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Precision Phage Therapy to Disarm Cytolytic *Enterococcus faecalis* in Severe Alcohol-Associated Hepatitis: A Biomarker-Paired, IND-Enabling Oral Cocktail

Project Summary / Abstract

Severe alcohol-associated hepatitis (AH) is a frequently fatal emergency with short-term mortality commonly cited at roughly 30–50% and only one marginally effective pharmacotherapy (corticosteroids), to which many patients are ineligible or non-responsive. A defined, gut-derived driver of disease has been identified: cytolysin-positive (cytolytic) *Enterococcus faecalis* secretes a two-subunit exotoxin that translocates to the liver and kills hepatocytes, worsening ethanol-induced injury, and fecal cytolysin positivity correlates with disease severity and mortality in patients (Duan et al., *Nature* 2019). Because the hepatotoxin is produced by a discrete bacterial subset rather than the whole microbiome, lytic bacteriophages offer surgical precision—depleting toxin producers while sparing commensals and avoiding the resistance-selection and collateral-dysbiosis liabilities of broad-spectrum antibiotics.

Our **central hypothesis** is that an orally delivered, rationally composed phage cocktail will reduce intestinal cytolytic *E. faecalis* burden and hepatic cytolysin, attenuate liver injury, and do so durably because phage escape carries a steep colonization fitness cost. We build on a defined oral 3-phage cocktail—sewage-isolated, covering the majority of tested cytolysin-positive strains, and characterized as safe with a predominantly anti-inflammatory, tissue-restoring immune profile (Garcia Mendes et al., *Viruses* 2022)—and on evidence that resistance arises via IS256-mediated events but impairs gut colonization rather than producing rebound disease (Fujiki et al., *Microbiology Spectrum* 2025). **Aim 1** defines host range against a contemporary, geographically diverse cytolytic *E. faecalis* panel and locks a coverage-maximizing, resistance-suppressing cocktail. **Aim 2** tests efficacy and dissects the phage–host–liver axis (biodistribution, resistance dynamics) in humanized, ethanol-fed mice. **Aim 3** generates the IND-enabling safety, manufacturing (CMC), and companion-diagnostic (stool cytolysin qPCR) package needed to support an FDA investigational-phage submission. The expected outcome is a defined, manufacturable, biomarker-paired phage product positioned for a precision first-in-human trial restricted to cytolysin-positive patients—a treat-the-responder strategy

for a high-mortality, therapy-poor indication squarely within the NIAAA mission.

Specific Aims

Severe AH is a frequently fatal emergency with one marginal therapy. Duan et al. (*Nature* 2019) showed that cytolytic *E. faecalis* causally drives ethanol-induced liver injury through a secreted cytolysin that reaches the liver and kills hepatocytes; that fecal cytolysin positivity tracks with disease severity and mortality; that a substantial fraction of AH patients (on the order of ~60% in the index cohort) carry cytolysin-positive *E. faecalis*; and that phage targeting of these bacteria abolished ethanol-induced liver disease in humanized mice. Garcia Mendes et al. (*Viruses* 2022) advanced a defined oral 3-phage cocktail—isolated from sewage, covering the majority of tested cytolysin-positive strains, safe after chronic-binge ethanol, with a predominantly anti-inflammatory immune signature—and judged it suitable for clinical-trial testing. Fujiki et al. (*Microbiology Spectrum* 2025) showed that phage resistance arises through IS256 insertion-sequence events (disrupting *xylA*, mutating *epaR*, and deleting *galE* in the Epa polysaccharide pathway) but at a steep fitness cost: reduced intestinal adherence, increased bile-salt sensitivity, and attenuated gut colonization.

Central hypothesis: A rationally composed, orally delivered phage cocktail will durably deplete cytolytic *E. faecalis* and lower hepatic cytolysin in cytolysin-positive hosts, attenuating liver injury, with escape variants self-limited by their colonization fitness cost.

Aim 1 — Define and optimize cocktail coverage against contemporary cytolytic *E. faecalis*.

Assemble a diverse clinical-isolate panel and a sewage-derived phage library; quantify host range by efficiency-of-plating and growth-suppression; and rationally lock a 3-phage composition that maximizes coverage of cytolysin-positive strains while suppressing resistance in co-culture.

Outcome: a coverage map and a justified, locked cocktail.

Aim 2 — Establish efficacy and the phage–host–liver mechanism in humanized ethanol-fed mice.

In fecal-transplanted, chronic-plus-binge ethanol-fed mice, test whether the optimized oral cocktail reduces intestinal cytolytic *E. faecalis*, lowers hepatic cytolysin, and attenuates steatosis, hepatocyte death, and inflammation, while mapping phage biodistribution and IS256-mediated resistance dynamics in vivo. *Outcome:* quantitative efficacy and mechanism data with prespecified effect-size thresholds.

Aim 3 — Generate IND-enabling safety, CMC, and a companion cytolysin diagnostic.

Complete repeat-dose oral safety/toxicology, define manufacturing and release specifications toward clinical-grade material, and validate a stool cytolysin qPCR to identify the cytolysin-positive subset most likely to benefit. *Outcome:* a regulatory package and theranostic suitable for an FDA investigational-phage submission.

Impact. Success delivers the first precision, biomarker-paired microbiome therapeutic for an often-fatal liver emergency—disarming a hepatotoxin at its bacterial source while sparing the gut, and treating only patients predicted to respond.

Significance

Severe AH is among hepatology's deadliest acute conditions, with short-term mortality commonly reported in the ~30–50% range and only corticosteroids as marginally effective therapy; many patients are steroid-ineligible or steroid-non-responsive. This therapeutic vacuum motivates mechanism-based approaches. Duan et al. (*Nature* 2019) converted a diffuse "dysbiosis" narrative into a discrete molecular target by showing that cytolytic *E. faecalis* is causal rather than a bystander: its secreted two-subunit cytolysin reaches the liver, directly kills hepatocytes, and exacerbates ethanol-induced injury, and fecal cytolysin positivity correlates with severity and mortality. Critically, pathogenic activity is confined to a defined bacterial subset, making precision depletion—rather than global microbiome disruption—both biologically rational and clinically attractive. Broad-spectrum antibiotics cannot achieve this selectivity and risk fueling resistant enterococci; lytic phages remove the toxin producers while leaving surrounding commensals intact.

This program is squarely within the NIAAA mission—alcohol-associated organ injury and its gut–liver mechanisms—and directly addresses the Institute's priority of translating mechanistic insight into therapeutics for alcohol-associated liver disease. (The work also informs broader liver-disease and gut-microbiome science of interest to NIDDK.) By coupling a precision therapeutic to a cytolysin biomarker, the project targets a high-mortality, therapy-poor indication with a strategy that is mechanistically specific, manufacturable, and clinically deployable.

Innovation

The innovation is **precision rather than breadth**: instead of suppressing the microbiome, we eliminate a single toxin-producing population with lytic phages. Three features distinguish the approach.

- **Theranostic by design.** An oral phage cocktail is paired with a stool cytolysin qPCR companion test, enabling clinicians to screen and treat only the cytolysin-positive subset—a true treat-the-responder strategy that is, to our knowledge, novel for alcohol-associated liver disease.
- **Resistance turned into an ally.** Fujiki et al. (*Microbiology Spectrum* 2025) showed that phage escape arises via IS256 events (disrupting *xylA*, mutating *epaR*, deleting *galE*) but at a steep fitness cost—reduced intestinal adherence and increased bile-salt sensitivity—so

resistance tends to coincide with impaired gut colonization rather than treatment failure. We design cocktail composition and dosing to exploit this trade-off.

- **A defined, locked, biomarker-paired product.** Rather than an ad hoc mixture, the program delivers a composition-locked cocktail with release specifications and a paired diagnostic, de-risking the path to a precision first-in-human trial.

Should three phages prove insufficient against contemporary strains, we will broaden coverage through iterative re-composition and expanded isolation (Aim 1, Pitfalls); any engineering-based extension would be pursued only as a clearly delineated contingency, not a core dependency.

Approach

Aim 1 — Define and optimize cocktail coverage against contemporary cytolytic *E. faecalis*

Rationale. Individual phages have narrow host ranges; durable, broadly active therapy requires a rationally composed cocktail. Duan et al. assembled a sewage-derived phage panel and used a multi-phage mix targeting cytolytic *E. faecalis*; Garcia Mendes et al. advanced a defined 3-phage oral cocktail covering the majority of tested cytolysin-positive strains. We update coverage against contemporary, diverse strains because cytolysin prevalence and clinical relevance may be cohort- and geography-dependent.

Design. We will (1) assemble a panel of cytolytic and non-cytolytic *E. faecalis* clinical isolates spanning multiple geographies, confirming cytolysin genotype and phenotype for every strain; (2) isolate and expand lytic phages from sewage and curate a candidate library enriched for broad-host-range phages; (3) quantify host range by efficiency-of-plating and growth-suppression (OD-based) assays; (4) score candidate 3-phage combinations for coverage breadth and resistance suppression in time-kill co-culture; and (5) lock a lead composition.

Quantitative criteria / go–no-go. Lock requires a 3-phage combination covering a prespecified majority threshold of the cytolytic panel by efficiency-of-plating and demonstrating reduced resistance emergence versus best single phage over a defined co-culture window. **[ILLUSTRATIVE]** thresholds (e.g., $\geq 70\%$ panel coverage) will be finalized at submission.

Expected outcomes. A coverage map for the strain panel and a justified, locked 3-phage cocktail predicted to cover most clinically relevant cytolytic strains while limiting escape.

Pitfalls & alternatives. If three phages give insufficient breadth, we iterate composition from the broad-host-range subset or expand isolation; persistent coverage gaps would trigger the delineated

engineering contingency (Innovation) in collaboration. **Rigor/authentication:** all strains and phages are sequence-verified, banked, and authenticated; cytolysin status is confirmed orthogonally (genotype + functional assay).

Aim 2 — Establish efficacy and the phage–host–liver mechanism in humanized ethanol-fed mice

Rationale. Duan et al. showed in humanized (fecal-transplanted), ethanol-fed mice that phage targeting of cytolytic *E. faecalis* abolished ethanol-induced liver disease; we reproduce and extend this with the optimized cocktail and add biodistribution and resistance read-outs.

Design. Mice will be humanized with feces from confirmed cytolysin-positive donors, fed the chronic-plus-binge ethanol regimen, and randomized to oral optimized cocktail versus vehicle, with non-cytolytic-colonized and uncolonized comparators. **Primary endpoint:** intestinal cytolytic *E. faecalis* burden. **Secondary endpoints:** hepatic cytolysin; steatosis, hepatocyte death, ALT/AST, and inflammatory markers; phage biodistribution to serum, spleen, and liver; and characterization of the immune response (expected predominantly anti-inflammatory and tissue-restoring per prior work). We will track IS256-mediated resistant variants and test the predicted colonization/bile-salt fitness cost in vivo.

Statistical plan & rigor. Group sizes are set by power analysis to a prespecified effect size on the primary endpoint [ILLUSTRATIVE]; analyses use mixed models with predefined comparisons. **Sex as a biological variable:** both sexes are enrolled and sex is included as a covariate, powered to detect sex-by-treatment interaction signals. Randomization, blinded histology scoring, prespecified endpoints, biological-replicate minimums, and confirmation of colonization before dosing are built in.

Expected outcomes. Demonstration that the cocktail lowers intestinal cytolytic burden and hepatic cytolysin and attenuates injury, with resistance—when it arises—linked to impaired colonization rather than rebound disease.

Pitfalls & alternatives. Variable engraftment is mitigated by pre-dosing colonization confirmation and stratified analysis. If resistant variants retain colonization, we adjust phage combination and dosing schedule. A go/no-go precedes Aim 3 scale-up: efficacy must meet the prespecified threshold on the primary endpoint.

Aim 3 — Generate IND-enabling safety, CMC, and a companion cytolysin diagnostic

Rationale. Additional safety testing and manufacturing scale-up remain prerequisites before first-in-human phage trials, and a companion biomarker is needed to enrich for likely responders.

Design. We will (1) perform repeat-dose oral safety/toxicology of the locked cocktail in animals, including endotoxin and purity assessment; (2) define manufacturing, purification, and release specifications (identity, titer, sterility, endotoxin) toward clinical-grade material consistent with the clinical-grade cocktail described by Garcia Mendes et al.; and (3) develop and validate a stool cytolysin qPCR companion assay (analytical sensitivity/specificity, reproducibility, limit of detection) for ICU-compatible screening.

Expected outcomes. A preclinical safety dossier, a draft CMC/release package, and a validated cytolysin qPCR—collectively supporting an FDA investigational-phage submission and a precision trial design.

Pitfalls & alternatives. If a single fixed formulation is constrained by strain diversity, we will define a controlled, specification-bound multi-component strategy. **Scientific-premise risk:** cytolysin-positive prevalence and its prognostic weight may be cohort- and geography-dependent, and some populations may show lower incidence; this is precisely why the qPCR-based enrichment is integral rather than optional. Multi-site specimen screening will establish a feasible addressable population and directly test premise robustness.

Timeline

[ILLUSTRATIVE] Year 1: Aim 1 strain/phage library and host-range mapping; cocktail lock (go/no-go #1). Years 1–3: Aim 2 efficacy, biodistribution, and resistance studies (go/no-go #2 on efficacy). Years 2–4: Aim 3 safety/toxicology and CMC. Years 3–5: cytolysin qPCR validation, dossier assembly, and pre-IND/FDA engagement.

Budget Justification (modular R01-style sketch)

[ILLUSTRATIVE] Modular direct costs of \$250,000/year [ILLUSTRATIVE] over 5 years [ILLUSTRATIVE]. Personnel: PI (microbiome/hepatology), Co-I (phage biology), Co-I (GI/hepatology clinician), a postdoctoral fellow, a research technician, and a part-time regulatory/CMC consultant. Supplies: phage isolation/propagation, strain-panel acquisition, sequencing, histology, immunoassays, and qPCR development. Animal costs: humanized ethanol-fed mouse cohorts (Aim 2) and toxicology (Aim 3). Other: biostatistics, pre-IND consulting, and publication. Specific dollar allocations across categories are [ILLUSTRATIVE] and will be finalized at submission.

Vertebrate Animals

Animal work is proposed in Aims 2 and 3. Humanized, ethanol-fed mouse models will test efficacy, mechanism, phage biodistribution, and safety, consistent with the humanized-mouse paradigm established by Duan et al. and Garcia Mendes et al. **Justification:** no in vitro system reproduces the gut–liver translocation axis central to this mechanism. **Minimization:** power-based group sizes, both sexes, randomization, blinded endpoints, and shared controls reduce animal use. All procedures follow an IACUC-approved protocol with appropriate housing, anesthesia/analgesia where applicable, and humane endpoints. Estimated animal numbers are [ILLUSTRATIVE] pending final power calculations.

Human Subjects / Clinical Trial

No human efficacy trial is conducted within this R01; the work is IND-enabling. Aim 3 develops a stool cytolysin qPCR using de-identified or consented clinical specimens under IRB oversight. We will engage FDA regarding the investigational-phage regulatory pathway to inform a future first-in-human, biomarker-enriched trial in cytolysin-positive severe AH patients. Any subsequent clinical study will proceed under a separate IND and IRB approval with informed consent. Enrollment figures for future trials are [ILLUSTRATIVE].

Team & Environment

- **Contact PI** — [Name, Institution] [ILLUSTRATIVE]: gut–liver axis and alcohol-associated liver disease.
- **Co-Investigator** — [Name, Institution] [ILLUSTRATIVE]: bacteriophage genomics and isolation.
- **Co-Investigator** — [Name, Institution] [ILLUSTRATIVE]: hepatology/clinical AH and ICU specimen access.
- **Regulatory/CMC consultant** — [Name] [ILLUSTRATIVE]: phage manufacturing, release testing, and FDA/IND strategy.
- **Biostatistician** — [Name] [ILLUSTRATIVE].
- **Environment** [ILLUSTRATIVE]: an institution with BSL-appropriate phage facilities, a humanized-mouse/ethanol-feeding core, GMP-aware manufacturing partners, and a hepatology service—to be populated with real names and institutions at submission.

References

1. Duan Y, Llorente C, Lang S, Brandl K, Chu H, Jiang L, et al. (incl. Fouts DE, Schnabl B). Bacteriophage targeting of gut bacterium attenuates alcoholic liver disease. *Nature*. 2019;575(7783):505–511. DOI: 10.1038/s41586-019-1742-x. <https://pubmed.ncbi.nlm.nih.gov/31723265/> · <https://www.nature.com/articles/s41586-019-1742-x>
2. Garcia Mendes B, Duan Y, Schnabl B. Immune Response of an Oral *Enterococcus faecalis* Phage Cocktail in a Mouse Model of Ethanol-Induced Liver Disease. *Viruses*. 2022;14(3):490. <https://pubmed.ncbi.nlm.nih.gov/35336897/>
3. Fujiki J, Nakamura T, Kreimeyer H, Llorente C, Fouts DE, Schnabl B. Insertion sequence-mediated phage resistance contributes to attenuated colonization of cytolytic *Enterococcus faecalis* variants in the gut. *Microbiology Spectrum*. 2025; doi:10.1128/spectrum.03303-24 (PMID: 40231830). <https://pmc.ncbi.nlm.nih.gov/articles/PMC12054073/>

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<https://phagecocktails.com/grant/steal/alcohol-hepatitis-enterococcus>