Previous studies have reported positive relationships between salivary levels of MS and caries prevalence or future caries increment and salivary MS level have been identified as an important part of caries risk assessment.¹⁻¹⁰ Although salivary MS were proven to be part of reliable caries risk assessment criteria in those studies, there were some subjects with high MS infection who did not develop caries lesions. In these studies, there was no consideration of possible virulence variations in different MS strains. A study by Bowden also showed that high numbers of *S. mutans* associated with the development of a lesion at one site may not result in caries at a second susceptible site with high levels of the same organism in the same subject.¹¹ This indicated that there might be factors other than MS quantity alone contributing to caries risk.

Recent studies on bacterial genetic polymorphism have found that MS strains are characterized by a high degree of genetic diversity.¹²⁻¹⁸ MS infection, however, is usually transmitted amongst family members, most commonly mother and child, who therefore share common MS strains.¹⁹⁻³⁷ Caries-active subjects tend to have multiple strain MS infections compared to the caries-free subjects.^{14,18,21} Three major virulence characteristics of these bacteria are likely to be important, namely acidogenicity (ability to produce acid), aciduricity (ability to live in acid) and glucan synthesis (ability to form extra-

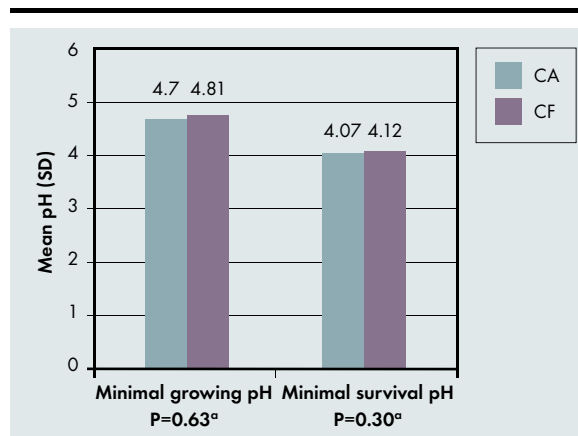


Figure 1. The bar graph presents the mean and SD of the minimal growing pH and minimal survival pH of mutans streptococcus strains isolated from caries-active (CA) subjects vs. caries-free (CF) subjects.

*Student t test

cellular polysaccharides when feeding on carbohydrates such as sucrose). In vitro, differences in cariogenic potential (aciduricity, acidogenicity, and glucan synthesis) of different MS strains have been observed.³⁸⁻⁴²

Only a few previous studies have compared the cariogenic potential of MS isolates from caries-active and caries-free subjects. MS isolates from caries-active subjects have been reported to produce more extracellular water insoluble glucan than MS isolates from caries-free subjects.⁴³⁻⁴⁶ Conflicting results have been reported on aciduricity, acidogenicity, and biofilm formation of MS and subjects' caries status.^{40,44,46,47} Although these results suggest that some MS strains may exhibit strong virulence characteristics that enhance their ability to colonize, survive, and induce caries formation, further studies are needed to investigate the relationship between MS virulence factors and caries status.

The aim of the present study was to compare cariogenic virulence factors, namely aciduricity, acidogenicity, and extracellular polysaccharide synthesis ability of MS strains isolated from caries-active and caries-free adults.

Methodology

Subjects and Strains of Mutans Streptococci

After approval by the Committee on Human Subject Research of the University of California, San Francisco, two adult populations from an ongoing NIH-funded study "Caries Management by Risk Assessment" were utilized in this study. Seventeen caries-active subjects with 1-7 frank cavities and more than five DMFT, and 12 caries-free subjects with no new cavities or restorations due to caries during the last five years were recruited. The authors chose these criteria to eliminate clinically observable caries activity for at least five years prior to the present study. MS were enumerated from stimulated whole saliva cultured on Mitis Salivarius Sucrose Bacitricin agar, MSSB. Five randomly selected typical MS colonies were isolated from each MS-positive subject and confirmed as MS by a sugar fermentation test. These strains were stored in TSB (trypticase soy broth) with 20 percent glycerol at -80°C until further study of MS aciduricity, acidogenicity, extracellular polysaccharide synthesis, and genetic diversity. *S. mutans* 25175 and *S. sobrinus* 6715 were used as reference strains in all assays.

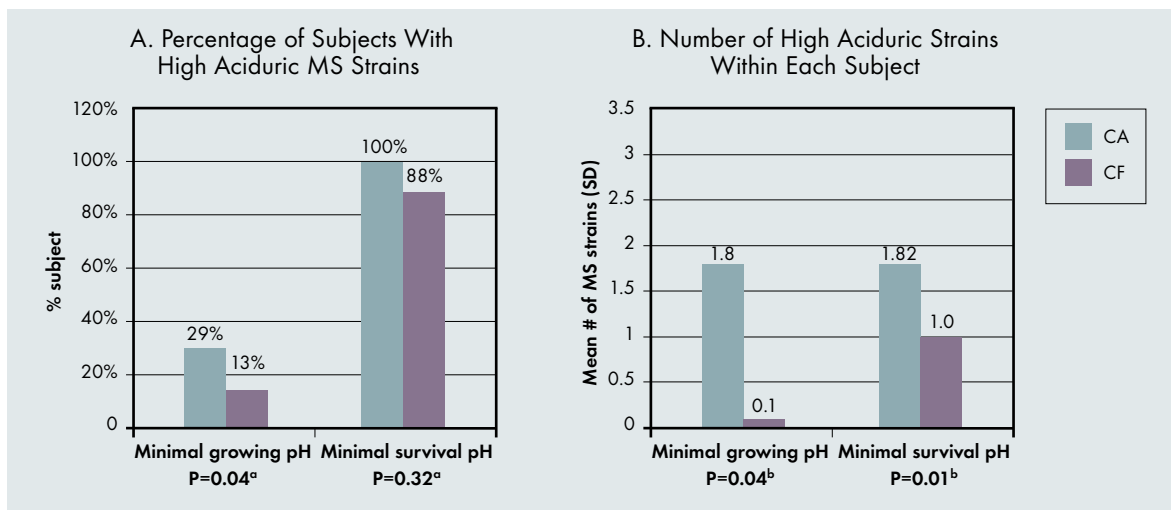


Figure 2. Panel A presents percentage of subjects who had MS strains with the minimal growing pH and the minimal survival pH below average. Panel B presents the mean and SD of number of MS strains, which had the minimal growing pH and the minimal survival pH below average, within each subject. *Statistically significant; ^aFisher exact test; ^bMann-Whitney test

Mutans Streptococci Aciduricity and Acidogenicity Assays

Mutans streptococci aciduricity was evaluated by two parameters, namely the bacterial minimal growth pH and the minimal bacterial survival pH. The MS strains were recovered on MSSB plates and then grown overnight in pH 7 TPY (tryptone peptone yeast) broth. The overnight cultures were then inoculated into TPY broths at pH 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0 for 48 hours. Aciduricity of each bacterial strain was assessed by visible bacterial growth in TPY broth after 48 hours. Broth with no visible bacterial growth was plated on BHI to check for the bacterial survival. The lowest pH with visible bacterial growth in broth was defined as the MS minimal growth pH and the lowest pH with MS growth on BHI (brain heart infusion) plates was defined as the MS minimal survival pH.

To evaluate acidogenicity, the initial and final pH of each MS strain was measured with an Accumet pH meter 900 (Fisher Scientific, USA) before and after 48-hour incubation in BHI broth with 5

percent glucose under anaerobic conditions. The pH drop between initial and final reading was defined as its acidogenicity measurement.

Extracellular Polysaccharide Synthesis Assays

For polysaccharide extraction, MS strains were inoculated into BHI broth with 5 percent sucrose and incubated at 37°C for 48 hours. The cultures were centrifuged (10,000g) for 30 minutes at 4°C and the supernatant was collected for water soluble polysaccharide (WSP) extraction. The pellet was washed two times with PBS (phosphate buffered saline) and supernatants were combined for WSP extraction. The pellets were dissolved in 0.5N NaOH for 30 minutes in a 60°C water bath and used for water insoluble polysaccharide (WISP). The NaOH incubation was repeated twice and the supernatants were collected by centrifugation and combined with the previous portions for WISP extraction. Three volumes of ethanol was added to culture supernatants or NaOH extracts for WSP or

WISP and they were stored in the cold overnight to precipitate the polysaccharides. The precipitate was collected by centrifugation and dissolved in 5 ml deionized water for WSP and 0.5 N NaOH for WISP.^{48,49} An Anthrone-sulfuric acid colorimetric microassay was used to quantify the polysaccharide in the extracts. For the anthrone-sulfuric acid colorimetric assay, 200 µl of extracted WPS or WISP was mixed with 5 ml of anthrone solution. The samples were heated in a boiling water bath for 12 minutes, and then cooled to room temperature. From each sample, 200 µl of was transferred into a 96-well microtiter plate and the absorbance was measured at 620 nm using a UV/visible Microplate Spectrophotometer.^{50,51} Polysaccharide concentrations were measured against glucose standard solutions.

Statistical Analysis

The statistical analysis tests were performed at the strain level (between all strains from CA vs. CF) and at the subject level (subjects with strains with

virulence characteristics above average between the CA and CF groups). To compare the aciduricity, acidogenicity, and extracellular polysaccharide synthesis between CA and CF MS strains, the data distribution was analyzed first for continuous data. If the data showed a normal distribution, the student t test was used. Otherwise, the Mann-Whitney nonparametric test was used. To compare the number of subjects with more virulent strains above average between the CA and CF group, Fisher's exact test was used. The average is defined as the overall mean of each virulence factor for all the strains. The Mann-Whitney test was used to compare the number of strains that were more virulent than average within each subject.

Results

Mutans colonization levels and MS isolates from CA and CF subjects

All CA subjects had high MS levels over 10,000 CFU/ml of saliva, while only eight of 12 CF subjects had MS infection with only two CF subjects having MS over 10,000 CFU/ml (detailed results not shown). As previously reported, 43 different MS genotypes were identified from the 17 CA adults by AP-PCR (arbitrarily primed polymerase chain reaction) 37 genotypes were *S. mutans* and six were *S. sobrinus*.¹⁸ Only 14 MS genotypes were identified from the CF adults and they were all *S. mutans*.

Mutans Streptococci Aciduricity and Acidogenicity

For aciduricity measurements, the mean (SD) minimal growth pH of MS strains from CA and CF groups was 4.70 (0.36) and 4.81 (0.15), respectively, with a range of 4.0-5.5. The mean (SD) survival pH of CA and CF MS

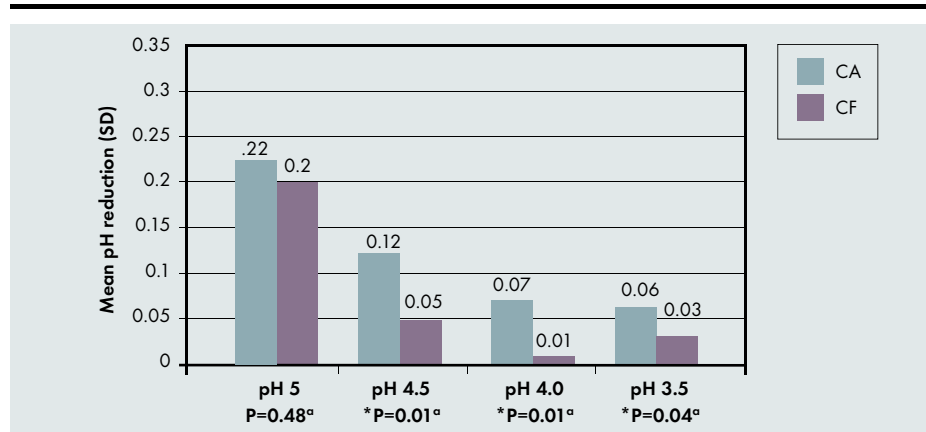


Figure 3. The bar graph presents the mean and SD pH reduction of mutans streptococcus strains isolated from caries-active (CA) and caries-free (CF) subjects.

*Statistically significant; ^aMann-Whitney test

strains were 4.06 (0.29) and 4.11 (0.36), respectively, with a range of 3.5-5.0 (Figure 1). There were no statistically significant differences in the mean aciduricity between CA and CF MS strains at strain level (t test, P>0.05). As shown in Figure 2, at subject level, significant more CA subjects (29 percent) had MS strains with minimal growth pH below average than CF subjects (13 percent, Fisher's exact test, P<0.05). All CA subjects had MS strains with minimal survival pH below average compared with 88 percent in the CF group (Fisher exact test, P>0.05). The mean (SD) of MS strains with minimal growth or survival pH below average in CA subjects was 1.80 (1.13) and 1.82 (0.64), while it was 0.13 (0.35) and 1.00 (0.53) in CF subjects, respectively, which were statistically significant (Mann-Whitney test, P<0.05).

For acidogenicity, there was no statistically significant difference between CA and CF strains on acid production in broth with pH ≥ 5.0 (Figure 3). However, MS strains from CA subjects produced significantly more acid at pH<5 (Mann-Whitney, P<0.05). No CF subjects were

found to have MS strains with acid production ability at pH below 4.5 while 59 percent and 53 percent of the CA subjects still had one or more MS strains capable of generating acid at pH 4.0 and 3.5 respectively (Fisher's exact test, P<0.01) (Figure 4).

Mutans Streptococci Extracellular Polysaccharide Synthesis

MS isolates from CA subjects produced significantly more WISP with mean (SD) as 0.47 (0.58, CA) and 0.23 (0.13, CF) (Mann-Whitney test, P<0.01) while no statistically significant difference was found in WSP between the two groups with mean (SD) as 3.85 (2.93, CA) and 3.76 (2.38, CF), respectively (Figure 5).

More CA subjects had MS strains with WSP and WISP synthesis ability above average than CF subjects but there was no statistically significant difference between the two groups (Figure 6). Similarly, CA subjects also tended to have more MS strains with WSP and WISP synthesis ability above average but there was no statistically significant difference between the two groups.

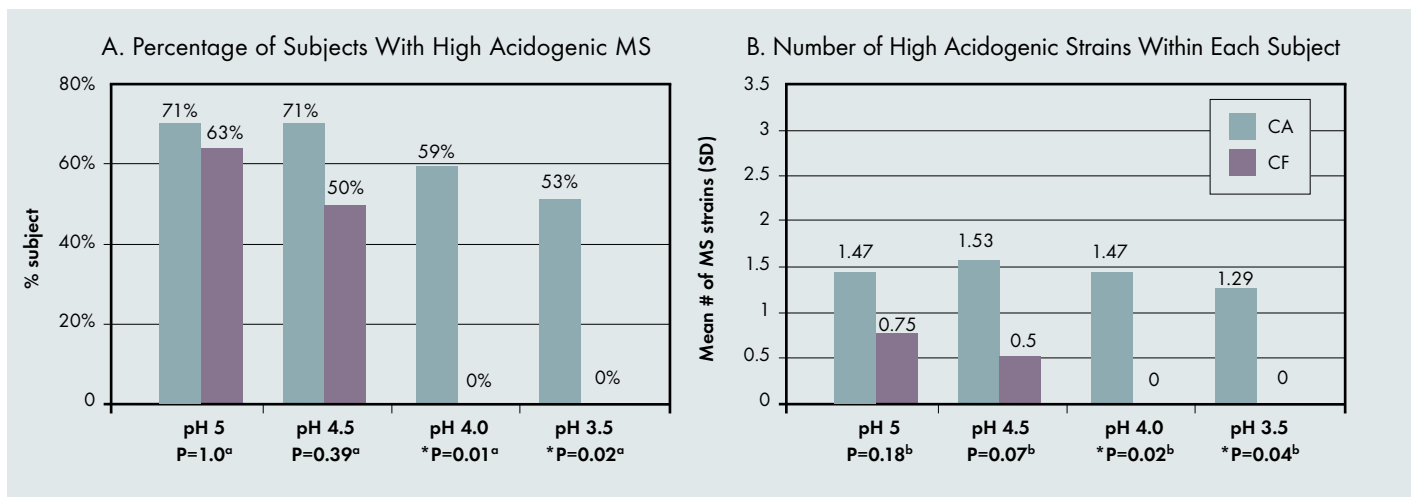


Figure 4. Panel A presents the percent of subjects with acid production ability at pH below 4.5 in caries-active (CA) and caries-free (CF) groups. Panel B presents the number of MS with acid production ability at pH below 4.5 within each subject.

*Statistically significant; ^aFisher exact test; ^bMann-Whitney test

Discussion

Acidogenicity is one of major cariogenic virulence factors of the mutans streptococcus group. Mutans streptococci can metabolize a wide variety of carbohydrates and generate acid as byproducts. Early studies showed that acid production by *S. mutans* and *S. sobrinus* was significantly higher than other oral streptococci at neutral pH.³⁹⁻⁴¹ Moreover, rapid acid production by some MS strains persisted at lower than neutral pH.^{38,39,41} The major acid product of MS when metabolizing sucrose and glucose is lactic acid.⁴⁷ Rapid acid production by MS enabled them to decrease plaque pH on the tooth surface dramatically and to initiate demineralization of tooth enamel or dentin. Although previous studies suggested variability in acidogenicity and aciduricity among different MS strains, no correlation between acid production of MS and caries status was found by Kohler and Napimoga, who compared MS acid production in broth at pH 5.5

and neutral pH, respectively.^{38-41,46,47} In the authors' study, no significant difference was found between CA and CF group either at the overall strain level or subject level at pH \geq 5.0. This agreed with the results of Kohler and Napimoga. However, MS isolates from CA subjects produced significantly more acid in TPY broth at pH \leq 4.5 than those from CF subjects. When the acidogenicity was analyzed at the subject level, none of the CF subjects had MS strains producing acid at pH \leq 4.0, whereas more than half of the CA subjects were infected with multiple strains being able to continue acid production below pH \leq 4.0. Very importantly in this study, the authors found that MS in the CA group were able to produce acid at pH values well below 5.5 where enamel and dentin can dissolve rapidly.

Aciduricity is another cariogenic virulence factor of the MS group. Previous studies showed that *S. mutans* and *S. sobrinus* also had greater acid tolerance than other oral bacteria.^{38,39}

The high aciduricity of MS species enables them to survive and flourish during an acidic environmental challenge generated by them or by other species. It is the acidification of the local environment by the acid end-products of mutans streptococci that inhibits the survival of other competing bacterial species, enabling mutans streptococci to maintain their niche in the oral flora, incidentally causing dental caries in its host.⁵² To the best of the authors' knowledge, no data on the relationship of aciduricity of MS clinical isolates and caries status has been published. The authors' results show that MS clinical isolates from the adults in this study were very aciduric. Most MS isolates were still able to grow at pH \leq 5.0 and survive at pH \leq 4.5 while *S. sanguinis* 10556 and *S. gordonii* 10558 stopped growing at pH 5.5, and were killed at pH 5.5 or 5.0 (unpublished data). Although no significant difference in mean aciduricity was found amongst MS strains from the CA and CF groups, the CA

group (29 percent) had significantly more subjects infected by MS strains with minimal growth $\text{pH} \leq 4.5$, and all CA subjects had MS strains that survived at $\text{pH} \leq 4.0$. Moreover, CA subjects were also infected with multiple high aciduric strains compared to CF subjects. These results indicate that cariogenic potential of MS strains might vary between subjects and that the presence of multiple strains in CA patients may contribute to their increased caries risk.

MS clinical isolates in the present study were able to synthesize extracellular water soluble polysaccharide and extracellular water insoluble polysaccharide using sucrose as the substrate. The WSP serves as an energy store to extend acid production when the carbohydrate source is limited. The WISP is the major matrix component that promotes dental plaque formation, which in turn enhances MS accumulation on tooth surfaces. However, no statistically significant differences in WSP between CA and CF groups at neither strain nor subject level were found in the present study. This finding suggests that WSP synthesis does not play a critical role in caries risk. However, CA MS isolates produced significantly more WISP than CF isolates, which is consistent with other studies.⁴³⁻⁴⁶ The finding further supports the importance of WISP formation by MS in cariogenicity.

Conclusions and Clinical Relevance

Overall, the authors' results showed that MS strains from CA were more virulent in aciduricity, acidogenicity, and polysaccharide synthesis than those in the CF group. Furthermore, more CA subjects were infected by multiple strains with aciduricity, acidogenicity, and polysaccharide

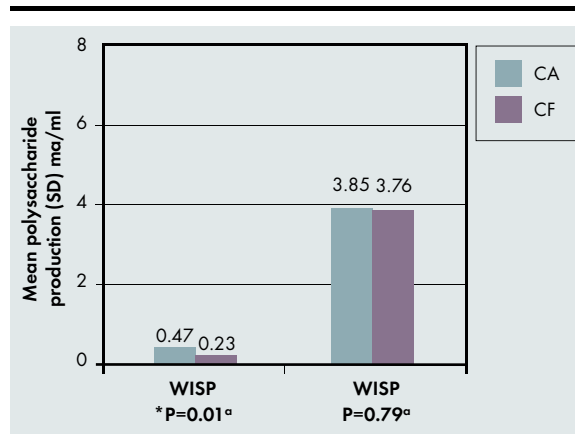


Figure 5. The bar graph presents the mean and SD of extracellular water soluble polysaccharide (ESP) and water insoluble polysaccharide (WISP) of mutans streptococcus strains isolated from caries-active (CA) and caries-free (CF) subjects.

*Statistically significant; ^aMann-Whitney test

synthesis ability above average. The results indicate that differences in acidogenicity, aciduricity, and polysaccharide synthesis in strains of MS at least partially account for caries activity. The results emphasize that dental caries is a bacterial disease and that there are strains of bacteria that are more virulent than others, some highly virulent. These highly virulent strains were transmitted to the child initially, most likely from the parent or caregiver. Currently it is not possible to identify these strains in the clinical setting. However, in order to deal with children with high caries activity, it is necessary to control the bacterial activity and the transmission of the multiple virulent strains. When a high caries challenge is identified by ongoing caries activity antibacterial treatments, such as chlorhexidine rinses, for the older child and for parents or caregivers of younger children are likely to help. In the future, it is likely that better antibacterial treatments will be available for elimination of cariogenic bacteria in children as well as adults, and potentially that chairside identification of these strains will be possible as part of routine dental practice.

CDA

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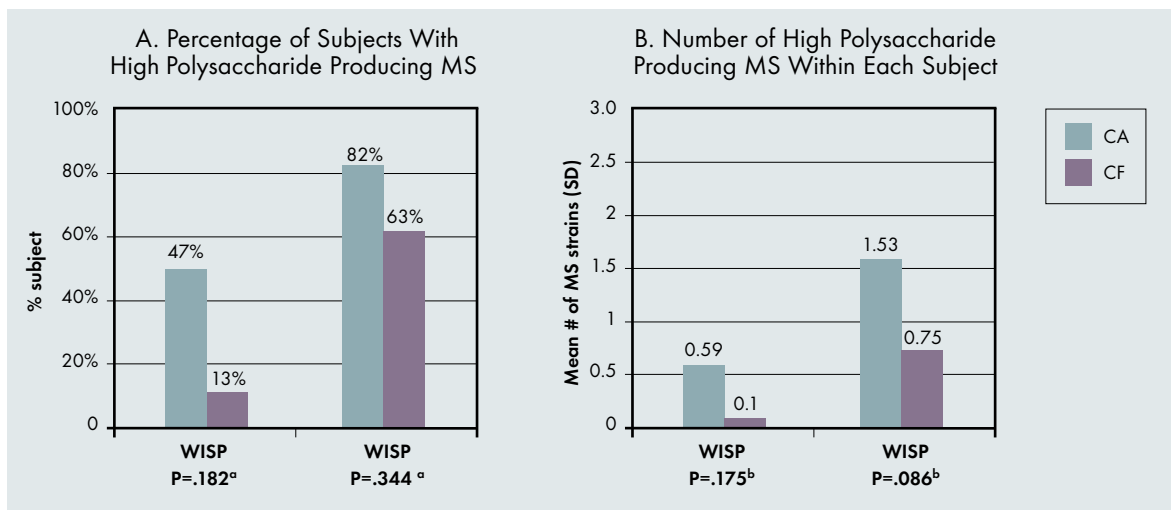


Figure 6. Panel A presents the percentage of subjects with polysaccharide production ability above average in caries-active (CA) and caries-free (CF) groups. Panel B presents the number of MS with polysaccharide production ability above average.

*Statistically significant; ^aFisher exact test; ^bMann-Whitney test

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To request a printed copy of this article, please contact / John D.B. Featherstone, MSc, PhD, Department of Preventive and Restorative Dental Sciences, University of California, San Francisco, Box 0758, 707 Parnassus Ave., San Francisco Calif. 94143.