// Phage-Based Precision Targeting of *Streptococcus mutans* for Caries Prevention

A Resistance-Hardened Bacteriophage Cocktail Targeting *Streptococcus mutans* for Microbiome-Sparing Caries Prevention

Project Summary / Abstract

Dental caries is among the most prevalent chronic diseases worldwide and is driven largely by *Streptococcus mutans*, an acidogenic organism that ferments dietary sugars into acid and builds the glucan-rich biofilm that demineralizes the tooth surface. Every mainstream control measure — fluoride, chlorhexidine, and mechanical debridement — is non-selective: it acts broadly across the oral flora, depleting protective commensals without specifically removing the cariogenic pathogen, and antibiotic approaches add resistance and dysbiosis liabilities. Bacteriophages are uniquely suited to this problem because they are strain-specific, amplify at the infection site, and can lyse *S. mutans* within plaque biofilm while sparing the surrounding microbiome.

Anti-*S. mutans* phage therapy is now established preclinically. The lytic phage SMHBZ8, isolated from human saliva, prevented carious lesions both in vitro on dissected murine hemi-mandibles and in vivo in a murine caries model, in suspension and in a sustained-release varnish formulation. The independently isolated phage φAPCM01 inhibited *S. mutans* in artificial saliva and reduced viable cells within established biofilms by at least 5 log CFU/mL. However, *S. mutans* defeats single phages by losing or altering the rhamnose-glucose cell-wall polysaccharide that serves as the phage M102 adsorption receptor, and by acquiring CRISPR spacers matching phage genomes — a direct rationale for multi-phage cocktails rather than monophage therapy. We will (1) assemble and characterize a complementary lytic cocktail selected for non-overlapping receptor usage and resistance profiles; (2) demonstrate biofilm penetration, killing, and resistance suppression in defined artificial-saliva and ex vivo tooth/jaw systems against pre-specified quantitative thresholds; and (3) confirm caries-preventive efficacy and microbiome-sparing selectivity in an established rodent caries model. The deliverable is a defined, mechanism-rationalized phage cocktail with the preclinical efficacy, selectivity, and safety package needed to support an investigational pathway toward a microbiome-sparing caries-control product.

Specific Aims

Dental caries remains one of the most widespread chronic diseases globally, and it is driven by a single dominant, biofilm-embedded pathogen, *S. mutans*, which metabolizes dietary sugars into acid and constructs the glucan-rich biofilm that demineralizes the tooth. This makes caries an unusually clean target for narrow-spectrum phage therapy. Yet the evidence base is entirely preclinical and rests almost wholly on **single phages**, which *S. mutans* can defeat through receptor loss and CRISPR adaptation. **Our central hypothesis is that a rationally assembled cocktail of lytic phages with orthogonal receptor dependencies will suppress resistance and clear biofilm-embedded *S. mutans* more durably than any single phage, while leaving the broader oral microbiome compositionally intact.** We will test this with three aims.

Aim 1 — Build and characterize a complementary anti-*S. mutans* phage cocktail with non-redundant resistance profiles. We will determine host range, efficiency of plating, adsorption kinetics, one-step growth, and receptor dependence for lytic *S. mutans* phages (M102-, φAPCM01-, and SMHBZ8-type lineages) against a defined panel of clinical serotype c and non-c strains. We will select 2–3 lytic phages whose infectivity depends on distinct receptor determinants so that escape from one phage does not confer cross-resistance. *Deliverable / success criterion:* a sequenced, integrase- and toxin-free cocktail covering ≥90% of panel strains with ≥2 members active on each strain via mapped, complementary receptor pathways.

Aim 2 — Demonstrate biofilm penetration, killing, and resistance suppression in physiologically realistic tooth/jaw models. We will quantify how the cocktail penetrates and collapses established *S. mutans* biofilms — reducing metabolic activity and viable counts — in artificial saliva and on ex vivo tooth/dissected hemi-mandible substrates, and measure the rate at which phage-resistant mutants emerge under cocktail versus single-phage pressure. *Deliverable / success criterion:* ≥3 log CFU greater biofilm kill and ≥10-fold lower resistant-mutant emergence frequency for the cocktail versus the best single phage.

Aim 3 — Establish caries-preventive efficacy and microbiome-sparing selectivity in an in vivo rodent caries model. We will test whether the cocktail, in suspension and in a sustained-release oral formulation, reduces *S. mutans* burden and carious lesion development in an established murine caries model while preserving overall oral-microbiome composition by 16S rRNA profiling. *Deliverable / success criterion:* significant reduction in lesion scores and *S. mutans* burden versus vehicle, at least matching single-phage controls, with no significant loss of overall (non-*mutans*) community diversity.

Impact. Success would yield the first defined, resistance-hardened *S. mutans* phage cocktail with the preclinical package needed to pursue human translation — a concrete step toward one of dentistry's first everyday, microbiome-sparing antibacterials. Go/no-go decision points (below) gate progression between aims so that in vivo resources are committed only against a validated, resistance-suppressing

cocktail.

Significance

Caries imposes an enormous, inequitably distributed burden, and the disease is mechanistically distinctive: it is defined by a single dominant acidogenic pathogen, *S. mutans*, which ferments dietary sugars into acid and constructs the glucan-rich biofilm that drives demineralization [4]. Conventional therapy fails to specifically target the pathogen while tending to eradicate commensal bacteria [2]: fluoride, chlorhexidine, and mechanical debridement act broadly across the oral flora, and antibiotic approaches add resistance and dysbiosis liabilities. There is no approved narrow-spectrum agent that removes *S. mutans* specifically.

Phages are uniquely matched to this problem. They are strain-specific, amplify at the site of infection, and can act on *S. mutans* inside plaque biofilm while sparing the surrounding microbiome [2,3]. The preclinical literature now supports feasibility on three independent fronts: the lytic phage φAPCM01 inhibits *S. mutans* in broth and artificial saliva at low multiplicity of infection and reduces viable cells within established biofilms by at least 5 log CFU/mL [3]; SMHBZ8, isolated from human saliva, prevented carious lesions in an in vitro dissected-jaw model and in an in vivo murine caries model, in both suspension and a sustained-release varnish formulation [1,2]; and the prototype phage M102 is a sequenced lytic phage specific for serotype c *S. mutans* whose biology defines the receptor and resistance landscape of the system [3,4]. By converting these single-phage proofs of concept into a defined, resistance-hardened cocktail with rigorous efficacy and selectivity data, this project directly advances NIDCR's interest in precision oral-microbiome interventions and supplies the translational foundation the field currently lacks.

Innovation

The field's central weakness is reliance on single phages, which *S. mutans* defeats by losing or altering the rhamnose-glucose cell-wall polysaccharide that constitutes the M102 adsorption receptor, and by acquiring CRISPR spacers matching phage genomes [3,4]. Our innovation is to design **against** these now-characterized defenses rather than to test another monophage:

1. **Orthogonal-receptor cocktail design.** We select cocktail members for distinct receptor dependence, so receptor-loss escape from one phage leaves the bacterium susceptible to another — converting a single point of failure into a combinatorial barrier.
2. **The biofilm matrix as barrier *and* target.** We treat the water-insoluble exopolysaccharide matrix as a defined obstacle to be measured and overcome, evaluating combinations — including phage-encoded lytic enzymes already present in the M102-type lysis cassette [4] —

that improve access to biofilm-embedded cells.

3. **Formulation-ready delivery.** We advance beyond a laboratory suspension to the sustained-release oral formulation already shown to retain anti-caries efficacy [1], moving toward a clinically deployable rinse, lozenge, or varnish concept.

Together this reframes anti-caries phage therapy from single-agent proof of concept to a deliberately resistance-hardened, formulation-ready cocktail with pre-specified quantitative success criteria.

Approach

Rigor of prior research. The three pillars we build on are peer-reviewed and independently generated by separate groups: φAPCM01 biofilm killing in artificial saliva [3]; SMHBZ8 in vitro and in vivo caries prevention in suspension and sustained-release form [1,2]; and the M102 genome and receptor/CRISPR resistance biology [3,4]. Each prior finding is reproduced as an internal benchmark before we extend it.

Rigor & reproducibility (all aims). Experiments use ≥3 biological replicates on independent days; investigators scoring caries and analyzing microbiome data are blinded to treatment; key biological resources (phage stocks by full-genome sequence and restriction profile; bacterial strains by serotype PCR and whole-genome sequence) are authenticated and banked. Quantitative endpoints and statistical plans are pre-specified per aim (below). Sex as a biological variable is addressed in Aim 3 by enrolling both sexes.

Aim 1 — Build and characterize a complementary anti-*S. mutans* phage cocktail with non-redundant resistance profiles

Rationale. *S. mutans* phages are highly serotype- and strain-specific: M102 is a lytic phage specific for serotype c strains, and its adsorption depends on the glucose side chain of the rhamnose-glucose cell-wall polysaccharide receptor; resistance arises from loss/alteration of that receptor and from CRISPR spacer acquisition matching the phage genome [3,4]. A cocktail of phages using **distinct** receptor determinants is therefore mechanistically the correct unit of therapy.

Experimental design. Against a defined panel of clinical *S. mutans* isolates (serotype c and non-c) plus reference strains known to be SMHBZ8-resistant (e.g., LM7, OMZ175-type), we will assay host range, efficiency of plating, adsorption kinetics, and one-step growth for available lytic phages (M102-, φAPCM01-, and SMHBZ8-type lineages [1,2,3,4]). Receptor dependence will be probed with receptor-defective spontaneous mutants and adsorption-competition assays. All candidate phages will be whole-genome sequenced to confirm a strictly lytic lifestyle (no integrase, repressor, or recognizable toxin/virulence genes) and to relate genotype to host range. We will then select 2–3 lytic

phages with complementary host ranges and demonstrably distinct receptor requirements.

Quantitative success criterion. Cocktail covers ≥90% of panel strains with ≥2 members active per strain through mapped, non-overlapping receptor pathways.

Potential pitfalls & alternatives. *Available phages may converge on the same receptor.* We will mitigate by isolating additional lytic phages from human saliva (the source of both SMHBZ8 and φAPCM01 [2,3]) and by screening for phages selected on receptor-variant hosts. Where receptor convergence is unavoidable, we will exploit the lytic enzymes encoded within the M102-type lysis cassette [4] as a receptor-independent killing module evaluated in Aim 2 — keeping the cocktail strictly lytic and avoiding any lysogeny risk.

Go/no-go: proceed to Aim 2 only if a ≥2-member, sequenced, integrase-/toxin-free cocktail with complementary receptor usage is in hand.

Aim 2 — Demonstrate biofilm penetration, killing, and resistance suppression in physiologically realistic tooth/jaw models

Rationale. Caries is a biofilm disease. Lytic *S. mutans* phages can penetrate and collapse established biofilms — φAPCM01 reduced biofilm metabolic activity and lowered viable cells by ≥5 log CFU/mL in artificial saliva [3] — but the water-insoluble exopolysaccharide matrix is both a barrier and a target. A cocktail must therefore be tested in biofilm, not merely planktonically, and explicitly against resistance emergence.

Experimental design. We will grow *S. mutans* biofilms in artificial saliva supplemented with sucrose (the φAPCM01 benchmark condition [3]) and on ex vivo tooth/dissected hemi-mandible substrates (the SMHBZ8 in vitro caries model [1]), then treat with the cocktail versus each constituent phage. *Endpoints:* viable counts (CFU), metabolic activity, and biofilm biomass/architecture. *Resistance suppression:* we will compare the frequency and time-course of phage-resistant mutant emergence under single-phage versus cocktail pressure, and characterize escape mutants for receptor-polysaccharide defects and CRISPR spacer acquisition [3,4]. We will also test whether adding the M102-type lytic-enzyme module [4] improves penetration of the exopolysaccharide matrix.

Quantitative success criteria. Cocktail achieves ≥3 log CFU greater biofilm kill than the best single phage, and ≥10-fold lower resistant-mutant emergence frequency, in the artificial-saliva primary system; the ex vivo jaw model confirms direction of effect.

Potential pitfalls & alternatives. *Ex vivo substrates are variable;* the defined artificial-saliva biofilm assay is the quantitative primary system and the tooth/jaw substrate is confirmatory. *If the matrix blocks access,* we prioritize lytic-enzyme/EPS-targeting combinations, treating the matrix as an

explicit target rather than an unavoidable barrier.

Go/no-go: proceed to in vivo Aim 3 only if both Aim 2 quantitative criteria are met.

Aim 3 — Establish caries-preventive efficacy and microbiome-sparing selectivity in an in vivo rodent caries model

Rationale. The most advanced anti-caries phage result is in vivo: SMHBZ8 prevented carious lesions in a murine caries model in both suspension and sustained-release form [1]. Translating a *cocktail* requires reproducing caries prevention in vivo while demonstrating the microbiome-sparing selectivity that motivates the entire approach.

Experimental design. Using an established murine caries model with *S. mutans* challenge and a cariogenic diet [1], animals (both sexes) will receive the cocktail in suspension or sustained-release oral formulation versus vehicle and single-phage controls. *Primary endpoints:* carious lesion scoring (clinical and µCT, as validated for SMHBZ8 [1]) and *S. mutans* burden. *Selectivity endpoint:* 16S rRNA profiling of the oral microbiome to test whether the cocktail depletes *S. mutans* while leaving the broader community compositionally intact. Group sizes [ILLUSTRATIVE: n = 12 per arm] will be fixed by power analysis on lesion scores during final design, with sex included as a covariate.

Quantitative success criteria. Significant reduction in lesion scores and *S. mutans* burden versus vehicle (at least matching single-phage controls), with no significant reduction in overall (non-*mutans*) community diversity.

Potential pitfalls & alternatives. *Oral phage residence may be short, and salivary/host factors may neutralize phages;* the sustained-release formulation directly addresses residence [1], and dosing frequency will be optimized. *If in vivo efficacy lags in vitro results,* we will adjust dose/MOI and formulation and re-confirm cocktail coverage against the *S. mutans* population recovered in vivo, guarding against in-host resistance.

Timeline

[ILLUSTRATIVE] Total project period 5 years. **Year 1:** Aim 1 host-range, receptor, and genome characterization; cocktail selection; Go/no-go #1. **Years 2–3:** Aim 2 biofilm killing, ex vivo tooth/jaw testing, and resistance-suppression studies; formulation development; Go/no-go #2. **Years 3–4:** Aim 3 in vivo efficacy and 16S selectivity studies. **Year 5:** Confirmatory in vivo work, manufacturing/quality groundwork, and assembly of the preclinical package to support an investigational pathway.

Budget Justification (modular R01-style sketch)

[ILLUSTRATIVE] Requested at the modular level: [ILLUSTRATIVE] $250,000 direct costs per year for [ILLUSTRATIVE] 5 years. **Personnel:** PI (microbiology/phage biology) [ILLUSTRATIVE] 2.4 calendar months; Co-I (cariology/oral microbiome) [ILLUSTRATIVE] 1.2 months; one postdoctoral phage biologist and one research technician (Aims 1–2) [ILLUSTRATIVE] 12 months each; a co-investigator with rodent caries-model expertise supporting Aim 3. **Other costs:** clinical-isolate panels and phage isolation/sequencing; biofilm and ex vivo tooth/jaw consumables; sustained-release formulation development; rodent caries-model per-diems, µCT, and histology; 16S microbiome sequencing. Equipment is largely in place; no major instrumentation is requested. A modular budget is justified because costs are dominated by personnel and standard microbiology, biofilm, sequencing, and small-animal expenses rather than unusual one-time purchases.

Vertebrate Animals

Animal work is proposed in Aim 3. **Use and justification.** An established murine caries model is required because caries is a biofilm- and host-diet–dependent disease whose prevention cannot be fully assessed in vitro; this model has previously demonstrated phage-mediated caries prevention with SMHBZ8 [1]. Mice receiving *S. mutans* challenge and a cariogenic diet will be treated with phage cocktail, single-phage, or vehicle, with caries scoring (clinical and µCT), bacterial burden, and 16S microbiome endpoints. **Sex as a biological variable.** Both sexes will be enrolled in balanced numbers, with sex included as a covariate. **Minimization and welfare.** Group sizes [ILLUSTRATIVE: n = 12 per arm] will be the minimum supported by power analysis on lesion scoring; humane endpoints, analgesia where indicated, and standard husbandry will follow IACUC-approved protocols. No procedures beyond those required for caries induction, treatment, and tissue/microbiome sampling are planned.

Human Subjects / Clinical Trial

Not applicable to the proposed work; all studies are in vitro, ex vivo, and in small animals, consistent with the field's current preclinical stage. For translational planning only: a first-in-human study of an investigational phage cocktail would proceed under FDA oversight, and individual compassionate use could be pursued through an FDA emergency/expanded-access investigational new drug (eIND) route, with full IRB review and informed consent. Such clinical work is explicitly outside the scope and budget of this R01 and would be the subject of a separate future application.

Team & Environment

This project requires assembled expertise in phage biology, cariology/oral microbiology, biofilm science, and small-animal caries modeling. Template roles to fill with real names and institutions:

- **Principal Investigator** — phage isolation, characterization, and cocktail design (lead Aims 1–2). [PI name / institution]
- **Co-Investigator, Cariology & Oral Microbiome** — biofilm and tooth/jaw models, 16S analysis, in vivo caries scoring (Aims 2–3). [Co-I name / institution]
- **Co-Investigator, Phage Formulation** — sustained-release oral delivery development. [Co-I name / institution]
- **Collaborators / Scientific Advisors** — investigators with established anti-*S. mutans* phage programs whose work underpins this proposal: the groups that isolated and characterized SMHBZ8 [1,2] and φAPCM01 [3], and investigators with M102 phage-genomics expertise [4]. [Confirm roles / letters of support]

Environment. The host institution should provide BSL-appropriate phage microbiology, biofilm and ex vivo tooth/jaw capability, an AAALAC-accredited animal facility with an established rodent caries model, and sequencing/bioinformatics core access — resources standard at oral-health research centers and sufficient to execute all three aims.

References

1. Wolfoviz-Zilberman A, Kraitman R, Hazan R, Friedman M, Houri-Haddad Y, Beyth N. Phage Targeting *Streptococcus mutans* In Vitro and In Vivo as a Caries-Preventive Modality. *Antibiotics (Basel).* 2021;10(8):1015. https://doi.org/10.3390/antibiotics10081015
2. Ben-Zaken H, Kraitman R, Coppenhagen-Glazer S, Khalifa L, Alkalay-Oren S, Gelman D, Ben-Gal G, Beyth N, Hazan R. Isolation and Characterization of *Streptococcus mutans* Phage as a Possible Treatment Agent for Caries. *Viruses.* 2021;13(5):825. https://doi.org/10.3390/v13050825
3. Dalmasso M, de Haas E, Neve H, Strain R, Cousin FJ, Stockdale SR, Ross RP, Hill C. Isolation of a Novel Phage with Activity against *Streptococcus mutans* Biofilms. *PLoS One.* 2015;10(9):e0138651. https://doi.org/10.1371/journal.pone.0138651
4. van der Ploeg JR. Genome sequence of *Streptococcus mutans* bacteriophage M102. *FEMS Microbiol Lett.* 2007;275(1):130-138. https://doi.org/10.1111/j.1574-6968.2007.00873.x