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# A Potential Biofilm Metabolite Signature for Caries Activity - A Pilot Clinical Study

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## Abstract

**Background:** This study's aim was to compare the dental biofilm metabolite-profile of caries-active (N=11) or caries-free (N=4) children by gas chromatography-mass spectrometry (GC/MS) analyses.

**Methods:** Samples collected after overnight fasting, with or without a previous glucose rinse, were combined for each child based on the caries status of the site, re-suspended in ethanol and analyzed by GC/MS.

**Results:** Biofilm from caries-active sites exhibited a different chromatographic profile compared to caries-free sites. Qualitative and quantitative analysis suggested a special cluster of branched alcohols and esters present at substantially higher intensity in biofilms of caries-active sites.

**Conclusions:** This pilot study indicates that there are metabolites present in the biofilm which have the potential to provide a characteristic metabolomics signature for caries activity.

**Keywords:** Dental-caries; Metabolites; Biomarker; Caries-activity; Gas chromatography-mass spectrometry

## Introduction

Despite our knowledge of the basic concepts of dental caries, the most common chronic childhood disease [1], the interactions within the dental biofilm microbiota, which may serve as an important caries risk indicator [2-5] are not well understood. Although dental caries is clearly a microbial regulated disease, it is not a traditional transmissible infectious disease as it does not meet Koch's key postulates, that an infectious organism presence or absence is related to presence or absence of the disease. Presently, data concerning caries risk indicators are primarily based on the number and proportion of *Streptococcus mutans* which seem to augment caries prediction only when used in very young children [6].

The understanding of the correlation between metabolite signals of dental biofilm and caries is in its infancy. Takahashi and his group [7] focused on the central carbon metabolism, the Embden-Meyerhof-Parnas (EMP) pathway, the pentose-phosphate pathway, and the tricarboxylic cycle in supragingival biofilm and specific oral bacteria, *S. mutans*, *Streptococcus sanguinis*, *Actinomyces oris*, and *Actinomyces naeslundii*. The investigators used capillary electrophoresis and mass spectrometry to elucidate metabolic regulation in dental biofilm through the comparison of metabolite profiles between supragingival dental biofilm and representative biofilm bacteria preceding and following a glucose rinse [7,8]. Their study indicated that the central carbon metabolism is basically functioning in the microbiota of *in vivo* supragingival biofilm, and these pathways are most likely similar to the integration of metabolic pathways determined in representative *in vitro* biofilm bacteria, *Streptococcus* and *Actinomyces*. Furthermore, they found that rinsing with glucose resulted in lactate production in supragingival plaque, demonstrating an increase of metabolites upstream and a decrease of metabolites downstream (except pyruvate) of the EMP pathway. Another study by this group demonstrated that metabolite analyses can be used to detect metabolic regulation in *in vivo* supragingival plaque [8].

Understanding the physiological traits of bacteria in oral biofilms is key for the identification of new preventative or therapeutic measures to control dental caries [9]. Findings indicate that around 200 bacterial species/phylogenies can be found in the oral cavity by 16S DNA analysis [5,10] and that there may be pathogenic communities in the dental biofilm microbiome that significantly correlate with dental caries status [10,11]. Recently, a core functional microbiota of almost 60 species was identified [12]. The metabolic changes that occur in the diverse oral biofilm during the transition from a non-pathogenic to a pathogenic state may also prove to be important disease indicators.

Modern management of dental caries involves determining the caries risk status of the patient; detecting and assessing caries lesions at an earlier stage; making a diagnosis as to whether the disease is actually present; establishing a prognosis; applying intervention strategies focused on preventing, arresting and possibly reversing the caries process; and delaying restorative treatment to only when absolutely necessary [13]. Identification of at-risk active sites is a core component for the principles for the modern management of dental caries [13] and this is the biggest challenge faced by dentists. Currently there is no means to accurately determine if a site will develop a lesion, or if a lesion will progress to cavitation. The goal of this preliminary study was to identify potential site-specific metabolite profiles in caries-free and

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caries active areas (areas with a lesion progressing towards cavitation).

It was hypothesized that caries lesion activity is a reflection of the activity of the overlaying dental biofilm; therefore, children that had caries-active lesions can be distinguished from children with no caries activity based upon the metabolite signal of their biofilm. A signature associated with dental caries status should enable a more accurate determination of lesion activity at the time of a dental examination. The results indicate that there is a potential distinct metabolite profile in the biofilm of caries active lesions.

## Materials and Methods

A convenience sample of 15 children from public schools in Aguas Buenas, Puerto Rico who were enrolled in a parent study previously described [14-16] was recruited in June 2011 as approved by the IRB committees from Indiana University (IU-IRB #0608-15) and University of Puerto Rico (UPR-IRB#A1340107). Parental consent was obtained along with assent. For inclusion in the study, children had to have been enrolled in the parent study, be between 10-15 years of age, have no medical problem that contra-indicated participation, have been characterized as caries-active (at least one lesion progressed to cavitation in the prior year) or caries-free (no history of lesions or fillings) per the International Caries Detection and Assessment System (ICDAS) and allow collection of dental biofilm. The children were requested not to brush their teeth or eat after the last meal (dinner) the evening before dental biofilm collection. All children were seen in the morning and dental biofilm samples were collected after overnight fasting (day 1) and after a 1 minute rinse with 15 ml of 20% glucose rinse (day 2). Dental biofilm samples were collected from 15 children, 11 characterized as caries-active (ICDAS 1-3) and 4 characterized as caries-free (ICDAS 0). On average there were 4 active sites (C-A) per caries-active child, with two children having only one C-A site and one child having 10 C-A sites, and there were 10 samples collected from caries-free sites (C-F) for each child (caries-active or caries-free child). At each collection day, for each child, biofilm samples were combined in two different collection vials according to the surface's caries status (C-A or C-F), re-suspended in ice-cold 100% ethanol and analyzed regarding their metabolite profile by GC/MS after sorptive extraction at the Indiana University Institute for Pheromone Research.

## GC/MS Method

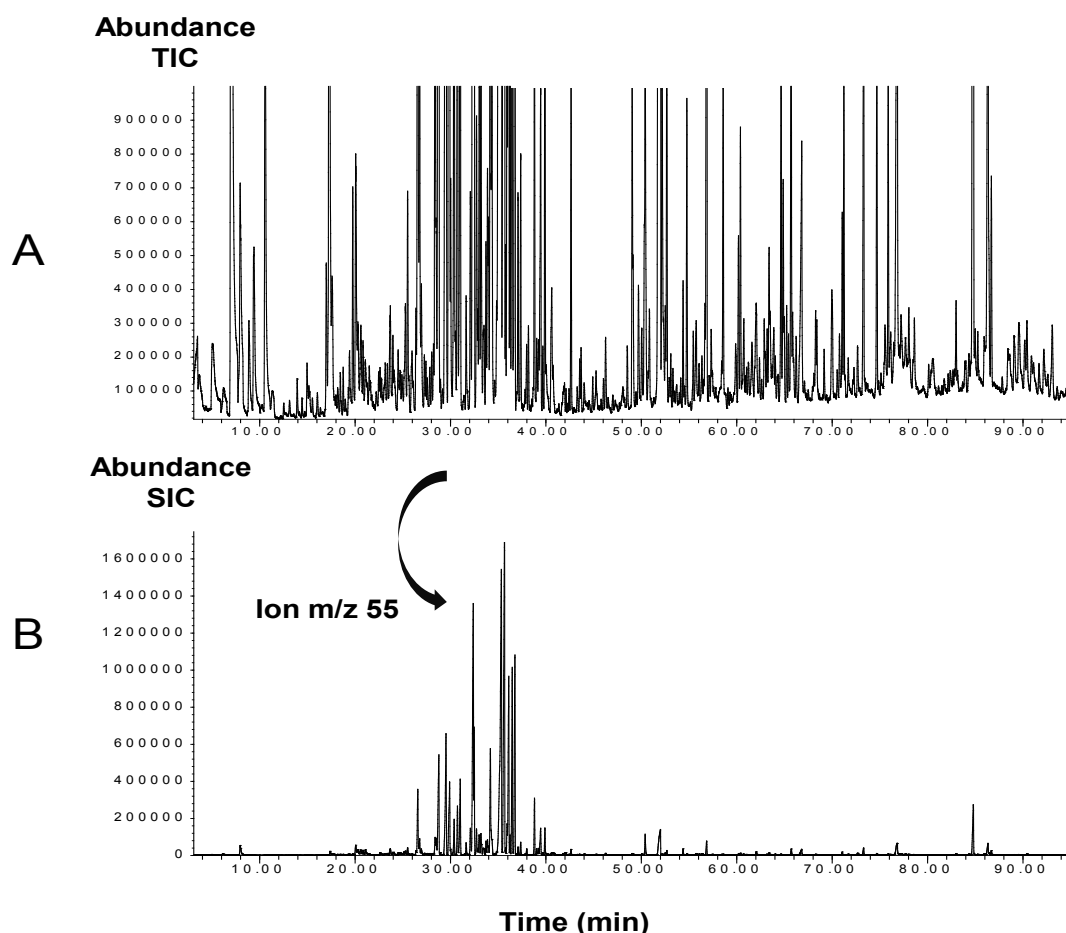
The dental biofilm was weighed and re-suspended in 800  $\mu$ l of 100% ethanol (Koptec, King of Prussia, PA) and stored at -20°C prior to the metabolite profiling using previously described methods [17, 18]. Each sample vial containing dental biofilm and ethanol was emptied to a 20 ml glass scintillation vial and the vial was filled to 20 ml with high-purity water (OmniSolv® EM Science, Gibbstown, NJ). The sample was ultrasonicated for 15 minutes in the presence of 50 mg of ammonium sulfate (99.999%, Sigma-Aldrich, St. Louis, MO). Previous analyses have shown that ammonium sulfate amplifies the extraction recoveries of volatile compounds from biofilm samples, as has been previously seen with other difficult biological sample matrices. Subsequently, 8 ng of the internal standard (7-tridecanone from Sigma-Aldrich, St. Louis, MO) in 5  $\mu$ l of methanol (J.T. Baker, Center Valley, PA) and a Twister™ stir bar (10 mm long, 0.5 mm film thickness, 24  $\mu$ l polydimethylsiloxane volume, Gerstel GmbH (Mülheim an der Ruhr, Germany) was added in each vial for the 60 min sorptive extraction process (at 800 rpm) to enrich small organic metabolic products from the ethanol-water phase (20 ml) into the polymer phase (24  $\mu$ l). After the extraction, the stir bars were placed in the Thermal Desorption

Autosampler (TDSA) tubes for the GC/MS analysis. Samples were thermally desorbed in a TDSA automated system in a splitless mode, followed by injection into the GC column with a cooled injection assembly, CIS-4 (operating at the temperature range from -80 to 270°C). The separation capillary column in the analyses was DB-5MS (30 m  $\times$  0.25 mm, i.d., 0.25  $\mu$ m film thickness) from Agilent Technologies Inc. (Wilmington, DE). The GC unit operated in the constant-flow mode. The GC/MS instrument used for the compound identification was the Agilent 6890 N gas chromatograph connected to the 5973i MSD mass spectrometer. Positive electron ionization (EI, 70 eV) mode was used with the scanning rate of 2.41 scans/s over the mass range of 41-350 amu. Total ion chromatograms (TICs) were measured for all the samples. Post-run selected ion chromatograms (SICs) at ion m/z 55 for the analytes of interest and the ion m/z 113 for the internal standard were recorded (Figure 1). The MSD transfer line temperature was set at 280°C. The ion source and quadrupole temperatures were set at 230°C and 150°C, respectively.

## Statistical Analyses

The C-A and C-F sites were compared for metabolite profile considering differences in peak area ratios of the ion m/z 55 for the compounds 1-6 (Figure 2) in a cluster of branched alcohols and esters (26-45 min) and the ion m/z 113 for the internal standard (7-tridecanone, 50.01 min) in the corresponding run. After the peak area ratios were calculated and normalized further per unit weight (mg), multivariate Wilcoxon Signed Rank (WSR) tests [19] were applied for simultaneous assessment of the six compounds in four null hypotheses: (H1) there were no differences in peak area ratios for C-A sites and C-F sites among caries-active children without glucose rinse; (H2) there were no differences in peak area ratios for C-A sites and C-F sites among caries-active children with glucose rinse; (H3) there were no differences in peak area ratios for C-A sites in caries-active children with and without glucose rinse; and (H4) there were no differences in peak area ratios for C-F sites in all children with and without glucose rinse. A sensitivity analysis was conducted for the last hypothesis by applying the multivariate WSR test to peak area ratios from only caries-active children. The multivariate tests provide a degree of Type I error control. For each hypothesis, univariate WSR tests are conducted for the six individual compounds only if the corresponding multivariate test is statistically significant at the 0.05 level, which would indicate significant differences for one or more of the six compounds. The univariate tests use a Bonferroni-corrected significance level such that a p-value less than 0.05/6=0.0083 is considered statistically significant (S), a p-value>0.05 is not statistically significant (NS), and a p-value between 0.0083 and 0.05 is considered almost statistically significant (AS). Due to the small sample size, p-values for all WSR tests were based on the permutation distribution of the test statistic under the null hypothesis [20].

As a secondary analysis, multivariate linear models are fitted separately to the six compounds to simultaneously estimate the magnitudes of the effects of glucose rinse (yes/no) and caries presence (C-A vs. C-F) on the peak area ratios. In these models, a child contributes up to four peak area ratio values corresponding to glucose rinse (yes/no) and caries presence (yes/no). Due to skewness, the log<sub>10</sub> transformation was applied to the peak area ratio after adding a 1 to address the presence of a zero outcome. All models were fit using SAS v. 9.3 Proc Mixed with Kenward-Roger degrees-of-freedom correction for small sample sizes [21] and a Kronecker-product covariance structure [22] to account for repeated measures (i.e., the quad-variate



**Figure 1:** A: A representative GC/MS TIC (total ion chromatogram) of the volatile compound profile extracted from a dental biofilm sample. B: A simplified selected ion chromatogram (SIC) for the post-run filtered ion  $m/z$  55 from the TIC in the graph A above.

outcome). Because of small sample sizes, the models did not include the interaction of glucose rinse and caries presence.

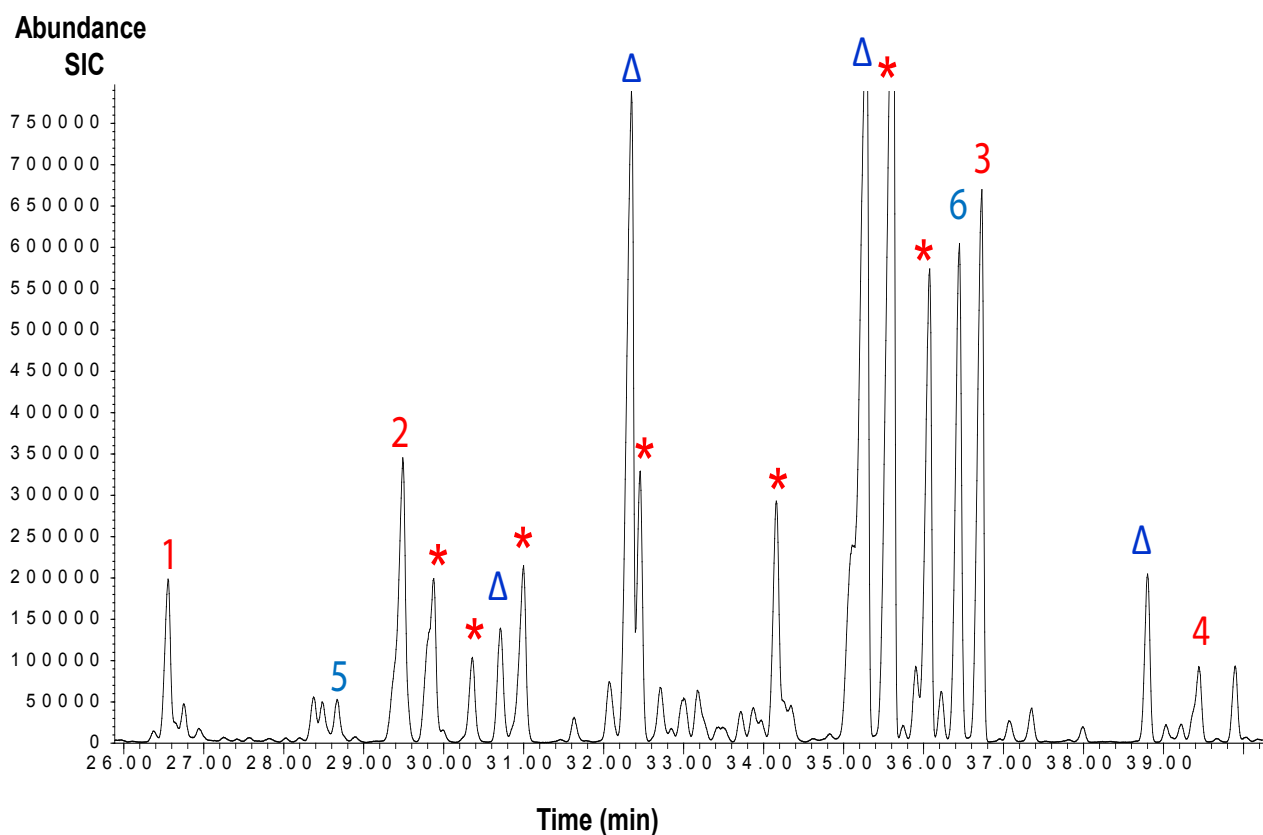
Additionally Wilcoxon Rank Sum tests with exact  $p$ -values determined from the permutation distributions of the respective test statistics were used to compare the average scores from sound sites from caries-active children and the average scores from sites from caries-free children. The comparisons were made for the four alcohols and the two esters. Two tests were done for each of the 6 compounds; one based on the data without glucose rinse and another based on the data after glucose rinse (total=12 tests).

## Results

During the sample preparation for the analyses two C-A samples were lost. Demographics and clinical data of the subjects are presented in Table 1. Samples from C-A sites weighed on average 0.0012 g (range 0.0001 to 0.0025 g) and from C-F sites on average 0.0098 (range 0.034 to 0.0288 g). Reproducibility of the GC/MS method was 8.50% (relative standard deviation, RSD,  $n=10$ ) calculated for the internal standard peak area of the ion  $m/z$  113 at retention time of 50.01 min total ion chromatograms (TICs) for each sample were post-run simplified by initially filtering the data by several different selected ions (SICs)

as shown in Figure 1 for the ion  $m/z$  55. The described GC/MS data by filtering by the ion  $m/z$  55 showed most clearly that dental biofilm collected from C-A sites with or without a previous glucose rinse exhibited a differential metabolic profile compared to samples from C-F sites (Figure 3). This was consistent across all subjects independent of the number of C-A sites present (1 or 10). The qualitative data analysis suggests that a special cluster of branched alcohols and esters is present at substantially higher intensity in biofilms of C-A sites versus C-F sites. This finding was demonstrated quantitatively when the peak areas of these compounds were normalized against an internal standard and per unit sample weight showing more pronounced metabolite amounts in samples from C-A sites, either in fasting conditions (V2,  $p=0.0205$ ) or after glucose rinse (V3,  $p=0.0033$ ) (Figure 4). Tentative identification of the compounds in the Figure 2 was based on the NIST library spectra (NIST Mass Spectral Search Program for the NIST/ EPA/ NIH Mass Spectral Library. Version 2.0 a., 2002). Possible compound structures include isooctanol (Rt 26.52 min), 2-ethyl-1-hexanol acetate (Rt 28.74), 3-methyl-1-heptanol or 6-methyl-1-heptanol (Rt 29.49 min), 2-propenoic acid octyl ester (Rt 32.38 min), 4,8-dimethylnonanol (Rt 39.40 min). However, the library matches were relatively low with 40-60 % probability.

In particular, the multivariate WSR test for H1 indicates that there



**Figure 2:** A detailed representative GC/MS post-run ion m/z 55 selected ion (SIC) metabolite profile of the dental biofilm obtained from the lesion region. 1,2,3,4: Tentatively identified branched alcohol peak areas which were used for quantitative comparisons; 5,6: tentatively identified ester peak areas which were used for quantitative comparisons. \*additional tentatively identified branched alcohols, Δadditional tentatively identified esters.

Caries status	N	Gender		MEAN (sd)			
		Male	Female	Age (years)	# of active caries lesions	# of caries free sites	DMFT
Caries-Free children	4	3	1	8.5 (1.29)	0	126.50 (8.43)	0 (0)
Caries-Active children	10	5	5	10.5 (1.78)	2.80 (3.22)	87.90 (24.92)	6 (3.2)

**Table 1:** Children demographics based on caries activity.

was an overall significant difference between C-A and C-F sites before glucose rinse ( $p=0.021$ ). Similarly, the multivariate WSR test for H2 indicates that there was a significant difference between C-A and C-F sites after glucose rinse ( $p=0.019$ ). For H3, the overall difference in peak area ratios with and without glucose rinse for C-A sites was not significant ( $p=0.412$ ). For H4, there was a significant difference between peak area ratios taken before and after glucose rinse ( $p\text{-value}=0.028$ ) for C-F sites among all children. However, in the sensitivity analysis for C-F sites from CA children only, the change from visit 2 (before glucose rinse) to visit 3 (after glucose rinse) was not significant ( $p\text{-value}=0.135$ ).

According to the univariate WSR tests, C-A and C-F sites had significance differences before glucose rinse in alcohol 1 and alcohol 2 (with the same  $p=0.005$ ), while adjusting for multiple testing the tests were almost significant for the two other alcohols and 2 esters. After glucose rinse, C-A and C-F sites had significance differences in alcohols 1, 2 and 3, and esters 1 and 2 (same  $p\text{-value}=0.005$ ). The corresponding test was almost significant for Alcohol 4 ( $p\text{-value}=0.013$ ). Finally, the univariate tests indicate that, the change from visit 2 to visit 3 for C-F sites (from all participating children) was significant only for Alcohol 1

( $p\text{-value}=0.006$ ) and the changes for the remaining three alcohols and ester 2 were almost significant. The change was not significant for ester 1.

For sound sites the comparison between the four alcohols and two esters indicated that there were no statistically significant differences between sound sites from caries-active children and caries-free children.

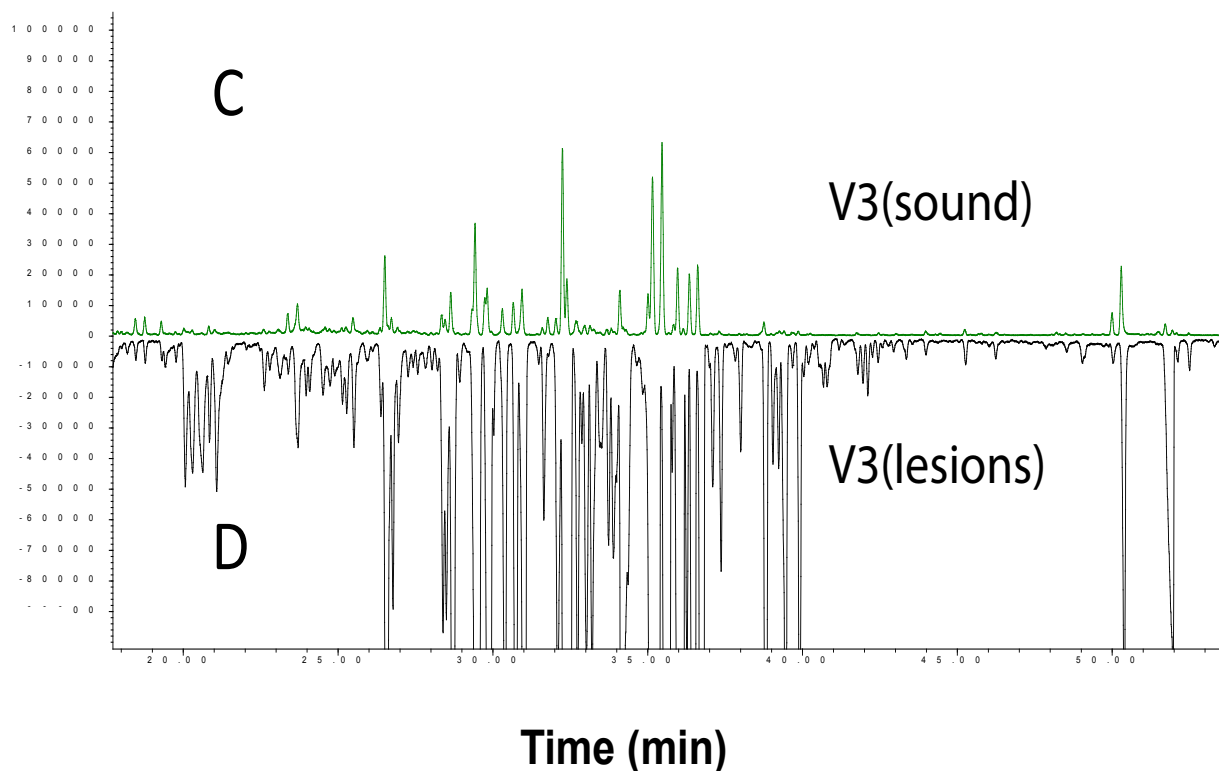
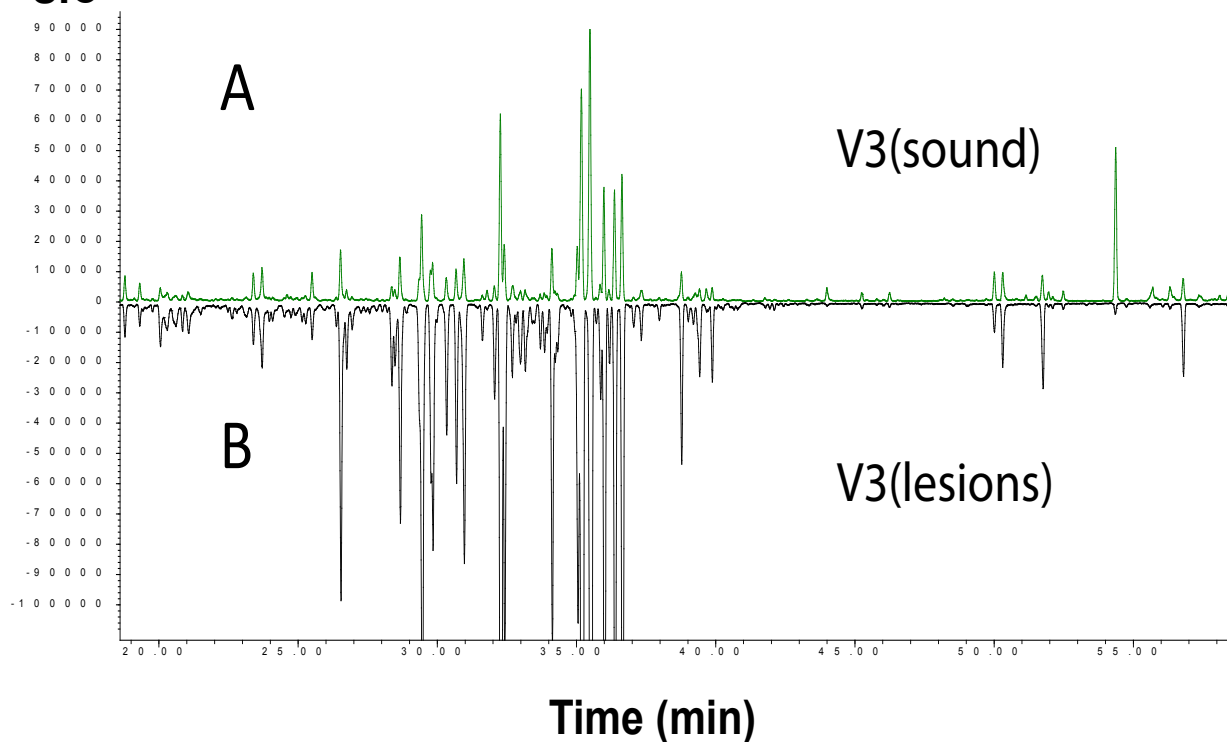
Table 2 shows that, on average, the differences in intensity of the normalized peak areas between C-A samples and C-F samples ranged from 0.40 to 0.70 on the  $\log_{10}$  scale.

**Discussion**

It is known that the intake of dietary carbohydrates induces ecological shifts in dental biofilm microbiota. The low-pH environment created due to fermentation of these carbohydrates transits dental biofilm microbiota from a dynamic stability stage to an acidogenic/aciduric stage which might result in carious lesion initiation and progression [23]. These frequent events of acidification in dental biofilm induce an Acid Tolerance Response (ATR) in the microbiota raising the proportions of acid tolerant microorganisms [24]. In this

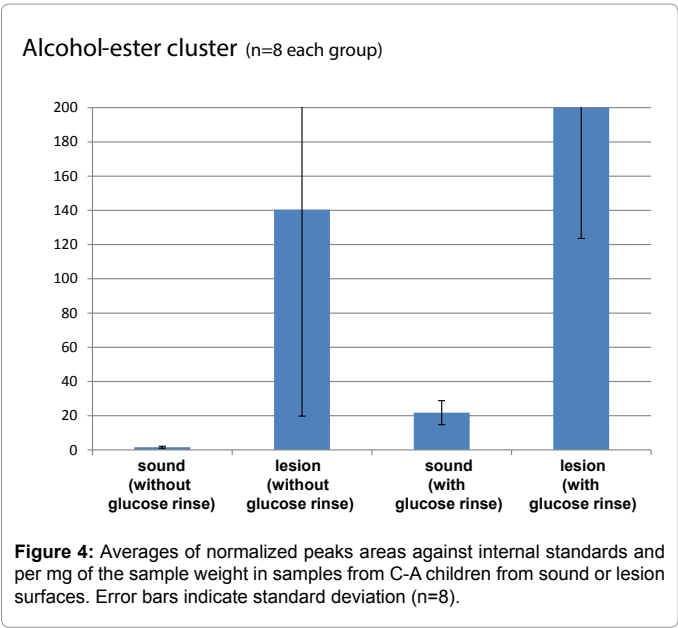
**Abundance**

**SIC**



**Figure 3:** Inversely overlaid selected ion  $m/z$  55 chromatograms at C-F (sound) and C-A (lesions) sites of two subjects after glucose solution rinse. Y-axis shows the abundance (intensity of the signal) and x-axis shows the elapsed time. A, B: subject 1; C, D: subject 2.





Effect	Estimate	SE	P-value
<b>Alcohol 1</b>			
Intercept	2.91	0.40	<0.001
GR	-1.03	0.41	0.026
CA	-1.72	0.40	0.001
<b>Alcohol 2</b>			
Intercept	3.81	0.47	<0.001
GR	-1.24	0.51	0.027
CA	-2.18	0.46	0.0003
<b>Alcohol 3</b>			
Intercept	4.40	0.54	<0.001
GR	-1.53	0.59	0.023
CA	-2.28	0.52	0.001
<b>Alcohol 4</b>			
Intercept	2.07	0.376	<0.001
GR	-0.73	0.366	0.066
CA	-1.32	0.394	0.005
<b>Ester 1</b>			
Intercept	2.62	0.345	<0.001
GR	-0.40	0.441	0.379
CA	-1.79	0.329	<0.001
<b>Ester 2</b>			
Intercept	4.24	0.525	<0.001
GR	-1.49	0.574	0.022
CA	-2.22	0.509	0.001

Table 2: Estimates from multivariate models with transformation.

context, it is accepted that there is an increase in the proportion of mutans streptococci and non-mutans streptococci aciduric bacteria [23]. Within these microorganisms, *S. mutans* are considered one of the primary etiological factors of dental caries [25], since they are able to mount an effective ATR that protect them from low-pH environmental conditions. Interestingly, it has been reported that a shift of *S. mutans* cell-wall fatty-acid composition occurs under low-pH conditions: there are increased proportions of long-chain monounsaturated fatty-acids leading to decreased cell-wall permeability and enhanced acid tolerance. This is a rapid process and the turnover in the lipidic composition of the

cell-wall occurs in about 20 minutes after environmental acidification [26]. This alteration allows *S. mutans* to remain metabolically active even under acidic conditions and might explain how dental biofilm microbiota as a whole responds to an acid stress environment.

Considering that a carious site is the result of the entire acid-tolerant consortium metabolic activity, it is feasible that the detected special compound cluster (Figures 2 and 3) is related to released cell-wall lipids. That cluster represents alcohols residues that are closely related to lipid macromolecules. Therefore, our results are in line with earlier findings of [26,27] showing the whole biofilm acid adaptation by means of advanced analytical methods. Additionally, the metabolite profile of C-A sites are clearly different compared to C-F sites. Furthermore, it is interesting to note the absence of difference in metabolite profiles of sound sites between C-A and C-F individuals. That finding confirms that carious lesions are site-specific and that their development depends on the metabolic interactions of overlaying dental biofilm microbiota. Granting this is a small sample size, it is noteworthy that in every analyzed biofilm sample, the metabolite profile difference was recorded in the surfaces with lesions versus the sound surfaces. This was observed even for two subjects with only one C-A lesion each. Additional work is necessary to identify the metabolites within the cluster that appear to be related to caries activity. Also, the possible microbiological pathways associated with these compounds need to be further clarified.

Although the relationship between diet, frequency of exposure to sugars and dental caries has been clearly shown, it is important to consider that dental caries is a dynamic process arising as a result of a network of complex metabolic and synergistic and/or antagonistic interactions amongst the dental biofilm microbiota. Under these circumstances, bacterial acid adaptation likely plays an important role in the transition of biofilms from a healthy to a pathogenic state.

Conclusions

Gathering information on metabolite profiles will enable future research to address the functional significance of specific biochemical and regulatory pathways that are potentially associated with and contribute to the onset and development of dental caries. This pilot study indicates that there is likely a characteristic metabolomic signature for caries activity. Further studies are warranted to identify the metabolite signals in biofilm which can ultimately translate into a signature associated with at-risk sites with implications in caries risk assessment, caries diagnosis and caries management.

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References

1. US Department of Health and Human Services (2000) Oral health in America: a report of the Surgeon General.

2. Al-Ahmad A, Wunder A, Auschill TM, Follo M, Braun G, et al. (2007) The in vivo dynamics of Streptococcus spp., Actinomyces naeslundii, Fusobacterium nucleatum and Veillonella spp. in dental plaque biofilm as analysed by five-colour multiplex fluorescence in situ hybridization. J Med Microbiol 56: 681-687.

3. Corby PM, Lyons-Weiler J, Bretz WA, Hart TC, Aas JA, et al. (2005) Microbial risk indicators of early childhood caries. *J Clin Microbiol* 43: 5753-5759.
4. Takahashi N, Nyvad B (2008) Caries ecology revisited: microbial dynamics and the caries process. *Caries Res* 42: 409-418.
5. Aas JA, Griffen AL, Dardis SR, Lee AM, Olsen I, et al. (2008) Bacteria of dental caries in primary and permanent teeth in children and young adults. *J Clin Microbiol* 46: 1407-1417.
6. Twetman S, Fontana M (2009) Patient caries risk assessment. *Monogr Oral Sci* 21: 91-101.
7. Takahashi N, Washio J, Mayanagi G (2010) Metabolomics of supragingival plaque and oral bacteria. *J Dent Res* 89: 1383-1388.
8. Takahashi N, Washio J (2011) Metabolomic effects of xylitol and fluoride on plaque biofilm in vivo. *J Dent Res* 90: 1463-1468.
9. Jakubovics NS, Kolenbrander PE (2010) The road to ruin: the formation of disease-associated oral biofilms. *Oral Dis* 16: 729-739.
10. Ling Z, Kong J, Jia P, Wei C, Wang Y, et al. (2010) Analysis of oral microbiota in children with dental caries by PCR-DGGE and barcoded pyrosequencing. *Microb Ecol* 60: 677-690.
11. Tanner AC, Mathney JM, Kent RL, Chalmers NI, Hughes CV, et al. (2011) Cultivable anaerobic microbiota of severe early childhood caries. *J Clin Microbiol* 49: 1464-1474.
12. Peterson SN, Meissner T, Su AI, Snesrud E, Ong AC, et al. (2014) Functional expression of dental plaque microbiota. *Front Cell Infect Microbiol* 4: 108.
13. Zero DT, Zandona AF, Vail MM, Spolnik KJ (2011) Dental caries and pulpal disease. *Dent Clin North Am* 55: 29-46.
14. Ferreira ZA, Santiago E, Eckert G, Fontana M, Ando M, et al. (2010) Use of ICDAS combined with quantitative light-induced fluorescence as a caries detection method. *Caries Res* 44: 317-322.
15. Fontana M, Santiago E, Eckert GJ, Ferreira-Zandona AG (2011) Risk factors of caries progression in a Hispanic school-aged population. *J Dent Res* 90: 1189-1196.
16. Ferreira ZA, Santiago E, Eckert GJ, Katz BP, Pereira de OS, et al. (2012) The Natural History of Dental Caries Lesions: A 4-year Observational Study. *J Dent Res* 91: 841-846.
17. Soini HA, Klouckova I, Wiesler D, Oberzaucher E, Grammer K, et al. (2010) Analysis of volatile organic compounds in human saliva by a static sorptive extraction method and gas chromatography-mass spectrometry. *J Chem Ecol* 36: 1035-1042.
18. Soini HA, Schrock SE, Bruce KE, Wiesler D, Ketterson ED, et al. (2007) Seasonal variation in volatile compound profiles of preen gland secretions of the dark-eyed junco (*Junco hyemalis*). *J Chem Ecol* 33: 183-198.
19. Sen PK (1988) Multivariate Median and Rank Sum Tests. *Encyclopedia of Biostatistics*.
20. Preisser JS, Sen PK, Offenbacher S (2011) Multiple Hypothesis Testing for Experimental Gingivitis Based on Wilcoxon Signed Rank Statistics. *Stat Biopharm Res* 3: 372-384.
21. Kenward MG, Roger JH (1997) Small sample inference for fixed effects from restricted maximum likelihood. *Biometrics* 53: 983-997.
22. Galecki A (1994) General class of covariance structures for two or more repeated factors in longitudinal data analysis. *Communications in Statistics-Theory and Methods* 23: 3105-3119.
23. Takahashi N, Nyvad B (2011) The role of bacteria in the caries process: ecological perspectives. *J Dent Res* 90: 294-303.
24. Marsh PD (2003) Are dental diseases examples of ecological catastrophes? *Microbiology* 149: 279-294.
25. Tanzer JM, Livingston J, Thompson AM (2001) The microbiology of primary dental caries in humans. *J Dent Educ* 65: 1028-1037.
26. Fozo EM, Quivey RG, Jr. (2004) Shifts in the membrane fatty acid profile of *Streptococcus mutans* enhance survival in acidic environments. *Appl Environ Microbiol* 70: 929-936.
27. Lemos JA, Abranches J, Burne RA (2005) Responses of cariogenic streptococci to environmental stresses. *Curr Issues Mol Biol* 7: 95-107.