Journal of Oral Microbiology

co-action PUBLISHING

ORIGINAL ARTICLE

Acidogenicity and acid tolerance of *Streptococcus oralis* and *Streptococcus mitis* isolated from plaque of healthy and incipient caries teeth

Jeffrey A. Banas[1]*, Min Zhu[1], Deborah V. Dawson[1], Derek R. Blanchette[1], David R. Drake[1], Hongjie Gu[1], Ryan Frost[1], Grant McCaulley[1] and Steven M. Levy[2]

[1]Iowa Institute for Oral Health Research, University of Iowa College of Dentistry, Iowa City, IA, USA; [2]Department of Preventive and Community Dentistry, University of Iowa College of Dentistry Iowa City, IA, USA

Background: Non-mutans low pH oral streptococci are postulated to contribute to caries etiology.
Objective: This study was undertaken to investigate whether the acidogenicity and acid tolerance of clinical strains of *Streptococcus oralis* and *Streptococcus mitis* correlate with health or early-stage enamel caries.
Design: *S. oralis* and *S. mitis* were isolated from plaque samples taken from the occlusal surfaces of second molars sampled at two different visits 4 years apart. All sites were sound at Visit 1; subjects were segregated into one of three groups based on the status of the site at Visit 2 and caries elsewhere in the dentition. Strains of *S. oralis* and *S. mitis* were evaluated for acidogenicity and acid tolerance, and the results correlated with the clinical status of the sites from which they were isolated. Mutans streptococci (MS) isolated from the plaque samples were also quantified, and the presence or absence of growth on pH 5.5 media or on media selective for bifidobacteria was recorded.
Results: No significant positive correlations were found between the acidogenicity properties of the *S. oralis* and *S. mitis* clones and caries at either visit. Similar results were obtained for acid tolerance of *S. oralis* clones but were inconclusive for *S. mitis* clones. A statistically significant positive correlation between MS levels and caries (or future caries) was evident at both visits, but there were no statistical correlations with the growth on pH 5.5 media or media selective for bifidobacteria.
Conclusions: The low pH potential likely varies considerably among oral streptococcal species and is least likely to be found among strains of *S. mitis*. Accordingly, the concept and constitution of 'low pH streptococci' may need to be re-evaluated.

Keywords: *dental plaque*; *mutans streptococci*; *cariogenicity*; *oral microbiota*; *acidogenicity*; *acid tolerance*

*Correspondence to: Jeffrey A. Banas, Iowa Institute for Oral Health Research, University of Iowa College of Dentistry, N413 DSB, Iowa City, IA 52242, USA, Email: jeffrey-banas@uiowa.edu

To access the supplementary material to this article, please see Supplementary files under 'Article Tools'.

Received: 21 July 2016; Revised: 30 September 2016; Accepted: 4 October 2016; Published: 26 October 2016

The microbial etiology of dental caries remains a subject of debate. The role of the mutans streptococci (MS), in particular *Streptococcus mutans*, has drawn the greatest share of attention, though some argue that this focus is to the detriment of understanding how overall plaque ecology drives caries risk (1–4). What seems clear is that on a population level the link between *S. mutans* and caries is strong. But at the individual level, there may be alternative pathways to caries, even in a severe form. While it is possible that the contribution of *S. mutans* is overstated in those instances where it is isolated from carious lesions, it is also plausible that *S. mutans* is capable of driving caries development with the support of any number of lesser cariogenic species. Resolving the etiology of dental caries will require further pursuit in addressing questions such as species-specific numerical thresholds for making a biological impact, interspecies networks that impact caries risk, and intraspecies diversity that limits species-level assignment to caries risk. The project reported here addressed this latter

Citation: Journal of Oral Microbiology 2016, **8**: 32940 - http://dx.doi.org/10.3402/jom.v8.32940
(page number not for citation purpose)

concern by investigating variability in acidogenicity and acid tolerance among strains belonging to two species linked to the 'low pH streptococci'.

The 'low pH streptococci' refer to non-MS strains capable of reducing the pH of a glucose broth to a minimum of less than 4.4 (5–8). These strains are thought to be found among the species *S. oralis*, *S. mitis*, *S. anginosus*, and *S. gordonii* though other species have also been suggested (5, 8–10). While these species are among the primary colonizers of the enamel pellicle and generally considered of low cariogenic potential, the low pH subsets have been associated with caries in multiple studies (6, 8, 10–12). Since *S. mitis* and *S. oralis* are reported to be much more highly represented in saliva and dental plaque than *S. gordonii* and *S. anginosus* (13, 14), we isolated strains of *S. mitis* and *S. oralis* from plaque samples taken from the occlusal surfaces of second molars that were either healthy or that showed signs of early-stage decay. It was hypothesized that the relative acidogenicity and acid tolerance of these strains would correlate with the clinical status of the sites from which they were isolated.

Materials and methods

Bacteriological media and strain isolation

Site-specific plaque samples from the occlusal surfaces of second molars, banked as part of the Iowa Fluoride Study (15), were retrieved from −80°C storage, thawed, vortexed, and diluted 1:1,000, and 50 µl was inoculated onto Trypticase Yeast Extract Cystine (TYC) medium agar plates (16) of pH 7 and 5.5, and onto Difco™ Mitis-Salivarius agar (Becton, Dickinson and Co., Sparks, MD) for the selective growth of oral streptococci. Mitis-Salivarius agar with 500 µg/ml kanamycin, 1% potassium tellurite, and 50 U/ml bacitracin was used for the recovery and enumeration of MS. Plates were incubated for 48 h (pH7 TYC and Mitis-Salivarius plates) or 72 h (pH 5.5 TYC plates) in an aerobic atmosphere with 5% CO_2 at 37°C. Modified modified-trypticase–phytone–yeast medium (MMTPY) was used for the recovery of bifidobacteria (17). MMTPY plates were incubated within an anaerobic chamber (85% nitrogen, 10% carbon dioxide, and 5% hydrogen) at 37°C for 72 h.

Samples were collected from a total of 85 subjects (38 males and 47 females; 79 White, 1 Black, 3 Hispanic, 1 Asian, and 1 Native American). Each subject was placed within one of three groups. Samples from all groups were from sound second molars at age 13 (Visit 1). At age 17 (Visit 2), the same sites were still healthy in Group 1 ($n = 39$), but showed evidence of incipient decay (D1; enamel involvement) in Group 2 ($n = 19$) and Group 3 ($n = 27$). Groups 2 and 3 were distinguished from one another by the absence (Group 2) or presence (Group 3) of frank decay (≥ 3 D2 lesions) elsewhere in the dentition. The investigators involved in bacterial isolation and testing were blinded to the group identities of the samples.

A panel of ATCC-type strains of representative oral streptococci was used to confirm growth on media selective for streptococci and for confirming the utility of the acid assays employed. These strains were: *S. oralis* ATCC 35037, *S. parasanguinis* ATCC 15912, *S. gordonii* ATCC 33399, *S. mitis* ATCC 49456, *S. constellatus* ATCC 27823, *S. mutans* ATCC 25175, *S. sanguinis* ATCC 10556, *S. intermedius* ATCC 27335, *S. sobrinus* ATCC 33478, *S. anginosus* ATCC 33397, *S. vestibularis* ATCC 49124, *S. salivarius* ATCC 25975, and *S. cristatus* ATCC 51100.

Screening of strains

To select colonies from the TYC and Mitis-Salivarius plates in proportion to their presence among streptococci in the plaque sample, all colonies (12–24 from each plate) from a distinct sector of the plate were picked and subcloned for future storage and isolation of DNA. DNA was isolated using the DirectAmp™ Tissue Genomic DNA Amplification Kit (Denville Scientific, Holliston, MA). PCR identification of *S. oralis* and *S. mitis* was based on amplification of portions of the *gdh* gene that were previously found to be highly conserved among streptococci but contain variable regions that allowed species-specific amplification (18). PCR reactions with a primer set GDH1 (forward: 5′-ACAACTGAAACCTT TGCATCTGG-3′; reverse: 5′-CGGTCGCATCTGTA CGGTAA-3′) or GDH2 (forward: same as for GDH1; reverse: 5′-TCAAYTTCCAYGAYGCACCA-3′) were set up for 25 µl reactions using 2 µl DNA template and reaction components from the GoTaq® Green Master Mix (Promega, Madison, WI). Reaction conditions were 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 51°C for 30 s, and 72°C for 40 s. Reactions were concluded with 5 min at 72°C and held at 4°C. Positive and negative reactions were determined by agarose (1%) gel electrophoresis and DNA visualization using SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA). Reactions were performed independently a second time for confirmation of amplification.

All clones giving a PCR product with the GDH1 and/or GDH2 primer sets were evaluated for genotype identity using arbitrary-primed PCR. The rationale was to reduce the pool of isolates to be assayed for acid properties by identifying identical clones. Primer 434 (5′-GCACAA CAGTTCCCTGACTTGCAC-3′) (19) (1 µl) was mixed with 4 µl template DNA in a 20-µl reaction mixture using the GoTaq® Green Master Mix. Reaction conditions were 95°C for 2 min, followed by 45 cycles of 94°C for 1 min, 30°C for 1 min, and 72°C for 2 min. Reactions were concluded by 5 min at 72°C and held at 16°C. Amplification patterns were observed following agarose (2%) gel electrophoresis at 70 V for 1.5 h. A strain was designated a unique amplitype if any portion of its DNA banding pattern was non-identical to other strains isolated from the

Citation: Journal of Oral Microbiology 2016, **8**: 32940 - http://dx.doi.org/10.3402/jom.v8.32940

same tooth site. Amplitypes were not compared between different subjects.

Testing acidogenicity and acid tolerance

Suspensions of *S. oralis* and *S. mitis* genotypes were made in 1% peptone to an optical density (OD_{600}) of 0.100 ± 0.005 using growth from overnight incubation on trypticase soy agar with 5% sheep's blood.

To test acid tolerance, 40 µl of the bacterial suspension was added to 160 µl of Brain Heart Infusion (BHI) broth of pH 4.5, 4.75, 5.0, 5.25, 5.5, 6.0, 6.5, and 7.0 within a Falcon® flat-bottomed polystyrene 96-well plate (Corning Inc. Life Sciences, Durham, NC). After overnight incubation at 37°C, the OD_{600} was measured in a SpectraMax M2^e spectrophotometer (Molecular Devices, Sunnyvale, CA).

Acidogenicity was tested using a modified protocol of de Soet et al. (20). Briefly, 100 µl of the peptone-based bacterial suspension was added to each of the eight wells within a column of a Falcon® flat-bottom polystyrene 96-well plate and the plate warmed to 37°C within an incubator. At time 0, 20 µl of 37°C 10% glucose was added to the first well, and thereafter to six additional wells at 10-min intervals. The eighth well served as a control and received 20 µl of distilled water in lieu of glucose. The plate was then centrifuged for 5 min at 4,000 rpm and 80 µl from each well was transferred to a new 96-well plate containing 20 µl/well of 0.1% *p*-nitrophenol. The *p*-nitrophenol has a yellow color at neutral pH and loses intensity as the pH decreases. Therefore, the most acidogenic isolates will show the greatest reduction in OD_{400} during the time course of the assay. The reduction in intensity was calculated as the OD_{400} of the control well minus the OD_{400} at each particular time point.

Terminal pH was measured for selected strains that were grown in a 15-ml chemically defined medium (CDM; SAFC Biosciences, Lenexa, KS), which contained glucose as the sole carbohydrate, and incubated overnight at 37°C in an aerobic atmosphere with 5% CO_2.

Statistical analyses

The Kruskal–Wallis test was used to assess the differences of quantitative variables among the three groups separately for Visits 1 and 2. Based on the results of the Kruskal–Wallis test global comparisons, the Wilcoxon rank-sum test was used to assess pairwise comparisons of the three groups. The Spearman rank correlation test was used to detect a potential correlation between the log of MS isolated and group (based on severity of group caries status). Categorical pairwise comparisons of the three groups for pH 5.5 growth and recovery of bifidobacteria were performed using Fisher's exact test. A parallel set of analyses was performed in which Groups 2 and 3 were pooled allowing for the comparison of Group 1 with the combination of Groups 2 and 3 using the Wilcoxon rank-sum test. Trapezoidal area (area under the curve) was used as a summary of acidogenicity over time and of acid tolerance for a series of pH values. All tests adopted a significance level of 5% using SAS 9.4 (Cary, NC). Adjustment for multiple comparisons was not applied for this pilot effort.

Results

Preliminary experiments revealed an unexpectedly low recovery of presumptive *S. mitis* isolates on TYC agar. Therefore, strain selection for the recovery of *S. oralis* and *S. mitis* was made on both TYC and Mitis-Salivarius (without tellurite) agars to account for possible differences in growth and recovery. As a control, 13 different oral streptococcal species (ATCC-type strains) were tested for growth on both agars; all species grew though *S. pseudopneumoniae* growth on TYC and Mitis-Salivarius agars was noticeably less fulminant than on trypticase soy

Table 1. Acidogenicity and acid tolerance of presumptive *S. oralis* isolates

	Acidogenicity (mean area under the curve for change in pH indicator OD over time ± standard deviation)		Acid tolerance (mean area under the curve for growth OD at low pH ± standard deviation)	
	All isolates	Most acidogenic	All isolates	Most acid tolerant
		Visit 1		
Group 1	11.74 ± 4.93	15.13 ± 9.13	0.041 ± 0.020	0.049 ± 0.022
Group 2	13.42 ± 9.06	19.16 ± 17.50	0.039 ± 0.015	0.050 ± 0.026
Group 3	13.16 ± 9.41	15.85 ± 12.37	0.046 ± 0.050	0.051 ± 0.050
Groups 2 + 3	13.26 ± 9.16	17.17 ± 14.52	0.043 ± 0.040	0.051 ± 0.042
		Visit 2		
Group 1	12.99 ± 6.20	18.45 ± 15.99	0.039 ± 0.015	0.048 ± 0.025
Group 2	13.25 ± 11.69	16.74 ± 14.78	0.045 ± 0.033	0.058 ± 0.051
Group 3	15.42 ± 10.74	21.18 ± 15.99	0.050 ± 0.026	0.066 ± 0.047
Groups 2 + 3	14.50 ± 11.06	19.29 ± 15.45	0.048 ± 0.029	0.062 ± 0.048

Table 2. Acidogenicity and acid tolerance of presumptive *S. mitis* isolates

	Acidogenicity (mean area under the curve for change in pH indicator OD over time ± standard deviation)		Acid tolerance (mean area under the curve for growth OD at low pH ± standard deviation)	
	All isolates	Most acidogenic	All isolates	Most acid tolerant
		Visit 1		
Group 1	8.64 ± 2.75	10.39 ± 2.86	0.020 ± 0.009	0.023 ± 0.011
Group 2	8.88 ± 2.72	9.81 ± 2.02	0.013 ± 0.002	0.014 ± 0.003*
Group 3	8.23 ± 2.31	9.39 ± 1.74	0.015 ± 0.007	0.016 ± 0.008*
Groups 2 + 3	8.52 ± 2.45	9.58 ± 1.82	0.014 ± 0.005	0.015 ± 0.006*
		Visit 2		
Group 1	8.94 ± 3.47	10.55 ± 4.73	0.019 ± 0.008	0.022 ± 0.010
Group 2	7.71 ± 1.65	9.92 ± 2.78	0.019 ± 0.005	0.025 ± 0.008
Group 3	7.72 ± 3.01	8.22 ± 3.12	0.020 ± 0.008	0.020 ± 0.008
Groups 2 + 3	7.71 ± 2.58	8.81 ± 3.05	0.020 ± 0.007	0.022 ± 0.008

*The value is significantly different from Group 1 for that column and visit, $p \leq 0.05$.

agar with 5% sheep's blood. The rate of recovery of presumptive *S. mitis* strains was nearly identical for both agars (6.3% for TYC vs. 7.6% for Mitis-Salivarius), though presumptive *S. oralis* isolates were recovered at twice the rate on Mitis-Salivarius (25.5%) as on TYC (13.6%). In all, presumptive *S. oralis* strains were isolated from 81 of 85 subjects and presumptive *S. mitis* strains were isolated from 52 of 85 subjects. Genotyping by Arbitrary Primed - Polymerase Chain Reaction (AP-PCR) was done to streamline testing of acid properties by identifying genetically identical clones in samples from a given tooth site. Genotypes were not compared between different subjects. Using this definition, there were 623 presumptive *S. oralis* isolates that comprised 324 unique genotypes, and 226 presumptive *S. mitis* isolates that comprised 138 unique genotypes.

Unique genotypes were analyzed for acidogenicity and acid tolerance. Data analyses not only considered average values when multiple genotypes were obtained from the same plaque sample, but also considered the values for the most acidogenic and most acid tolerant genotypes. Since acidogenicity was calculated as the change in OD over time and acid tolerance was defined as the growth OD, larger values corresponded with greater proficiency for each of these acid properties. When comparisons were made across groups for Visits 1 and 2, there were no statistically significant differences for the presumptive *S. oralis* isolates (Table 1 and Supplementary Fig. 1). For presumptive *S. mitis*, there were statistically significant differences for acid tolerance between Group 1 and Groups 2 and 3, but only for Visit 1 (Table 2 and Supplementary Fig. 1). The differences were statistically significant for both the complete complement of isolates and for those representing the most acid tolerant strains. These differences represented a trend opposite of that predicted, since isolates from the healthy Group 1 sites displayed higher acid tolerance than those from 'future' caries sites in Groups 2 and 3.

To test the possibility that comparing averages of acidogenicity and acid tolerance masked differences that might be occurring at the level of individual subjects, data were also analyzed by a chi-squared analysis that inquired whether there was an equal distribution between groups based on subjects with a greater average or peak

Table 3. Chi-squared analysis of visit with higher acid tolerance among *S. mitis* isolates

Average acid tolerance			*p*	Peak acid tolerance				*p*
Group 1 vs. Groups 2 + 3			0.0266	Group 1 vs. Groups 2 + 3				0.0329
	Average acid tolerance				Maximal acid tolerance			
Group	V1 greater	V2 greater	Total	Group	Equal	V1 greater	V2 greater	Total
1	36 (92.31%)	3 (7.69%)	39	1	0 (0.00%)	37 (94.87%)	2 5.13%	39
2 + 3	34 (73.91%)	12 (26.09%)	46	2+3	1 (2.17%)	34 (73.91%)	11 (23.91%)	46
Total	70	15	85	Total	1	71	13	85

Citation: Journal of Oral Microbiology 2016, **8**: 32940 - http://dx.doi.org/10.3402/jom.v8.32940

acidogenicity or acid tolerance at Visit 1 or 2. A statistically significant difference was found only for *S. mitis* acid tolerance, using peak or average values, when Group 1 was compared with the pooled Groups 2 and 3. In this comparison, there was a significantly greater frequency of higher acid tolerance at Visit 2 from the caries subjects (Groups 2 and 3) (Table 3).

In general, the *S. oralis* strains were more acidogenic and more acid tolerant than the *S. mitis* strains. This pattern was also observed when testing ATCC-type strains of these two species. Perhaps significantly, the ATCC *S. mitis* and *S. oralis* strains were the least and second least acidogenic and acid-tolerant strains among the 13 oral streptococcal species tested in the acid assays (data not shown).

To consider other variables that could be linked with caries status, statistical correlations with recovery of MS, recovery of bifidobacteria (21), and recovery of bacteria on pH 5.5 medium were investigated for the different groups. Of these variables, the recovery of MS was statistically significant both in pairwise comparisons and correlation analyses (Fig. 1 and Tables 4 and 5). Interestingly, the correlation with mutan levels was statistically significant even for Visit 1, which was 4 years prior to the diagnosis of early decay. Still, of the 577 clones that were isolated on pH 5.5 media, only 42 (7.3%) were MS (Table 6). Even fewer were presumptive *S. oralis* (3.3%) and none were presumptive *S. mitis*. One-third (32.4%) of the pH 5.5 clones could be assigned to a common oral streptococcal species (within the limits of our PCR-based identification scheme), but there was no obvious preferential recovery of particular species from a given subject group. Further, there was no link between the apparent acid tolerance of the strains isolated from pH 5.5 media and their acidogenicity. We measured similar terminal pH values of *S. mutans* clones isolated on pH 5.5 media (pH 4.92; $n = 14$) and clones isolated on pH 7 media (pH 4.89; $n = 3$). The pH 7 clones were from subjects who were negative for pH 5.5 growth, suggesting that the *S. mutans* clones present in these subjects did not grow on pH 5.5 media.

Discussion

There were several interesting outcomes from this study. First, the hypothesized positive correlation between acidogenicity and acid-tolerance properties of *S. oralis* or *S. mitis* and caries was not observed. Second, while this study affirmed a positive correlation between MS levels and caries, there were no statistically significant correlations between the growth of bifidobacteria or growth on pH 5.5 media and caries. Other investigations of streptococci from white-spot carious lesions have also found positive correlations with MS levels but have called attention to their low proportional representation (6, 11, 22). As the MS were not the main focus of this study,

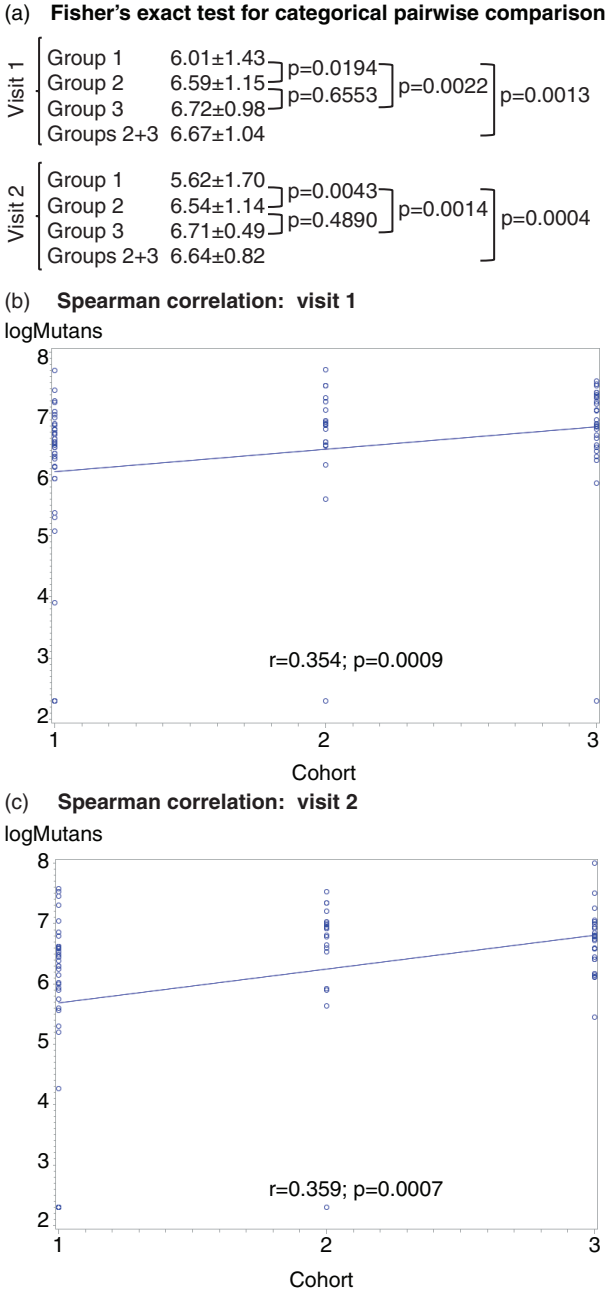

Fig. 1. (a) Tabular and (b, c) graphical data and statistical analyses for the $\log_{10}$ recovery of mutans streptococci from plaque samples obtained at Visits 1 and 2. Panel a shows that MS recovery was significantly lower in Group 1 compared with Groups 2 and 3 when paired individually or with data pooled for Groups 2 and 3. Panels b and c show the statistically significant positive correlation between MS recovery and caries at Visits 1 and 2, respectively (subject groups are labeled as cohorts on the graphs).

their proportional representation was not determined. However, it was observed that $<10\%$ of the clones recovered on pH 5.5 media were MS. The proportionally low representation of the MS is one of the main points

Table 4. Fisher's exact test for categorical pairwise comparisons: Visit 1

pH 5.5 growth	*p*	Bifidobacteria	*p*
Group 1 vs. Group 2	0.3666	Group 1 vs. Group 2	0.5141
Group 1 vs. Group 3	0.4330	Group 1 vs. Group 3	1.0000
Group 2 vs. Group 3	1.0000	Group 2 vs. Group 3	0.5135

	pH 5.5 growth				Bifidobacteria		
Group	No	Yes	Total	Group	No	Yes	Total
1	14 (35.90%)	25 (64.10%)	39	1	31 (79.49%)	8 (20.51%)	39
2	4 (21.05%)	15 (78.95%)	19	2	13 (68.42%)	6 (31.58%)	19
3	7 (25.93%)	20 (74.07%)	27	3	21 (77.78%)	6 (22.22%)	27
Total	25	60	85	Total	65	20	85

put forth to argue that the MS cannot be the dominant force in caries etiology. Nonetheless, the statistically significant differences in MS levels among the subject groups at Visit 1, while all sampled sites were still considered clinically sound, cannot be overlooked. We also measured the *in vitro* terminal pH of representative *S. mutans* clones isolated on pH 5.5 media and on pH 7 media from subjects negative for pH 5.5 growth. There was no significant difference. This result, along with the lack of a significant positive correlation between growth on pH 5.5 media and caries, leads us to speculate that the growth on low pH media could be related more to the acid adapted state of plaque bacteria at the time of sampling than to the overall acidogenic potential of the plaque microbiome. Consequently, quantifying growth on low pH media may not accurately assess cariogenic potential.

Another notable outcome was the relatively low recovery of *S. mitis* strains. *S. mitis* has been reported to be the most highly represented streptococcal species in coronal dental plaque (13, 14). It is possible that its distribution varies such that it is poorly represented on occlusal surfaces, poorly represented in the subject population sampled in this study, more susceptible to loss of viability when stored frozen, or simply misidentified in earlier studies due to the imprecision of identification methods.

There are several potential explanations for the lack of a significant correlation between acidogenicity and acid-tolerance properties and caries among the *S. oralis* and *S. mitis* strains recovered in this study. Foremost among these is that the data accurately reflect reality, at least for the sites tested in this study. In order for that to be the case, we have to be confident in the accuracy of species assignment. The PCR-based methodology developed for this study (18) replaced a biochemical strategy that proved inadequate, especially for the identification of *S. mitis*. Multi-locus sequence analysis (MLSA) based on seven genes (23) was used to test a random sample representing 10% of the presumptive unique *S. oralis* genotypes and 10% of the presumptive unique *S. mitis* genotypes, along with 20 random clones that were PCR-negative for *S. oralis* and *S. mitis*. All presumptive *S. mitis* clones were *S. mitis*/*S. pseudopneumoniae*, with nearly identical BLAST scores for each species for the selected loci. Specificity for *S. oralis* was approximately 78%, though MLSA could not distinguish between *S. oralis* and the unnamed strain designated VT162 (24). Non-

Table 5. Fisher's exact test for categorical pairwise comparisons: Visit 2

pH 5.5 growth	*p*	Bifidobacteria	*p*
Group 1 vs. Group 2	1.0000	Group 1 vs. Group 2	0.5023
Group 1 vs. Group 3	0.4580	Group 1 vs. Group 3	0.7571
Group 2 vs. Group 3	0.7657	Group 2 vs. Group 3	1.0000

	pH 5.5 growth				Bifidobacteria		
Group	No	Yes	Total	Group	No	Yes	Total
1	20 (51.28%)	19 (48.72%)	39	1	32 (82.05%)	7 (17.95%)	39
2	9 (47.37%)	10 (52.63%)	19	2	14 (73.68%)	5 (26.32%)	19
3	11 (40.74%)	20 (59.26%)	27	3	21 (77.78%)	6 (22.22%)	27
Total	40	45	85	Total	65	20	85

Citation: Journal of Oral Microbiology 2016, **8**: 32940 - http://dx.doi.org/10.3402/jom.v8.32940

Table 6. Species assignment of streptococcal isolates recovered on pH 5.5 medium

Subjects/visits per group(s)[b]	Number of clones isolated on pH 5.5 media from subjects in each group that were assigned to species by PCR[a]								
	S. mutans	*S. sobrinus*	*S. salivarius*	*S. gordonii*	*S. sanguinis*	*S. parasanguinis*	*S. oralis*	Milleri group	Unknown
Group 1 *N* = 41 visits *N* = 27 subjects	22	3	46	4	0	5	10	13	142
Groups 2 + 3 *N* = 55 visits *N* = 35 subjects	9	8	42	5	1	4	9	6	248
Group 2 *N* = 23 visits *N* = 16 subjects	6	2	16	3	1	0	7	3	90
Group 3 *N* = 32 visits *N* = 19 subjects	3	6	26	2	0	4	2	3	158

Strains that could not be assigned to a species by a PCR-based strategy were designated as Unknown. [a]None were positive for *S. mitis*, none were positive for *S. infantis*, and none were positive for *S. pseudopneumoniae*; [b]growth on pH 5.5 media was obtained for only a subset of subjects and sometimes for only one of the two visits.

S. oralis species belonged to *S. gordonii*, *S. intermedius*, *S. anginosus*, and *S. sanguinis*. Consequently, the *S. oralis* screen specificity rises to 84% if other species (*S. gordonii* and *S. anginosus*) traditionally linked to low pH strains are included (18). In order to be sure that the non-*S. oralis* strains were equally distributed among the different subject groups and therefore did not bias the acidogenicity and acid-tolerance results, we took advantage of the ability of *S. gordonii*, *S. intermedius,* and *S. anginosus* to utilize arginine. Using BBL Decarboxylase Arginine Broth, we tested all putative *S. oralis* clones and found the distribution of arginine utilizers to be random among the subject groups and consistent with the expected specificity of the *S. oralis* screen. Sensitivity was outstanding as none of the 20 random PCR-negative clones were identified as *S. oralis* or *S. mitis*. Overall, species assignment was excellent. Slightly more variability existed among presumptive *S. oralis* than presumptive *S. mitis*, but non-*S. oralis* clones included other species linked to low pH streptococci.

Given the inherent difficulty in distinguishing between oral streptococcal species, it is conceivable that low pH strains attributed to *S. oralis* and *S. mitis* in previous studies were, in fact, strains that belonged to other species. On the contrary, the *S. oralis* data from this study revealed a trend toward greater acidogenicity in subject groups 2 and 3, as well as greater acid tolerance in those groups at Visit 2. Since at least one report (6) describes the association of non-MS low pH streptococci with caries as weaker than that between MS and caries, it is possible that the sample size in this study was insufficiently large to detect a significant positive correlation, especially when limited to a single species. All low pH strains may need to be considered collectively in order to more readily detect a potential link with caries. To efficiently test acidogenicity and include a time-dependent variable, a pH-sensitive dye was used rather than direct pH measurement. Therefore, it is uncertain how many of the *S. oralis* and *S. mitis* clones would have achieved a low terminal pH in glucose broth. However, analysis of a panel of ATCC-type strains of oral streptococcal species found the *S. oralis* and *S. mitis* representatives to be the least proficient in both acid production and acid tolerance. In contrast, the *S. anginosus* representative was the most proficient in both acid properties, even exceeding *S. mutans* and *S. sobrinus*. The *S. gordonii* representative displayed the second highest level of acidogenicity in our assay. Thus, the inclusion of *S. anginosus* and *S. gordonii* strains among the putative *S. oralis* could be responsible for the greater variability in acidogenicity and acid tolerance within the *S. oralis* strain library. These results suggest that non-MS low pH strains could be more likely among species other than *S. oralis* and *S. mitis*.

The negative correlation between acid tolerance and future caries for the *S. mitis* in Visit 1 might lead one to speculate that greater acid tolerance allows more of this species to survive in low pH environments thereby precluding larger accumulations of more acidogenic species. This difference was not maintained at Visit 2 when comparing averages of acid tolerance between subject groups. In contrast, the frequency of Group 2 + 3 subjects with higher acid tolerance *S. mitis* isolates at Visit 2 was

significantly greater than for Group 1 when analyzed by chi-square. Thus, these two methods of data analysis seemingly offer different perspectives on the importance of acid tolerance among *S. mitis*. Future investigations with larger cohorts will be necessary to determine if these patterns hold. Additionally, investigations of acid tolerance may offer greater insight when coupled with a standardized protocol for acid adaptation.

In summary, this study of non-MS low pH streptococci did not find a significant positive correlation between strain-specific acidogenicity properties and caries. *S. mitis* strain variation appeared to be the least likely to contribute to caries etiology whereas a positive, though statistically insignificant, trend was observed for *S. oralis*. Correlations between caries and strain-specific acid tolerance were not found for *S. oralis* and were enigmatic for *S. mitis*. Thus, future study of non-MS low pH streptococci is warranted, but may be most effective when considering all non-MS low pH streptococci collectively and with a focus on acidogenicity. Further exploration of acid tolerance may yet yield new insights but may require analysis following an acid adaptive response. A related line of investigation should inquire whether various oral streptococcal species indeed harbor significant variations in acidogenicity and acid tolerance or whether the apparent variability is due to the difficulty in accurately distinguishing among oral streptococcal species. Such work could refine the concept and/or constitution of non-MS low pH streptococci as related to dental caries. Finally, a significant positive correlation between MS levels and caries was confirmed as it consistently is across a breadth of study designs. Consequently, it is premature to dismiss the significance of the MS until experiments can more adequately evaluate how acid potential and relative representation combine to shape caries risk.

Conflict of interest and funding

This work was funded by NIDCR award DE022605. Banked plaque samples were obtained from the Iowa Fluoride Study supported by NIH award DE09551, the NIH CTSA support program UL1RR024979, and the Roy J. Carver Charitable Trust Award 16-4687. The authors declare that this study was conducted without bias and that they have no conflict of interest with respect to reporting the data and conclusions.

References

1. Marsh PD. Microbial ecology of dental plaque and its significance in health and disease. Adv Dent Res 1994; 8: 263–71.
2. Kleinberg I. A mixed-bacteria ecological approach to understanding the role of the oral bacteria in dental caries causation: an alternative to *Streptococcus mutans* and the specific-plaque hypothesis. Crit Rev Oral Biol Med 2002; 13: 108–25.
3. Takahashi N, Nyvad B. The role of bacteria in the caries process: ecological perspectives. J Dent Res 2011; 90: 294–303.
4. Peterson SN, Snesrud E, Schork NJ, Bretz WA. Dental caries pathogenicity: a genomic and metagenomics perspective. Int Dent J 2011; 61: 11–22.
5. van Houte J, Sansone C, Joshipura K, Kent R. Mutans streptococci and non-mutans streptococci acidogenic at low pH, and *in vitro* acidogenic potential of dental plaque in two different areas of the human dentition. J Dent Res 1991; 70: 1503–7.
6. Sansone C, van Houte J, Joshipura K, Kent R, Margolis HC. The association of mutans streptococci and non-mutans streptococci capable of acidogenesis at a low pH with dental caries on enamel and root surfaces. J Dent Res 1993; 72: 508–16.
7. van Houte J. Role of micro-organisms in caries etiology. J Dent Res 1994; 73: 672–81.
8. van Ruyven FOJ, Linström P, van Houte J, Kent R. Relationship among mutans streptococci, 'low-pH' bacteria, and iodophilic polysaccharide-producing bacteria in dental plaque and early enamel caries in humans. J Dent Res 2000; 79: 778–84.
9. Kilian M, Mikkelsen L, Henrichsen J. Taxonomic study of viridans streptococci: description of *Streptococcus gordonii* sp. nov. and emended descriptions of *Streptococcus sanguis* (White and Niven 1946), *Streptococcus oralis* (Bridge and Sneath 1982), and *Streptococcus mitis* (Andrewes and Horder 1906). Int J Syst Bacteriol 1989; 39: 471–84.
10. Svensäter G, Borgström M, Bowden GHW, Edwardsson S. The acid-tolerant microbiota associated with plaque from initial caries and healthy tooth surfaces. Caries Res 2003; 37: 395–403.
11. van Houte J, Sansone C, Joshipura K, Kent R. *In vitro* acidogenic potential and mutans streptococci of human smooth-surface plaque associated with initial caries lesions and sound enamel. J Dent Res 1991; 70: 1497–502.
12. Aas JA, Griffen AL, Dardis SR, Lee AM, Olsen I, Dewhirst FE, et al. Bacteria of dental caries in primary and permanent teeth in children and young adults. J Clin Microbiol 2008; 46: 1407–17.
13. Tappuni AR, Challacombe SJ. Distribution and isolation frequency of eight streptococcal species in saliva from predentate and dentate children and adults. J Dent Res 1993; 72: 31–6.
14. Simón-Soro Á, Tomás I, Cabrera-Rubio R, Catalan MD, Nyvad B, Mira A. Microbial geography of the oral cavity. J Dent Res 2013; 92: 616–21.
15. Broffitt B, Levy SM, Warren J, Cavanaugh JE. Factors associated with surface-level caries incidence in children aged 9 to 13: the Iowa Fluoride Study. J Public Health Dent 2013; 73: 304–10.
16. De Stoppelaar JD, Van Houte J, de Moor CE. The presence of dextran-forming bacteria, resembling *Streptococcus bovis* and *Streptococcus sanguis*, in human dental plaque. Arch Oral Biol 1967; 12: 1199–202.
17. Beighton D, Gilbert SC, Clark D, Mantzourani M, al-Haboubi M, Ali F, et al. Isolation and identification of *Bifidobacteriaceae* from human saliva. Appl Environ Microbiol 2008; 74: 6457–60.
18. Banas JA, Zhu M, Dawson DV, Cao H, Levy SM. PCR-based identification of oral streptococcal species. Int J Dent 2016:3465163, doi: http://dx.doi.org/10.1155/2016/3465163
19. Rudney JD, Larson CJ. Identification of oral mitis group streptococci by arbitrarily primed polymerase chain reaction. Oral Microbiol Immunol 1999; 14: 33–42.
20. de Soet JJ, Nyvad B, Kilian M. Strain-related acid production by oral streptococci. Caries Res 2000; 34: 486–90.
21. Kaur R, Gilbert SC, Sheehy EC, Beighton D. Salivary levels of *Bifidobacteria* in caries-free and caries-active children. Int J Paediatr Dent 2013; 23: 32–8.

22. Nyvad B, Kilian M. Comparison of the initial streptococcal microflora on dental enamel in caries-active and caries-inactive individuals. Caries Res 1990; 24: 267–72.
23. Bishop CJ, Aanensen DM, Jordan GE, Kilian M, Hanage WP, Spratt BG. Assigning strains to bacterial species *via* the internet. BMC Biol 2009; 7: 3 doi: http://dx.doi.org/10.1187/1741-7007-7-3
24. Vecherkovskaya MF, Tetz GV, Tetz VV. Complete genome sequence of the *Streptococcus* sp. VT 162, isolated from the saliva of pediatric oncohematology patients. Genome Announc 2014; 2: e00647–14. doi: http://dx.doi.org/10.1128/genomeA.00647-14