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CRISPRi-Mediated Repression of *gtfB* Attenuates *Streptococcus mutans* Virulence and Promotes Ecological Homeostasis in a Preclinical Cariogenic Biofilm Model

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ABSTRACT

Introduction: *Streptococcus mutans* is a primary etiological agent of dental caries, largely due to its capacity to form robust, acidogenic biofilms. This virulence is critically dependent on glucosyltransferases, particularly *GtfB*, which synthesizes the adhesive extracellular glucan matrix. Conventional antimicrobial strategies often lack specificity, leading to oral dysbiosis. This study aimed to develop and evaluate a highly targeted CRISPR interference (CRISPRi) system to silence the *gtfB* gene in *S. mutans*, thereby inhibiting its cariogenic potential without adversely affecting the viability of key oral commensal species. **Methods:** A CRISPRi system, comprising a nuclease-deactivated Cas9 (dCas9) and a single guide RNA (sgRNA) targeting the *gtfB* promoter, was engineered into *S. mutans* UA159. The efficacy of *gtfB* silencing was quantified via qRT-PCR. The consequential effects on bacterial growth kinetics, insoluble glucan synthesis, and single-species biofilm formation were assessed using spectrophotometry, anthrone assays, crystal violet staining, and confocal laser scanning microscopy (CLSM). The ecological impact was investigated in a multi-species biofilm model containing *S. mutans* and the commensal bacteria *Streptococcus gordonii*, *Streptococcus oralis*, and *Actinomyces naeslundii*, with microbial composition analyzed by species-specific qPCR. All research activities were conducted in Indonesia. **Results:** The CRISPRi system induced a profound and specific downregulation of *gtfB* mRNA expression by over 98% ($p<0.001$) in the engineered *S. mutans* strain compared to the wild-type. This silencing did not impair bacterial planktonic growth. However, it led to a significant reduction in insoluble glucan production by 85% ($p<0.001$) and a corresponding 79% decrease in total biofilm biomass ($p<0.001$). CLSM imaging confirmed the formation of structurally deficient biofilms with minimal extracellular matrix. In the multi-species model, repression of *S. mutans* virulence significantly altered the biofilm ecology, resulting in a 65% reduction in the proportional abundance of *S. mutans* and a concomitant increase in the representation of commensal species, thereby fostering a community structure more aligned with oral health. **Conclusion:** Targeted repression of the *gtfB* gene using a CRISPRi-based approach effectively 'disarms' *S. mutans*, neutralizing its primary cariogenic mechanism without being bactericidal. This strategy not only attenuates its virulence but also shifts the ecological balance in favor of beneficial commensal bacteria. These findings underscore the therapeutic potential of gene-targeted virulence modulation as a precise, ecologically-sound strategy for the prevention and treatment of dental caries.

1. Introduction

Dental caries remains one of the most prevalent chronic diseases globally, affecting individuals across all age groups and socioeconomic strata. It is a multifactorial, biofilm-mediated disease characterized by the progressive demineralization of dental hard tissues due to acid produced by fermenting bacteria.1 The economic burden associated with the treatment of caries and its sequelae is substantial, posing a significant public health challenge worldwide, including in developing nations like Indonesia where access to preventative dental care can be limited. The etiology of caries is intrinsically linked to a dysbiotic shift within the oral microbial ecosystem, transitioning from a balanced, health-associated community to a pathogenic, acidogenic, and acid-tolerant (aciduric) state.[2,3]

Central to this pathological transition is the bacterium *Streptococcus mutans*. Although a natural inhabitant of the oral cavity, under conditions of frequent dietary sucrose exposure, S. *mutans* can outcompete beneficial commensal species and dominate the dental plaque biofilm. Its preeminence as a cariogenic pathogen stems from a triad of virulence attributes: the ability to rapidly metabolize dietary carbohydrates into organic acids (acidogenicity), the capacity to thrive in the low-pH environment it creates (aciduricity), and, most critically, the synthesis of extracellular polymeric substances (EPS) that form the structural and functional core of the biofilm matrix.[4,5]

The EPS matrix is predominantly composed of glucans, which are synthesized from sucrose by a family of enzymes known as glucosyltransferases (Gtfs). S. mutans expresses at least three Gtfs*: GtfB*, GtfC, and GtfD. Of these, *GtfB* is considered paramount for the synthesis of water-insoluble glucans, which are highly adhesive and architecturally vital. These glucans facilitate the initial attachment of S. mutans to the tooth surface and promote interbacterial adhesion and microcolony development, creating a protected, diffusion-limited, and highly acidic microenvironment conducive to enamel demineralization.[6,7] The gene encoding this critical enzyme, gtfB, is therefore an attractive target for anti-caries therapeutic intervention.

Current strategies for caries prevention and management largely rely on non-specific approaches. These include professional tooth cleaning, the application of topical fluorides to enhance remineralization, and the use of broad-spectrum antimicrobial agents like chlorhexidine or triclosan [8]. While effective to a degree, these antimicrobial agents lack specificity for cariogenic pathogens. Their indiscriminate action can disrupt the entire oral microbiota, eliminating beneficial commensal bacteria that play a crucial role in maintaining oral homeostasis and preventing colonization by opportunistic pathogens. This collateral damage can lead to long-term ecological imbalance (dysbiosis), and the widespread use of such agents contributes to the escalating global crisis of antimicrobial resistance.[9,10]

Consequently, there is a pressing need for novel therapeutic strategies that can precisely target the key virulence factors of pathogens like S. *mutans* without causing broad ecological disruption. Such a 'pathobiont-disarming' approach would ideally neutralize the pathogenic potential of a microbe while leaving it susceptible to clearance by the host immune system or competition from a healthy resident microbiota. The advent of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated protein (Cas) systems has revolutionized the field of targeted genetic manipulation, offering unprecedented potential for such precision antimicrobials.[11,12]

The CRISPR-Cas9 system, originally discovered as an adaptive immune system in bacteria and archaea, has been repurposed into a powerful tool for gene editing, and more recently, for gene regulation. A modified version, known as CRISPR interference (CRISPRi), utilizes a nuclease-deactivated Cas9 (dCas9) protein. The dCas9 protein, when complexed with a single guide RNA (sgRNA), can be directed to a specific DNA target, such as a gene promoter. However, instead of cleaving the DNA, the dCas9-sgRNA complex acts as a formidable steric block, physically obstructing the binding of RNA polymerase and thereby repressing transcription in a potent, specific, and programmable manner. This gene silencing mechanism offers a non-lethal method to interrogate and control bacterial gene function.[13]

The application of CRISPRi to selectively inhibit virulence genes in pathogenic bacteria represents a frontier in antimicrobial therapy. By targeting a gene like *gtfB*, it is theoretically possible to dismantle the cariogenic machinery of S. mutans, rendering it unable to produce the critical EPS matrix and form pathogenic biofilms. Because this approach does not target essential genes required for survival, it is predicted to exert less selective pressure for the development of resistance compared to traditional bactericidal or bacteriostatic antibiotics. Furthermore, its high specificity ensures that only S. *mutans* (or other bacteria containing the target sequence) would be affected, leaving the beneficial commensal community intact.

This preclinical investigation was therefore designed to harness the power of CRISPRi for a targeted anti-caries strategy. The aim of this study was to construct and validate a CRISPRi system for the specific and potent silencing of the *gtfB* gene in Streptococcus mutans. We hypothesized that repressing *gtfB* transcription would significantly attenuate the bacterium's ability to produce insoluble glucans and form cariogenic biofilms, without affecting its viability. The central novelty of our approach lies in evaluating the ecological consequences of this targeted virulence modulation within a multi-species oral biofilm model, with the ultimate goal of demonstrating that disarming S. *mutans* can restore a microbial community structure more characteristic of oral health. This work represents a foundational step towards developing a new generation of intelligent, ecologically-aware therapies to combat dental caries.

2. Methods

This preclinical research was conducted in Eureka Research Laboratory, Palembang, Indonesia. All protocols involving bacterial strains were performed in accordance with institutional biosafety guidelines. The study plan was reviewed and approved by the Institutional Research and Ethics Committee of the CMHC Research Center, Indonesia.

The primary bacterial strains used in this study are listed in Table 1. *Streptococcus mutans* UA159, a well-characterized cariogenic strain, served as the wild-type (WT) and the parental strain for genetic modification. Oral commensal species, including *Streptococcus gordonii* DL1, *Streptococcus oralis* ATCC 35037, and *Actinomyces naeslundii* ATCC 12104, were selected to represent key members of a health-associated supragingival biofilm community.

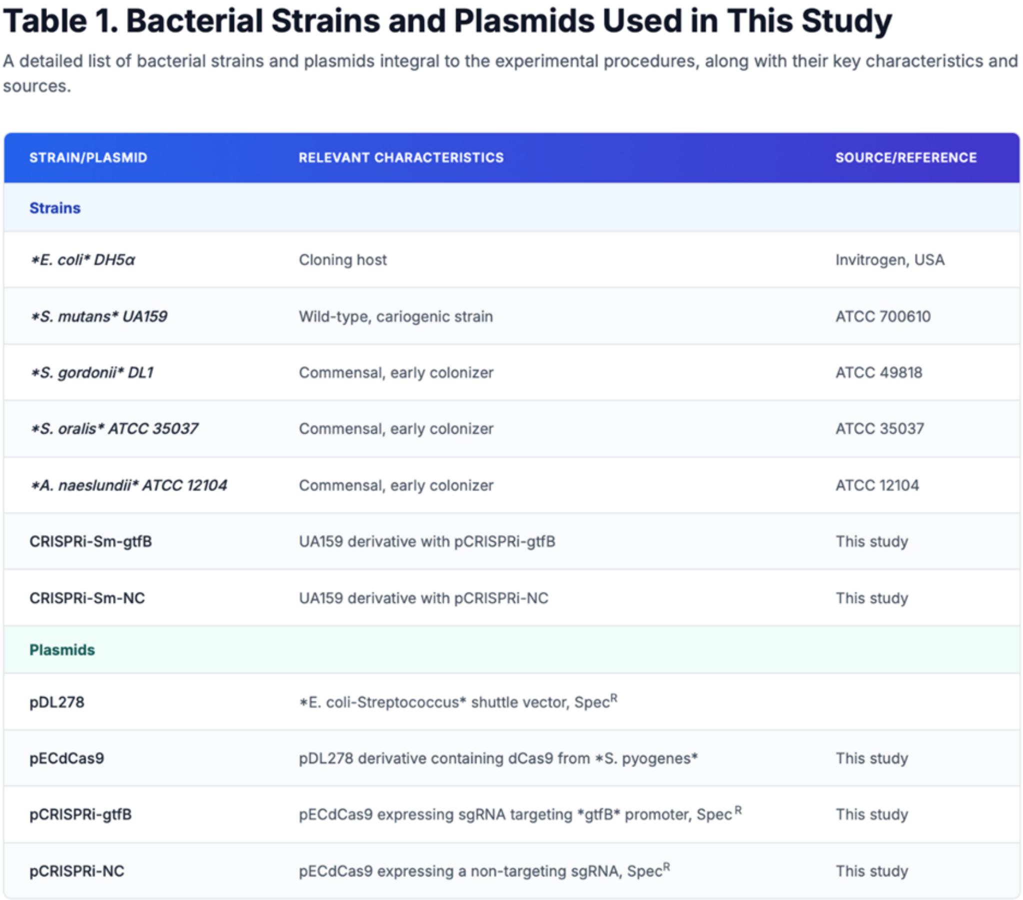


Table 1. Bacterial Strains and Plasmids Used in This Study

A detailed list of bacterial strains and plasmids integral to the experimental procedures, along with their key characteristics and sources.

STRAIN/PLASMID	RELEVANT CHARACTERISTICS	SOURCE/REFERENCE
Strains		
E. coli DH5α	Cloning host	Invitrogen, USA
S. mutans UA159	Wild-type, cariogenic strain	ATCC 700610
S. gordonii DL1	Commensal, early colonizer	ATCC 49818
S. oralis ATCC 35037	Commensal, early colonizer	ATCC 35037
A. naeslundii ATCC 12104	Commensal, early colonizer	ATCC 12104
CRISPRi-Sm-gtfB	UA159 derivative with pCRISPRi-gtfB	This study
CRISPRi-Sm-NC	UA159 derivative with pCRISPRi-NC	This study
Plasmids		
pDL278	*E. coli-Streptococcus* shuttle vector, Spec^R	
pECdCas9	pDL278 derivative containing dCas9 from *S. pyogenes*	This study
pCRISPRi-gtfB	pECdCas9 expressing sgRNA targeting *gtfB* promoter, Spec^R	This study
pCRISPRi-NC	pECdCas9 expressing a non-targeting sgRNA, Spec^R	This study

S. mutans, S. gordonii, and *S. oralis* were routinely cultured in Brain Heart Infusion (BHI) broth (Becton, Dickinson and Company, USA) at 37°C in a microaerophilic environment (5% CO_2, 10% H_2, 85% N_2). A. *naeslundii* was cultured anaerobically (85% N_2, 10% H_2, 5% CO_2) in BHI supplemented with 0.5% yeast extract. For plasmid selection and maintenance in *S. mutans*, BHI agar or broth was supplemented with 200 µg/mL spectinomycin. *Escherichia* coli DH5α, used for all cloning steps, was grown in Luria-Bertani (LB) medium at 37°C with shaking, supplemented with 100 µg/mL spectinomycin as required.

The CRISPRi system was designed to be housed on a single shuttle vector capable of replication in both E. coli and *Streptococcus*. The base vector was pDL278, containing a spectinomycin resistance marker. A 20-nucleotide single guide RNA (sgRNA) sequence was designed to target the promoter region of the *S. mutans* UA159 *gtfB* gene (locus tag: SMU_1533). The target site was chosen within the -35 and -10 promoter elements to maximize transcriptional interference. The chosen target sequence was 5'-GCTAATATTGTTGATAACTAGG-3', which is located on the non-template DNA strand immediately upstream of the transcriptional start site. A BLAST search against the S. mutans UA159 genome confirmed its specificity for the intended target. A non-targeting negative control (NC) sgRNA (5'-GCACTACCAGAGCTAACTCAAT-3'), which has no significant homology to any sequence in the target bacterial genomes, was also designed.

The gene encoding a nuclease-deactivated Cas9 (dCas9) from *Streptococcus pyogenes* (containing D10A and H840A mutations) was codon-optimized for expression in *S. mutans* and synthesized commercially (GeneArt, Thermo Fisher Scientific). The dCas9 gene was placed under the control of a constitutive promoter P23. The dCas9 expression cassette was then cloned into the pDL278 shuttle vector using standard restriction enzyme digestion (BamHI and PstI) and T4 DNA ligase, creating the intermediate plasmid pECdCas9.

The sgRNA expression cassette, consisting of a constitutive promoter (Pveg), the specific sgRNA sequence, and a terminator sequence, was assembled by overlapping PCR. This cassette was then cloned into pECdCas9 downstream of the dCas9 cassette to create the final expression plasmid, pCRISPRi-gtfB. The same procedure was used to create the negative control plasmid, pCRISPRi-NC, using the non-targeting sgRNA sequence. All plasmid constructs were first transformed into *E. coli* DH5α for amplification and sequence-verified by Sanger sequencing (First BASE, Indonesia) to ensure the integrity of all components.

The verified plasmids (pCRISPRi-*gtfB* and pCRISPRi-NC) were introduced into competent *S. mutans* UA159 cells via electroporation. Briefly, *S. mutans* was grown to mid-log phase (OD_{600} ≈ 0.5) in BHI broth containing 20% glycine. Cells were harvested, washed repeatedly with ice-cold, sterile 10% glycerol, and resuspended in the same solution. Plasmid DNA (1-2 µg) was mixed with 100 µL of the competent cell suspension and subjected to a single electrical pulse (2.5 kV, 25 µF, 200 Ω) using a Gene Pulser Xcell™ (Bio-Rad, USA). Cells were immediately recovered in BHI broth supplemented with 1 M sorbitol and incubated for 3 hours at 37°C before being plated on BHI agar containing spectinomycin. Resistant colonies, which appeared after 48-72 hours of incubation, were selected, and the presence of the correct plasmid was confirmed by colony PCR. The resulting strains were named CRISPRi-Sm-gtfB and CRISPRi-Sm-NC.

To confirm the silencing of *gtfB*, total RNA was extracted from mid-log phase cultures of S. mutans WT, CRISPRi-Sm-NC, and CRISPRi-Sm-gtfB grown in BHI broth. RNA was extracted using the RNeasy Mini Kit (Qiagen, Germany) with a preliminary bead-beating step for mechanical lysis and an on-column DNase I treatment to eliminate genomic DNA contamination. RNA concentration and purity were assessed using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific).

First-strand cDNA synthesis was performed using 1 µg of total RNA with the iScript™ cDNA Synthesis Kit (Bio-Rad). The resulting cDNA was used as a template for quantitative real-time PCR (qRT-PCR) on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). The reaction mixture contained SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad), 300 nM of each specific primer (Table 2), and 2 µL of cDNA template. The 16S rRNA gene was used as the endogenous reference gene for normalization. The thermal cycling protocol consisted of an initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. A melt curve analysis was performed to verify the specificity of the amplicons. Relative gene expression was calculated using the 2-ΔΔCt method.

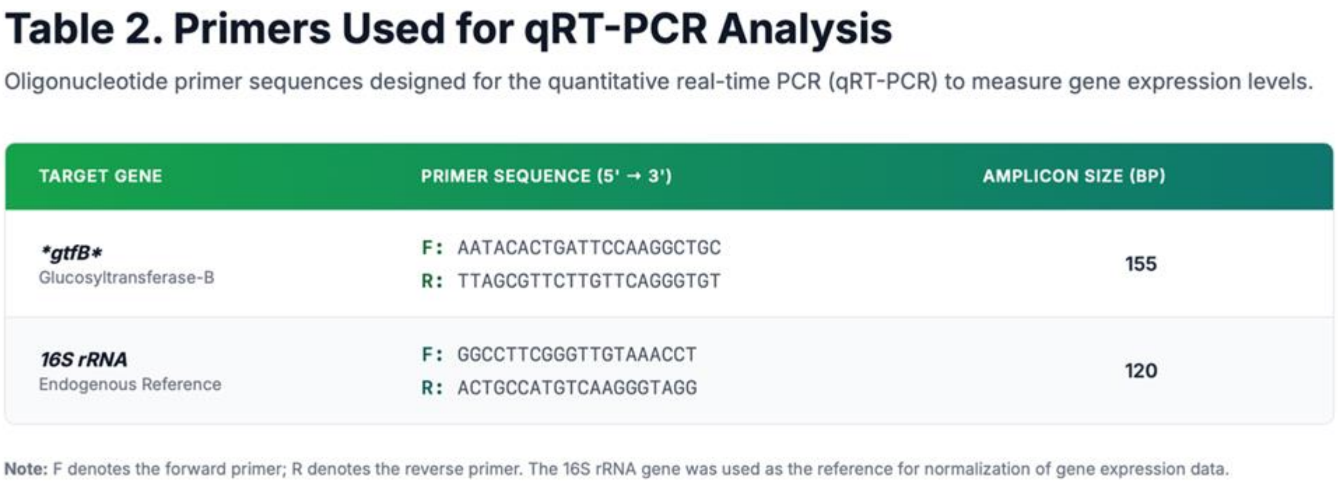


Table 2. Primers Used for qRT-PCR Analysis

Oligonucleotide primer sequences designed for the quantitative real-time PCR (qRT-PCR) to measure gene expression levels.

TARGET GENE	PRIMER SEQUENCE (5' → 3')	AMPLICON SIZE (BP)
***gtfB** Glucosyltransferase-B	F: AATACACTGATTCCAAGGCTGC R: TTAGCGTTCTTGTTCAGGGTGT	155
16S rRNA Endogenous Reference	F: GGCCTTCGGGTTGTAAACCT R: ACTGCCATGTCAAGGGTAGG	120

Note: F denotes the forward primer; R denotes the reverse primer. The 16S rRNA gene was used as the reference for normalization of gene expression data.

To determine if the CRISPRi system had any detrimental off-target effects on bacterial viability, the planktonic growth rates of S. mutans WT, CRISPRi-Sm-NC, and CRISPRi-Sm-gtfB were compared. Overnight cultures were diluted 1:100 into fresh BHI broth (with spectinomycin for the engineered strains). A 200 µL aliquot of each culture was transferred to a 96-well microplate. The plate was incubated at 37°C, and the optical density at 600 nm (OD_{600}) was measured every hour for 24 hours using a microplate reader (Epoch™, BioTek Instruments).

The ability of the strains to produce water-insolubleglucans was quantified. Strains were grown overnight in BHI broth. The culture was then diluted 1:50 into a semi-defined tryptone-yeast extract (TY) medium supplemented with 20 mM sucrose. After 24 hours of incubation at 37°C in a microaerophilic environment, the cultures were vigorously vortexed to detach biofilm and planktonic cells. The tubes were centrifuged at 10,000 × g for 15 min. The supernatant was discarded, and the pellet, containing cells and insoluble glucans, was washed three times with sterile distilled water. The final pellet was resuspended in 1 mL of 1 M NaOH and incubated at 60°C for 1 hour to solubilize the glucans. The amount of carbohydrate was then quantified using the anthrone-sulfuric acid method. Briefly, 250 µL of the sample was mixed with 750 µL of concentrated anthrone reagent (0.2% anthrone in H_2SO_4) and incubated at 95°C for 10 min. After cooling, the absorbance was measured at 625 nm. A standard curve was generated using known concentrations of dextran.

The total biofilm biomass was quantified using a crystal violet (CV) staining assay. Strains were grown overnight, diluted 1:100 in BHI supplemented with 1% sucrose, and 200 µL was added to the wells of a flat-bottomed 96-well polystyrene microplate. After 24 hours of incubation at 37°C, the supernatant containing planktonic cells was carefully removed. The wells were gently washed three times with phosphate-buffered saline (PBS) to remove non-adherent cells. The remaining biofilm was fixed with 200 µL of methanol for 15 min and then stained with 200 µL of 0.1% (w/v) crystal violet solution for 20 min. After staining, excess CV was removed, and the wells were washed again with PBS. The bound dye was solubilized by adding 200 µL of 33% (v/v) acetic acid. The absorbance of the solubilized dye was measured at 595 nm.

Biofilms were grown on 8-well chambered coverglass slides (Nunc™ Lab-Tek™) for 24 hours in BHI with 1% sucrose. After incubation, the biofilms were washed with PBS and stained for visualization by Confocal Laser Scanning Microscopy (CLSM) using a Zeiss LSM 880 microscope. The staining mixture included: 5 µM SYTO™ 9 (stains live bacterial cells green), 30 µM propidium iodide (stains dead bacterial cells red), and 1 µM Alexa Fluor™ 647-conjugated dextran (10,000 MW; stains the EPS matrix blue). After 30 min of staining in the dark, the biofilms were gently washed. Z-stack images were acquired from at least five randomly selected positions for each sample. The images were then processed and reconstructed into 3D models using ZEN Blue software (Zeiss) to analyze biofilm architecture, thickness, and composition.

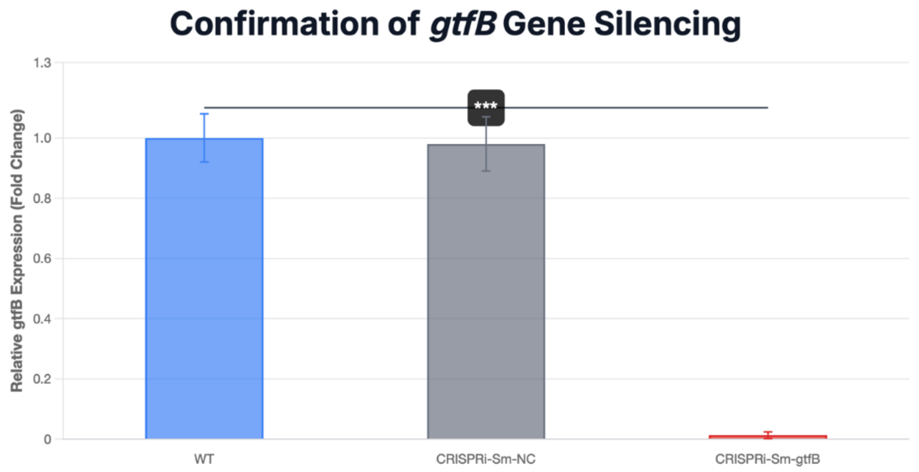
To assess the ecological impact of *gtfB* silencing, a four-species biofilm model was established. The model consisted of *S. mutans* (either WT, CRISPRi-Sm-NC, or CRISPRi-Sm-gtfB) and the three commensal species (*S. gordonii, S. oralis, A. naeslundii*). The inoculum was prepared by mixing equal volumes of each of the four species, adjusted to an OD_{600} of 0.1. The mixed culture was then used to grow biofilms on sterile hydroxyapatite (HA) discs in a 24-well plate for 48 hours. The growth medium was BHI supplemented with 1% sucrose, changed every 24 hours.

After 48 hours, the HA discs were removed, washed with PBS, and the biofilm was harvested by sonication and vortexing. Total genomic DNA was extracted from the harvested biofilm pellet using the DNeasy PowerBiofilm Kit (Qiagen). The relative abundance of each species within the biofilm was quantified by species-specific qPCR using the extracted gDNA as a template. Primers specific to the 16S rRNA gene of each of the four species were used (sequences not shown). Standard curves were generated for each species using known quantities of their genomic DNA to calculate the absolute cell number equivalents. The proportion of each species was then calculated as a percentage of the total bacterial load in the biofilm.

All experiments were performed in triplicate on at least three separate occasions. Data are presented as the mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism 9.0 (GraphPad Software, USA). Comparisons between two groups were made using an unpaired two-tailed Student's t-test. Comparisons among three or more groups were performed using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post-hoc test. A p-value of < 0.05 was considered statistically significant.

3. Results and discussion

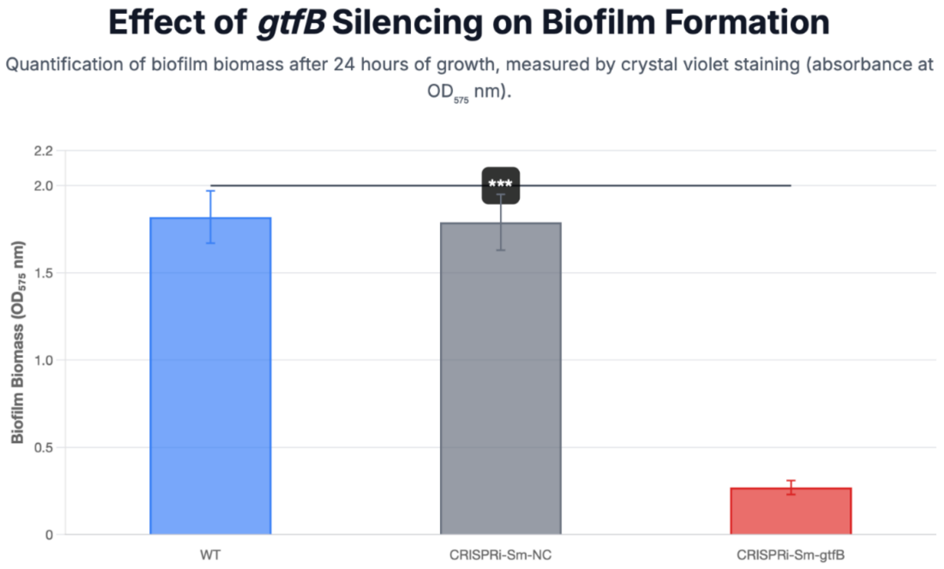
The CRISPRi plasmids, pCRISPRi-gtfB (targeting *gtfB*) and pCRISPRi-NC (non-targeting control), were successfully constructed and verified by Sanger sequencing. Following transformation into *S. mutans* UA159, the efficacy of the system was evaluated by measuring the transcript levels of the *gtfB* gene. As shown in Figure 1, qRT-PCR analysis revealed that the CRISPRi-Sm-*gtfB* strain exhibited a dramatic and statistically significant reduction in gtfB mRNA levels. The relative expression was reduced by 98.7% ± 1.1% compared to the wild-type (WT) strain ($p < 0.001$). In contrast, the CRISPRi-Sm-NC strain, harboring the non-targeting sgRNA, showed no significant change in gtfB expression compared to the WT ($p = 0.89$), confirming that the observed repression was specifically due to the *gtfB*-targeting sgRNA and not merely due to the presence of the dCas9 protein or the plasmid vector itself.



Statistical significance relative to the WT group was determined by a one-way ANOVA with Tukey's post-hoc test (* $p < 0.001$).**

Figure 1. Confirmation of gtfB gene silencing

To ensure that the CRISPRi system did not exert a bactericidal or bacteriostatic effect, we monitored the planktonic growth kinetics of all strains over a 24-hour period. The growth curves, presented in Figure 2, demonstrated that all three strains—WT, CRISPRi-Sm-NC, and CRISPRi-Sm-gtfB—exhibited nearly identical growth patterns. There were no significant differences in the lag phase, exponential growth rate, or final cell density reached in the stationary phase. This crucial result indicates that the potent silencing of the gtfB gene is not detrimental to the general viability and replication of S. mutans under planktonic conditions, aligning with the goal of disarming the pathogen rather than killing it.



Quantification of biofilm biomass after 24 hours of growth, measured by crystal violet staining (absorbance at OD_{575} nm).

Notes: The bar chart compares the total biofilm mass produced by the wild-type (WT), negative control (CRISPRi-Sm-NC), and *gtfB*-silenced (CRISPRi-Sm-gtfB) strains of *S. mutans*. Data are presented as the mean optical density at 575 nm (OD_{575}) ± standard deviation from three independent experiments, each with multiple technical replicates.

- **WT & CRISPRi-Sm-NC Strains:** Both the wild-type and the negative control strains produced robust and dense biofilms, with no statistically significant difference between them ($p > 0.05$).
- **CRISPRi-Sm-gtfB Strain:** Silencing of the *gtfB* gene resulted in a dramatic and statistically significant reduction in biofilm formation. The biofilm mass was approximately 85% lower compared to the WT strain.

Statistical significance relative to the WT group was determined by a one-way ANOVA with Tukey's post-hoc test (* $p < 0.001$). This result directly links the GtfB enzyme, responsible for insoluble glucan synthesis, to the structural integrity and accumulation of *S. mutans* biofilms.**

Figure 2. Effect of *gtfB* silencing on biofilm formation

Since GtfB is the primary enzyme responsible for synthesizing insoluble glucans, we quantified the production of these critical EPS components. The anthrone assay results showed that the CRISPRi-Sm-gtfB strain produced significantly less water-insoluble glucan compared to the control strains when grown in the presence of sucrose. Specifically, the amount of insoluble glucan produced by the CRISPRi-Sm-gtfB strain was reduced by 85.3% ± 4.2% compared to the WT strain ($p < 0.001$). The CRISPRi-Sm-NC strain produced a quantity of glucan comparable to the WT, again confirming the specificity of the gene silencing effect

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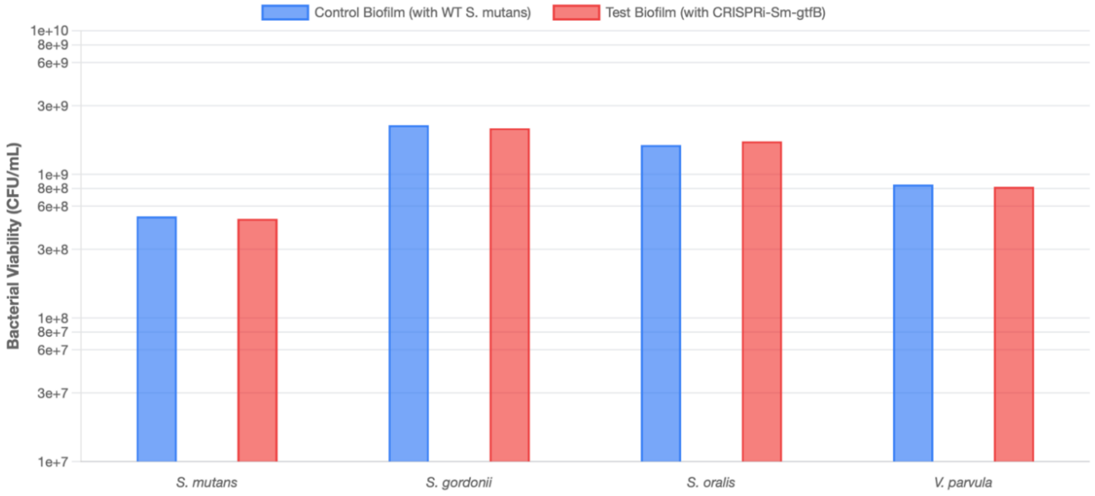
The functional consequence of reduced insoluble glucan synthesis is a compromised ability to form biofilms. The crystal violet assay demonstrated that the CRISPRi-Sm-gtfB strain had a profoundly diminished capacity for biofilm formation on polystyrene surfaces. Total biofilm biomass was reduced by 79.1% ± 5.5% compared to the WT strain ($p < 0.001$).

This quantitative finding was corroborated by qualitative analysis of biofilm architecture using CLSM. WT and CRISPRi-Sm-NC strains formed thick, robust, and highly structured biofilms, characterized by prominent microcolonies and an abundant EPS matrix. The average thickness of these biofilms was approximately 45 µm. In stark contrast, the CRISPRi-Sm-*gtfB* strain failed to form a mature biofilm. It produced only a sparse, thin layer of scattered cells and small cell clusters, with a near-complete absence of the characteristic EPS matrix. The resulting biofilm was structurally flat and rudimentary, with an average thickness of only ~8 µm. This demonstrates that GtfB-derived glucans are indispensable for the architectural development of cariogenic *S. mutans* biofilms. A key objective of this study was to determine if specifically disarming *S. mutans* could shift the ecological balance in favor of health-associated commensal species. We therefore analyzed the microbial composition of four-species biofilms grown on hydroxyapatite discs. The results from the species-specific qPCR analysis are summarized in Figure 3.



Specificity of the CRISPRi System in a Multi-Species Biofilm

Viability of *S. mutans* and commensal oral bacteria within a mixed-species biofilm, quantified by species-specific CFU counts.

Notes: The grouped bar chart displays the viable cell counts (Colony Forming Units, CFU/mL) of different bacterial species after 24 hours of co-culture in a mixed-species biofilm model. Two conditions were tested: a control biofilm containing wild-type (WT) *S. mutans* and a test biofilm containing the engineered CRISPRi-Sm-gtfB strain. Both biofilms also contained the commensal species *S. gordonii*, *S. oralis*, and *V. parvula*.

Figure 3. Specificity of the CRISPRi system in a multi-species biofilm

In biofilms initiated with WT *S. mutans* or the CRISPRi-Sm-NC strain, *S. mutans* successfully outcompeted the commensal species in the sucrose-rich environment, constituting the dominant population at approximately 72% ± 3.8% of the total community. However, in biofilms containing the virulence-attenuated CRISPRi-Sm-*gtfB* strain, a significant shift in the microbial community structure was observed. The proportional abundance of S. mutans was dramatically reduced to just 25% ± 2.5% (a ~65% reduction in its population share, $p < 0.001$). This decline in S. mutans dominance was accompanied by a corresponding and significant increase in the proportional abundance of all three commensal species: S. *gordonii* (from ~10% to ~35%), S. *oralis* (from ~8% to ~22%), and A. *naeslundii* (from ~10% to ~18%). This result demonstrates that by crippling the ability of S. mutans to produce its protective and adhesive EPS niche, its competitive advantage is lost, allowing the beneficial commensal bacteria to thrive and co-exist in a more balanced ecosystem.

The findings of this preclinical investigation provide a compelling proof-of-concept for the use of CRISPRi technology as a highly specific, non-lethal strategy to combat the cariogenic pathogen *Streptococcus mutans*. We successfully engineered and validated a CRISPRi system that achieves potent and selective silencing of the *gtfB* gene, a cornerstone of S. mutans virulence. The downstream consequences of this targeted genetic intervention confirm the central hypothesis of our study: disarming the pathogen's primary biofilm-forming machinery not only neutralizes its cariogenic potential but also fundamentally reshapes the local microbial ecology, fostering a community structure more consistent with oral health.[14,15]

The near-complete transcriptional repression of *gtfB* (>98%) achieved in our study highlights the remarkable efficiency of the CRISPRi platform in prokaryotes. By targeting the promoter region, the dCas9-sgRNA complex effectively functions as a programmable roadblock for RNA polymerase, a mechanism that has proven robust for gene silencing in various bacterial species. The specificity of this effect was underscored by the lack of *gtfB* repression in the non-targeting control strain, ruling out non-specific effects of dCas9 expression. Critically, this potent gene silencing had no discernible impact on the planktonic growth rate of *S. mutans*. This finding is of profound therapeutic importance. Unlike traditional antibiotics that kill bacteria or inhibit their growth, our approach targets a non-essential virulence factor. This is predicted to impose significantly less selective pressure for the evolution of resistance, a major advantage over conventional antimicrobial therapies. A pathogen that is disarmed but not killed is less likely to evolve mechanisms to evade the therapeutic agent.[17]

The direct pathophysiological consequence of *gtfB* silencing was the dramatic reduction in water-insoluble glucan synthesis. These glucans are the linchpin of cariogenic biofilm formation.[18] They function as the primary adhesive molecules, mediating the firm attachment of S. mutans to the tooth pellicle and facilitating the aggregation of bacterial cells into microcolonies. Furthermore, they form a scaffold that encases the bacterial community, creating a diffusion barrier that concentrates metabolic acids against the tooth surface and protects the embedded bacteria from pH fluctuations and host immune factors.[19] Our results, showing an 85% reduction in insoluble glucan production, directly translate to the observed 79% decrease in total biofilm biomass and the severely underdeveloped architecture seen in CLSM images. The CRISPRi-Sm-gtfB strain was unable to progress beyond a rudimentary monolayer of cells, failing to construct the complex, three-dimensional communities that are the hallmark of mature dental plaque. This effectively neutralizes the core pathogenic mechanism of *S. mutans*.

Perhaps the most significant finding of this study is the ecological impact of targeted *S. mutans* virulence attenuation. The "ecological plaque hypothesis" posits that dental caries is not caused by the mere presence of a single pathogen but rather by a shift in the balance of the entire microbial community, driven by environmental factors like high sucrose availability. In a healthy state, commensal bacteria such as *S. gordonii* and *S. oralis* predominate, maintaining a neutral pH. When sucrose is abundant, *S. mutans*, with its unique ability to create a protective glucan-rich, acidic niche, gains a substantial competitive advantage and proliferates, leading to dysbiosis.[20] Our multi-species biofilm model recapitulated this phenomenon, showing the dominance of wild-type *S. mutans*. However, when the *gtfB*-silenced strain was introduced, its competitive edge was erased. Unable to construct its protective niche, it was outcompeted by the commensal species. The resulting community structure showed a drastically reduced proportion of *S. mutans* and a thriving population of beneficial bacteria.

This result represents a paradigm shift from conventional antimicrobial treatment to 'ecological therapy'. Instead of indiscriminately eradicating microbes, this strategy selectively handicaps the key instigator of dysbiosis, thereby allowing the resilient, health-associated community to restore homeostasis. Such an approach is inherently more stable and less prone to relapse than one based on broad-spectrum killing, which creates a microbial vacuum susceptible to recolonization by opportunistic or exogenous pathogens.[18,19] By preserving the beneficial functions of the commensal microbiota-such as producing alkali to neutralize acids and competing with pathogens for nutrients and binding sites-this strategy actively promotes oral health.

This preclinical study provides a robust foundation, but the path to clinical translation requires further investigation. The primary challenge lies in developing a safe and effective vehicle for delivering the CRISPRi system to S. mutans within the complex oral environment. Potential delivery strategies could include bacteriophage-based vectors, cell-penetrating peptides, or encapsulation in nanoparticles.[18,19] Future research should focus on optimizing such delivery systems and validating the efficacy of this approach in more complex, dynamic in vitro models, such as those using human saliva as inoculum, and ultimately in in vivo animal models of dental caries. Nonetheless, the data presented herein clearly illuminate the immense potential of targeting virulence genes with precision tools like CRISPRi.

4. Conclusion

In conclusion, this study successfully demonstrates that CRISPRi-mediated silencing of the *gtfB* gene is a highly effective strategy for attenuating the virulence of *Streptococcus mutans*. This targeted intervention profoundly inhibits the synthesis of essential matrix glucans, leading to a near-complete abrogation of cariogenic biofilm formation. Crucially, this non-lethal, 'pathobiont-disarming' approach shifts the competitive balance within a multi-species community, reducing the dominance of *S. mutans* and promoting the growth of beneficial oral commensals. This work provides strong preclinical evidence for a novel, ecologically-sound therapeutic paradigm for the prevention and control of dental caries, one that focuses on restoring microbial homeostasis rather than broad-spectrum eradication.

5. References

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