Journal of Current Research in Oral Surgery
2023, Volume 3, Page No: 73-83

Available online at: www.tsdp.net

ISSN: 3062-3480

Original Article

Acid Tolerance Phenotype of Oral Biofilms in Severe Early Childhood Caries: Links to Microbial Composition and Fermentation Profiles

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Received: 09 March 2023; **Revised:** 24 May 2023; **Accepted:** 29 May 2023

ABSTRACT

As dental caries develop, oral biofilms experience shifts in both microbial makeup and phenotypic behavior. This investigation aimed to assess and compare the acid tolerance (AT) of dental plaque collected from two cohorts of children—one group presenting with severe caries (CA) and another entirely caries-free (CF)—and to link these findings with variations in microbial composition and metabolic activity within the biofilms. Plaque was obtained from 20 children aged 2–5 years in each cohort. Acid tolerance was determined by measuring bacterial viability after an acid exposure at pH 3.5, employing LIVE/DEAD® BacLight™ staining combined with confocal microscopy. AT was scored on a scale from 1 (minimal/no tolerance) to 5 (extensive tolerance). Metabolic end-products following a 20 mM glucose challenge for one hour were analyzed through Nuclear Magnetic Resonance (NMR). Microbial community profiles were generated using 16S rRNA Illumina sequencing. The CA group showed a markedly higher mean AT score (4.1) compared to the CF group (2.6, $p < 0.05$). Post–glucose pulse, CA plaques exhibited significantly elevated ratios of lactate to acetate, lactate to formate, lactate to succinate, and lactate to ethanol relative to CF samples ($p < 0.05$). Sequencing data identified 25 species enriched in CA plaque, among them taxa from *Streptococcus*, *Prevotella*, *Leptotrichia,* and *Veillonella* ($p < 0.05$).These findings indicate that pooled plaque from children with extensive caries displays an increased AT when compared with plaque from healthy peers, and that this characteristic aligns with differences in microbial activity and community composition. Consequently, the collective functional phenotype of plaque may serve as a potential marker for caries status. However, longitudinal research is required to determine whether shifts in AT over time can reliably forecast caries development before AT can be applied as a predictive clinical indicator.

Keywords: Oral biofilms, Severe early, Acid tolerance phenotype, Fermentation profiles, Microbial composition

How to Cite This Article: Tanaka Y, Bianchi MR, Ismail NS. Acid Tolerance Phenotype of Oral Biofilms in Severe Early Childhood Caries: Links to Microbial Composition and Fermentation Profiles. J Curr Res Oral Surg. 2023;3:73-83. https://doi.org/10.51847/W79flk2FuQ

Introduction

Dental caries is widespread, affecting roughly 35% of individuals worldwide. In the primary dentition of children under six years, it ranks as the 12th most common global health condition and significantly influences child wellbeing [1–3]. Furthermore, early caries occurrence is a strong indicator of future disease risk [4].

Caries initiation is driven by the metabolic activity of structured multispecies biofilms comprising bacteria, fungi, archaea, and viruses. The architecture of these microbial communities promotes protection, gene exchange, intercellular communication, and nutrient sharing, generating properties not evident when organisms are studied in isolation [5]. Biofilm formation also induces phenotypic adaptations, such as enhanced resistance to environmental stresses [6, 7].

Under healthy conditions, biofilms and the host maintain a dynamic equilibrium in which oral pH remains near neutral. Carbohydrate metabolism yields various organic acids, with the pathways and acid types

depending on microbial species or strain [8]. During caries progression, an increase in acid load—due to reduced salivary buffering, heightened sugar metabolism, or both—lowers the biofilm pH. This promotes demineralization of tooth tissues and shifts the microbial community toward species better suited for acidic niches [9]. Consequently, a rise in overall biofilm AT is expected to occur during the earliest phases of lesion formation.

Caries-active plaque typically shows reduced microbial diversity compared with plaque from caries-free individuals, implying that certain functional traits may be critical for lesion formation [5, 10]. A variety of bacterial and fungal species contribute to caries, though the roles of many other microorganisms remain unclear [11]. No single bacterial species uniquely defines caries activity, yet high levels of *Streptococci*, *Lactobacilli,* and *Scardovia* are frequently observed [10]. While *Streptococcus mutans*, *Scardovia wiggsiae,* and various oral *Lactobacillus* species possess strong intrinsic AT, other early colonizers can acquire an acid tolerance response (ATR) after adaptation to acidic conditions in vitro [12–14]. ATR may involve increased ATPase expression, modifications that reduce proton permeability, and chaperone production that safeguards DNA and proteins [15]. Environmental pH and sucrose availability also influence the proteomics of many oral bacteria, altering glycolysis, acidogenesis and AT [16, 17].

Although DNA sequencing technologies have revealed that aciduric and acidogenic microbes increase during caries onset, more research is needed to characterize the functional behavior of whole microbial communities during active disease to identify improved predictive biomarkers [8, 18]. Existing caries-risk tools include microbial tests, salivary measures, and past caries history, but current assessment models are insufficiently accurate, indicating the need for new approaches [19, 20]. Since AT is a key virulence factor in caries, heightened plaque AT could serve as an early indicator of disease activity. Previous studies have documented AT differences in isolated bacterial strains and plaque from active lesions. For instance, plaque collected from smooth, intact surfaces in children aged 3–5 years showed that *S. mutans* isolates from caries-active subjects exhibit higher AT than those from caries-free peers, though acidogenicity after a glucose pulse did not differ [21]. Another study involving a one-week daily sucrose rinse among adolescents recorded an increase in bacteria capable of growing at pH 5.5 [22], suggesting dietary sugars influence plaque phenotypes. However, comprehensive analyses of AT at the community level in young children with high caries activity remain limited.

Since plaque AT is expected to rise early during the initiation of caries and tends to remain fairly constant over short timeframes, this trait may represent a potential early-stage indicator for caries prediction [23]. Before AT can be evaluated as a diagnostic tool, it must first be demonstrated that children with pronounced caries show elevated AT, while those without disease exhibit little or none. Therefore, the central purpose of this study was to determine whether children with extensive decay present higher AT levels than caries-free peers. Alongside AT, we also analyzed the bacterial makeup of the plaque and examined metabolic outcomes following a glucose exposure to explore how specific microbes or acid-producing activity relate to AT.

Materials and Methods

Selection of study subjects

The study enrolled 50 children, all between 2 and 5 years of age. Based on a standard deviation of 1.06 [20], the required sample size to identify a 1-point difference in AT between groups—at 80% power and a 95% confidence level—was 18 per group. To ensure adequate representation, even if some samples had to be discarded, 25 children were recruited for each category.

Children in the caries-active (CA) cohort were recruited from the Specialist Clinic in Paediatric Dentistry, Faculty of Odontology, Malmö University, Malmö, Sweden, after referral due to substantial caries in their primary teeth. Eligibility required ≥3 decayed teeth (dt) [24] and classification as high caries risk according to Region Scania's risk-rating system (Low/Medium/High). Clinical assessments were carried out by a single trained paediatric dental specialist using a mirror, probe and proper illumination.

The caries-free (CF) group was recruited at the Public Dental Health clinic in Alvesta, Kronoberg County, Sweden. Inclusion criteria were dt = 0 [24] and designation as low risk following the Region Kronoberg risk protocol. One general dentist conducted all examinations with identical clinical equipment and lighting conditions.

Both Nordic and non-Nordic children were considered. Exclusion criteria included antibiotic use within the previous three months, ongoing medication, systemic illness, autoimmune conditions, or functional impairments that could influence oral health. No

dietary or oral hygiene advice was provided before sampling.

Ethics statement

Ethical approval was granted by the Swedish Ethical Review Authority (reference 2020-01187 and 2021-03680). Written consent was obtained from the legal guardian of each participant before sample collection.

Sample collection

Sampling was performed by the examining clinician during routine visits between January 2021 and December 2022. Plaque was taken from all buccal and lingual surfaces of both arches, including sound and decayed enamel and dentine, using a sterile plastic instrument. Material was pooled into an Eppendorf tube containing 500 µl of sterile UHQ-water. Samples reached the Section for Oral Biology and Pathology, Faculty of Odontology, Malmö University within 24 h. Upon arrival, volumes were verified and normalized to 500 µl when needed. Each pooled sample was vortexed for 2 × 30 s and split into three fractions: a 250 µl aliquot stored at −80°C for 16S rRNA sequencing, a 150 µl aliquot frozen at −80°C for metabolite analysis, and a 100 µl fraction reserved for AT testing.

Acid tolerance assessment

AT evaluation occurred within 24 h after lab arrival. The 100 µl sample was mixed, divided into two tubes, and centrifuged for 5 min at 1,300 rpm (Eppendorf Centrifuge 5415 D) at room temperature. After removing the supernatant, 25 µl of TYE medium (Tryptone–Yeast extract with 0.4 M glucose and 0.4 M phosphate/citrate buffer) adjusted to either pH 3.5 (AT condition) or pH 7.5 (viability control) was added. The mixture was pipetted gently and incubated aerobically at 37°C for 2 h.

LIVE/DEAD® BacLight™ stain (Molecular Probes, Eugene, OR, USA) was applied according to the manufacturer's guidelines, and samples were transferred into an Ibidi® µ-slide VI Ibi-treat flow cell. The flow cell was centrifuged at 1,000 rpm for 60 s and then imaged with a confocal laser scanning microscope (Nikon Eclipse TE2000, Nikon Corp., Tokyo, Japan) equipped with a 488 nm Ar laser. Images were captured using a Photometrics Prime 95B camera and Nikon NIS-Elements software. Ten randomly chosen fields of view were recorded from each specimen for later analysis.

Evaluation of sample viability through image-based methods

Viability assessments were carried out on materials stained with LIVE/DEAD® BacLight™, where intact cells emitted green fluorescence and membrane-damaged cells emitted red. To verify the condition of specimens transported from the clinic, control aliquots were held at pH 7.5. Among the 50 plaque samples originally obtained, only 42 of the control preparations retained viability levels above 90%. Consequently, 5 specimens from the CA cohort and 3 from the CF cohort were excluded at this point. A further two CF samples were removed because the available biomass for AT testing was insufficient (<5% of the field of view in confocal imaging).

Quantification of acid tolerance via microscopy

Forty plaque samples remained for assessment (20 in each clinical group). After exposure to pH 3.5, ten confocal images per sample were examined independently by two raters using a previously validated 1–5 AT scoring framework [23]. In this system, BacLight-stained cells retaining membrane integrity were interpreted as acid-tolerant, whereas those showing membrane injury were classified as non-tolerant. All image sets were anonymized so observers were blind to caries status. Average AT values for each sample were used to compare groups with the Wilcoxon signed-rank test, and significance was defined as $p < 0.05$. For samples where the two observers differed (never by more than 0.4), their mean score was taken forward for statistical analysis.

Glucose-triggered metabolic activation

A 250 µl portion of the homogenized, pooled plaque suspension was diluted with 1.2% NaCl to reach a final concentration of 0.9% NaCl, followed by mechanical mixing via vortexing and pipetting. A sterile 1 M glucose solution was added to yield 20 mM final glucose, and the mixture was incubated at 37°C for 60 min. The preparation was then chilled on ice for 5 min and spun at 14,000 rpm for 10 min. The supernatant was transferred into cryovials and frozen at −80°C for later NMR profiling.

NMR-based detection of microbial metabolites

Samples were thawed for 15 min at room temperature and centrifuged at 14,000 × g for 10 min at 4°C (Eppendorf 5804R, FA-45-30-11 rotor). From each supernatant, 585 µl was combined in a deep-well plate with 65 µl of buffer (400 mM potassium phosphate, pH 7.4, 1.548 mM TSP-d4, and 0.13% w/v sodium azide). The mixtures were agitated for 2 min at 12°C and 500 rpm (Thermomixer Comfort). Then, 575 µl from each well was loaded into 5 mm SampleJet tubes via a SamplePro Tube L (Bruker BioSpin). ^{1}H-NMR spectra were recorded on a 700 MHz Bruker Avance III system equipped with a 5 mm QCI

cryoprobe and SampleJet sample exchanger. The "noesygppr1d" sequence was used, acquiring 128 scans and 64k data points with a 30 ppm spectral width, 1.6 s acquisition time, 4 s relaxation delay, and receiver gain 181. A 0.3 Hz exponential line broadening was applied prior to the Fourier transform. Spectra were referenced to TSP-d4, processed in TopSpin 3.6.2, and metabolite concentrations were quantified in ChenomX 9.0 using TSP-d4 as the calibrant.

Extraction and preparation of DNA

DNA isolation followed a published workflow [20]. Briefly, 130 µl lysis buffer and 10 µl of an enzyme mixture—lysozyme (25 mg/ml, AppliChem A4972,0001), lysostaphin (1.25 KU/ml, Sigma-Aldrich SAE0091-2MG), mutanolisine (0.625 KU/ml, Sigma-Aldrich SAE0092-10KU), and zymolase (125 KU/ml, Sigma-Aldrich SAE0098-20KU)—were combined and incubated at 37°C for 1 h. This was followed by the addition of 20 µl 1% glucanex for 10 min at 60°C, then proteinase K treatment for 15 min at 65°C and 10 min at 95°C. Purification was performed using the MagNA Pure LC 2.0 instrument and the MagNA Pure LC DNA Isolation Kit III for Bacteria and Fungi (Cat. No. 03 264 785 001). DNA concentration was determined with a Qubit™ 3 Fluorometer. The V3–V4 region of the 16S rRNA gene was amplified using primers CCTACGGGNGGCWGCAG (forward) and GACTACHVGGGTATCTAATCC (reverse). Library preparation adhered to Illumina's Metagenomic Sequencing Library Preparation protocol (Part #15044223 Rev. A) and sequencing was conducted at FISABIO (Valencia, Spain) on an Illumina MiSeq platform using 2 × 300 bp paired-end reads. Resulting data were deposited in SRA under Bioproject PRJNA1157018.

Bioinformatic processing

Processing followed previously described analytical steps using DADA2 (v1.20.0) [25]. Reads that exceeded the permitted trimming threshold or contained more than 5 expected errors were removed. After dereplication and error modeling (loessErrfun), paired reads were merged with a minimum 15 bp overlap. Chimeras were removed, and ASVs were classified using the SILVA v.138.1 reference [26]. Group-level taxonomic differences were tested with ANCOMBC2 [27], followed by paired Wilcoxon tests (wilcox.test, R stats package) [28]. P-values were adjusted using FDR. Correlations between metabolite concentrations and microbial abundances were computed with mixOmics. Low-abundance and low-prevalence taxa were excluded from ANCOMBC2 and correlation analyses. For rarefaction and species-level richness/diversity, the minimum read depth used was 4.5×10^5.

Results and Discussion

Caracteristics of study population

Participants in the CA cohort had a mean age of 4.5 years (±0.97), consisting of 13 boys and 7 girls, whereas those in the CF cohort averaged 4.7 years (±0.57), with 11 boys and 9 girls. No meaningful statistical differences in age distribution were detected. At the point of sampling, the CF group displayed 0 decayed teeth, whereas the CA group showed an average of 8.0 (±3.2) decayed teeth **(Table 1).**

Table 1. Characteristics of the CA group regarding number of decayed teeth (dt).

Descriptive Statistics – Number of carious lesions (N = 20)	
Valid cases	20
Missing cases	0
Mean	8.0
Median	7.0
Standard deviation	3.2
Minimum	3
Maximum	15

The dt values spanned from 3 to 15, yielding a mean of 8.0 (±3.2).

Acid tolerance (AT)

All plaque samples entering the analysis retained viability rates exceeding 90% upon arrival. Acid tolerance was determined by examining membrane integrity following exposure to pH 3.5, using a validated 1–5 scoring system [23], where 1 denotes minimal AT and 5 denotes maximal AT. CA samples scored between 3.00–4.85, while CF samples ranged from 1.3–3.8. The average AT score of the CA cohort (4.1) was notably greater than that of the CF cohort (2.6), with an adjusted p-value of 8×10^{-6} **(Figure 1a).** Furthermore, the highest AT category (scores 4.0–5.0) was exclusively represented by CA samples, accounting for 75% of that group. Conversely, the lowest AT category (scores 1.0–2.0) appeared solely in the CF cohort, representing 70% of that population.

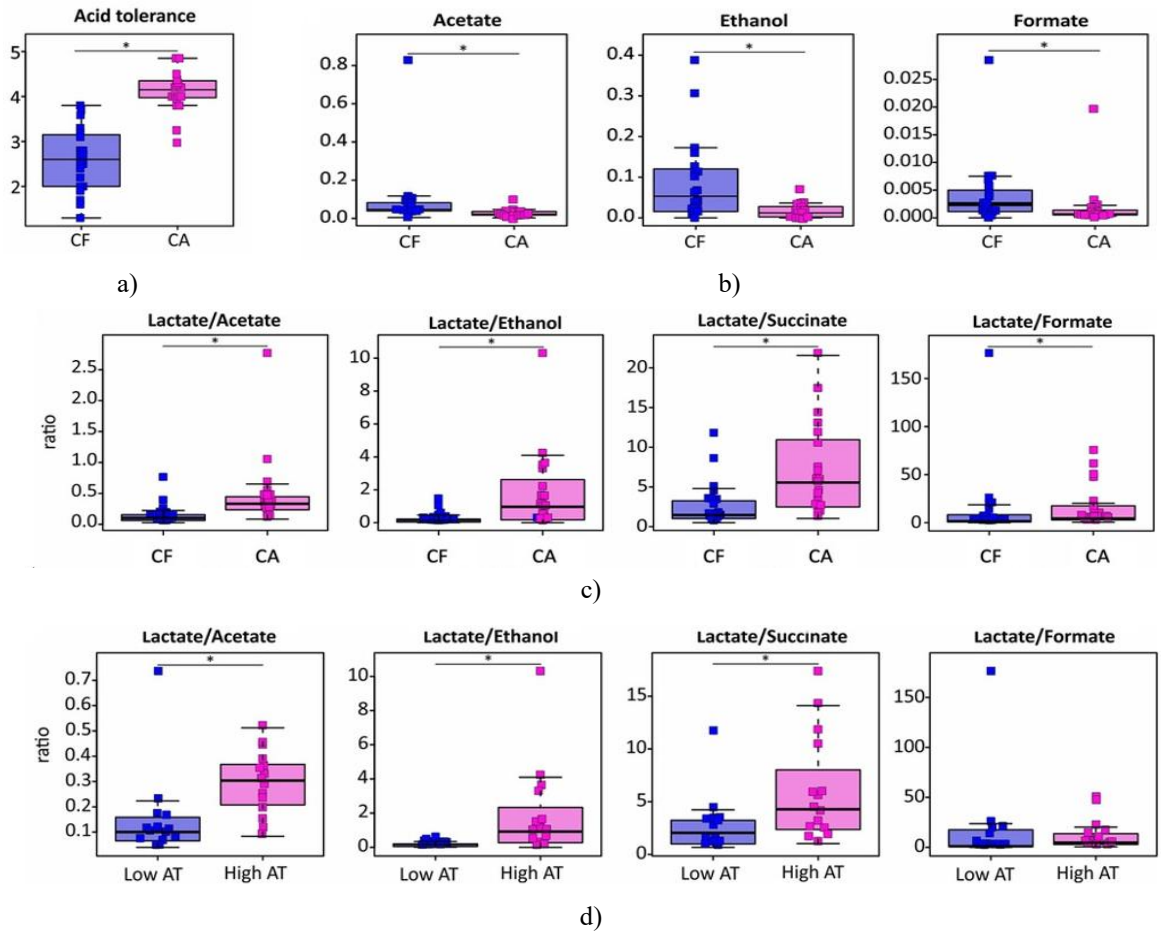


Figure 1. Influence of caries status on AT and metabolite levels after a 20 mM glucose stimulus. Boxplots for CA and CF groups display AT scores (a), metabolite concentrations (mM) (b), and lactate-to–other-acid ratios (c). Comparisons between low AT (scores 1–2) and high AT (scores 4–5) are also shown (d).

Organic acid production

Phenotypic behavior was further assessed after exposing plaque suspensions to 20 mM glucose for one hour. A total of 23 metabolites were observed in the NMR spectra. Glucose-derived end products included acetate, formate, propionate, succinate, ethanol, lactate and butyrate. Among these, acetate, formate and ethanol levels were significantly higher in CF samples relative to CA samples **(Figure 1b).** Total lactate production did not differ significantly between groups; however, lactate-to-formate, lactate-to-succinate, lactate-to-acetate and lactate-to-ethanol ratios were elevated in CA samples compared to CF samples **(Figure 1c)**. High-AT samples (scores 4–5) also showed higher ratios of lactate to succinate, acetate and ethanol compared with low-AT samples (scores 1–2), though the lactate-to-formate ratio did not differ **(Figure 1d).**

Bacterial composition

Across samples, sequencing yielded an average of 295,000 reads, and rarefaction analysis indicated full species-level coverage after approximately 114,000 reads. At the genus level, both groups exhibited broadly similar profiles. Streptococcus dominated both cohorts, accompanied by substantial levels of Leptotrichia, Neisseria, Capnocytophaga and Actinomyces.

Species-level analysis revealed significantly higher richness and diversity within the CA group compared with the CF group, with no difference in dominance (Chao1 index $p = 0.00023$; Shannon index $p = 0.024$; **Figure 2a**). In both cohorts, unassigned species of Streptococcus, Leptotrichia, Neisseria and Fusobacterium were highly represented, while Corynebacterium matruchotii and Lautropia mirabilis were the most abundant among the identified taxa **(Figure 2b)**. Within the Streptococcus genus, the CF group showed Streptococcus cristatus as the most abundant member **(Figure 2b)**, whereas in the CA group, S. cristatus, S. mutans and Streptococcus sobrinus predominated **(Figure 2c).**

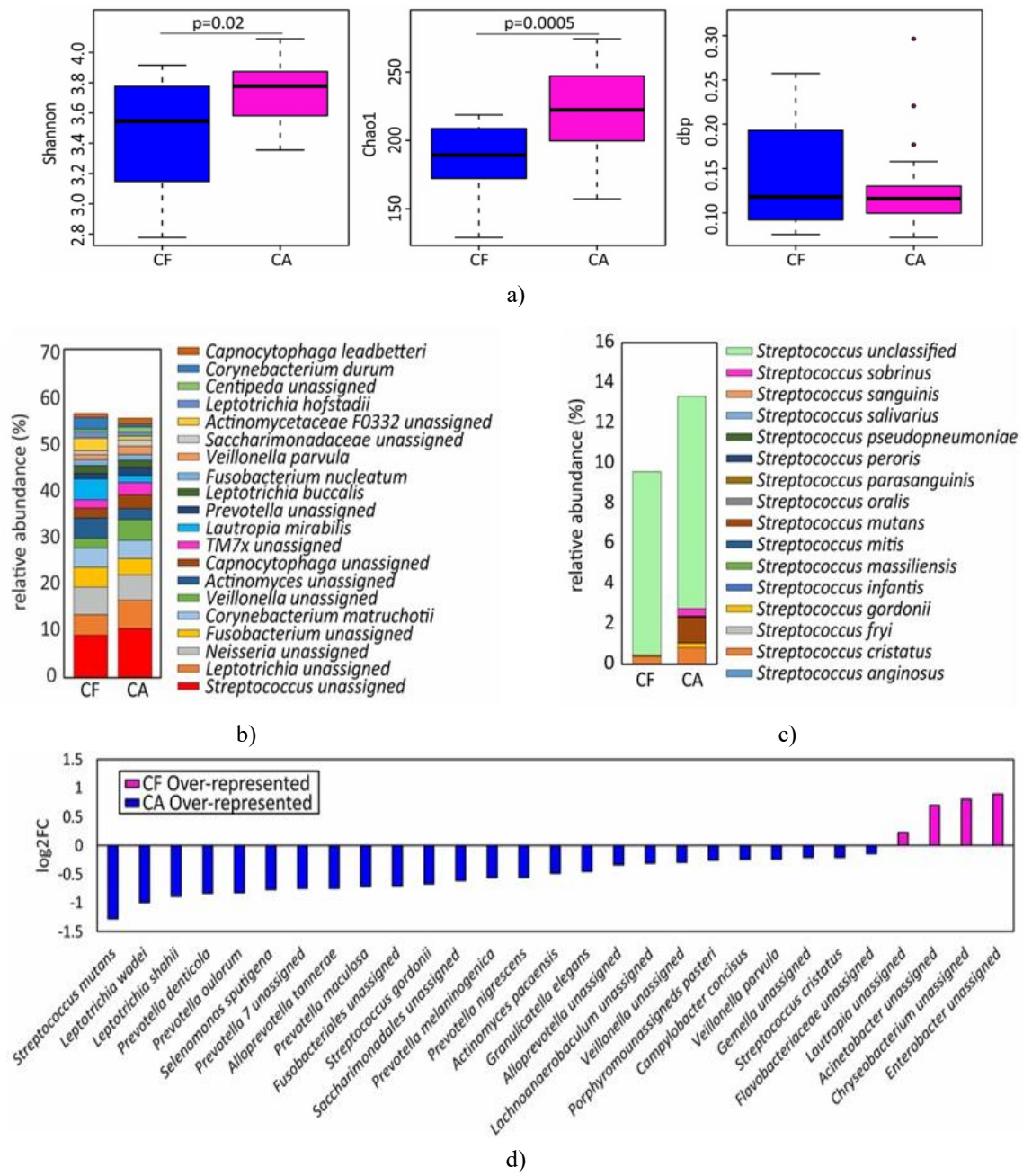


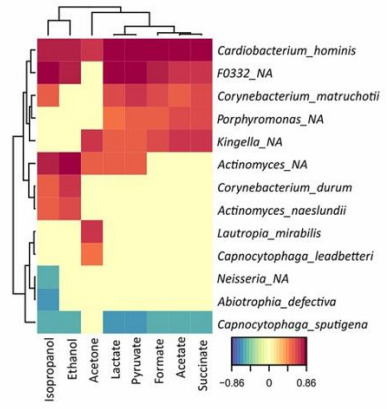
Figure 2. Variations in plaque microbiota associated with severe caries. (a) Boxplots of Shannon diversity, Chao1 richness and dbp dominance for CA and CF children. (b) Relative abundance of the top 20 species. (c) Abundance of Streptococcus-classified species. (d) Species differing between CF and CA groups presented as $\log_2$ fold change ($\log_2$FC).

When species present at more than 0.1% relative abundance were considered, 25 taxa appeared enriched in the CA cohort **(Figure 2d)**. Within this group, *S. mutans* was the predominant organism, occurring in 95% of CA samples but only 30% of CF samples (data not shown). Several additional organisms frequently linked to carious lesions—such as *Veillonella parvula* and two *Leptotrichia* species (*L. wadei* and *L. shahii*)—were likewise found at elevated levels in the CA group. Notably, multiple members of the proteolytic, obligately anaerobic genus *Prevotella* (*P. denticola*, *P. oulorum*, *P. maculosa*, *P. melaninogenica*, and *P. nigrescens*) displayed markedly higher relative abundances in CA individuals. *Scardovia wiggsiae* also trended upward in CA samples compared to CF, although this contrast did not meet the 5% significance cutoff.

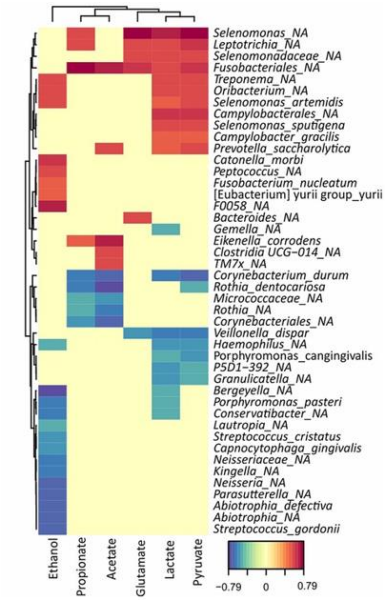
To determine whether specific microbes were linked to high or low AT scores, correlations between relative abundance and AT values were calculated. Somewhat unexpectedly, the strongest positive AT associations in the CA group involved an unclassified *Porphyromonas*

species and *Porphyromonas pasteri*. In contrast, CF subjects showed negative correlations between AT and unassigned *Leptotrichia*, *Fusobacterium*, and *Tannerella* taxa, along with *Lachnoanaerobaculum sabbureum* and *Corynebacterium matruchotii*. Conversely, non-classified members of *Rothia*, *Neisseria*, and *Streptococcus* correlated positively with AT among CF individuals.

Links between single metabolites and specific taxa revealed that unidentified *Fusobacteriales* in CA samples were most strongly associated with the generation of lactate, propionate, acetate, and pyruvate, while *Corynebacterium durum* showed the opposite pattern for these metabolites **(Figure 3b)**. Within the CF group, *Cardiobacterium hominis* exhibited the highest positive relationship with acid formation, whereas *Capnocytophaga sputigena* demonstrated a negative association with these metabolic end-products **(Figure 3a).**



a)



b)

Figure 3. Correlation patterns between microbial abundances and NMR-derived metabolite levels following a 20 mM glucose stimulus. The intensity of positive (red) and negative (blue) relationships is indicated for caries-free (a) and caries-active (b) participants.

Aligned with the concept that acid-resistant communities facilitate caries onset, AT values were higher among CA subjects than among CF subjects. This may in part reflect the expansion of inherently acid-adapted microorganisms like *S. mutans* in progressively acidic niches, alongside the gradual acid-tolerance shifts of additional oral residents as caries evolves [14]. Caries progression involves a continuum of microbial traits; for example, genes tied to acid stress responses tend to dominate earlier phases, whereas osmotic-stress-related and protease-encoding genes are more prominent in deeper dentinal lesions [29]. Because the plaque from each participant was pooled, functional characteristics linked to site-specific stages of lesion activity could not be evaluated. However, pooling also offered the advantage of simultaneously assessing microbial properties across a spectrum of lesion stages and unaffected surfaces within the CA group, where overall AT exceeded that of CF individuals. This outcome contrasts with Havsed *et al*. [20], who reported no significant CA–CF differences in plaque AT among adolescents, although the majority (7/10) of the highest AT values occurred in the CA subgroup.

Lactate output from glucose metabolism is regarded as a principal determinant of the low-pH environment characteristic of caries-associated biofilms [30]. To examine metabolic behavior and whether CA plaque generated more lactate than CF plaque, metabolite concentrations in the glucose-pulsed supernatants were quantified. In the present dataset, ethanol, acetate, and formate production was significantly higher in CF samples, whereas other organic acids did not differ between groups. Ratios comparing lactate to acetate, ethanol, formate, or succinate were, however, elevated in CA participants. These findings parallel those of Havsed *et al*. [20], who likewise observed that although individual acid levels did not differ, lactate-to-other-acid ratios were greater in CA samples.

Unclassified *Fusobacteriales* in the CA group exhibited strong associations with lactate and with propionate, acetate, and pyruvate. Although certain species such as *Fusobacterium nucleatum* can lower environmental pH during glucose metabolism in vitro [31], the correlation patterns shown here are more likely reflective of the metabolic characteristics of the overall plaque community rather than intrinsic features of the individual species. *Leptotrichia* and

Fusobacterium representatives, often detected in health as well as in periodontal lesions, attach to numerous oral taxa during biofilm maturation and therefore commonly appear in well-developed plaque and throughout the continuum of caries development [32, 33].

Although *Veillonella* species are known to convert lactate into acetate and succinate—and this genus was more abundant in the CA group—this shift did not translate into significantly elevated succinate or acetate levels in those samples. One possible explanation is that several other lactate-utilizing genera, including *Actinomyces*, *Prevotella*, and *Neisseria*, were present in both groups [5, 8, 13, 34]. It should also be noted that plaque specimens in this work were manipulated under aerobic conditions; while many oral bacteria can process glucose in the presence of oxygen, the metabolic outputs could have been slightly different under anaerobic handling due to altered biochemical pathways [8].

The complex microbial communities from both CA and CF subjects differed not only in species proportions but also in biofilm behavior. Because material from all buccal and lingual tooth surfaces was combined, the observed features reflect whole-mouth microbial activity. These findings are consistent with the contemporary view that dental caries arises from a consortium of interacting organisms rather than from a single pathogen, with ecological disruptions influencing both the structure and functionality of the biofilm [9, 10, 35].

Despite shared taxa between CA and CF groups, their relative abundances diverged. As anticipated, *S. mutans* appeared in markedly greater quantities in CA samples, paralleling previous reports examining biofilms from caries-active individuals [36, 37]. Roughly 85% of people with active decay carry *S. mutans*, though levels vary with population characteristics and sampling sites [35, 36], and its elevated representation often signals an acidic plaque milieu [38]. In line with earlier studies, *S. mutans* was also detected in CF children, albeit at significantly lower prevalence and abundance. Another organism with strong sugar-fermenting capacity, *Scardovia wiggsiae*, showed a higher proportion in CA samples, though without statistical significance. This species has been linked to deterioration in primary and permanent dentitions, and its ability to ferment carbohydrates efficiently at low pH enhances its advantage under acidic conditions [38].

Consistent with Havsed *et al*. [20], *Veillonella* taxa were more common in CA children. Given their capacity to metabolize organic acids as carbon sources [39] and the increased acid yield when they grow in combination with *S. mutans* [40], their elevated presence may indicate heightened availability of organic acids during caries progression. Likewise, the greater abundance of *L. shahii* and *L. wadei* in CA plaque aligns with earlier observations in pediatric populations [41]. These species can produce lactic acid from carbohydrate substrates, potentially aiding the pH decline that initiates enamel and dentin demineralization.

The unexpectedly high representation of *Prevotella* taxa in CA samples may reflect the advanced degree of carious activity among the participants. Plaque was collected from intact as well as carious surfaces, including dentin. Members of this genus are strongly proteolytic anaerobes, frequently associated with periodontal disease, yet they are also commonly isolated from dentinal lesions [41–43]. Their presence deep within dentin may relate to the tissue's rich organic matrix and the anaerobic conditions of advanced cavities. Moreover, their saccharolytic to moderately saccharolytic traits may also contribute to demineralization [29, 44, 45]. In a longitudinal investigation tracking pooled plaque from children aged 1–6, proteolytic genera such as *Prevotella* predominated as early as 1–3 years before observable caries [37].

Taxa often cited as indicative of healthy plaque in primary dentition, such as *Neisseria* spp. [17, 46]—did not appear at higher levels in CF children here. Because sampling took place immediately when consent was obtained, the request for participants to avoid oral hygiene for 24 hours beforehand may have allowed more mature biofilms to develop in the CF group.

Overall, the data reinforce that multifaceted bacterial interactions shape caries formation, altering both the community makeup and functional attributes of the biofilm. Greater levels of sugar-fermenting organisms and proteolytic anaerobes were evident in CA children, and the differences in metabolites produced after glucose exposure point to distinct metabolic strategies. Dental plaque AT—measured as survival following an acid challenge—was substantially higher in children with pronounced caries activity than in CF subjects. Evaluating plaque phenotype as a whole may thus offer a useful snapshot of caries status. Nevertheless, longitudinal analyses are required to clarify how AT evolves during the development of caries before it can be adopted as a predictive indicator.

Acknowledgments: We would like to thank Anders Bay-Nord at the Swedish NMR Centre for performing the NMR and in interpreting the results. A special

thanks to Madeleine Blomqvist for her laboratory expertise and contribution to image analysis.

Conflict of Interest: The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Financial Support: The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This project was funded by the Faculty of Odontology (Grant number: Rek2.2.1-2017/142), the Foresight Programme (Grant number: LED 1.4-2017/404) and the Eklund Foundation Scholarship for the promotion of Odontological Research at Malmö University (Grant number: FO 2023/395).

Ethics Statement: The studies involving humans were approved by Swedish Ethical Review Authority, reference 2020-01187 and 2021-03680. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

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