> **License: CC0 (public domain).** Fork it, rename it, submit it. Every [ILLUSTRATIVE] figure is a placeholder to replace.

A GRAS-Track, Clean-Label Phage Cocktail for Biocontrol of *Listeria*, *Salmonella*, and *E. coli* O157:H7 on Ready-to-Eat Foods and Food-Contact Surfaces

Funder: USDA-NIFA / USDA SBIR (Phase I feasibility → Phase II scale-up)

Project Summary / Abstract

Listeria monocytogenes, *Salmonella enterica*, and Shiga toxin–producing *Escherichia coli* O157:H7 are leading causes of foodborne illness, recalls, and death, and they persist precisely where conventional controls are weakest: on ready-to-eat (RTE) foods, fresh produce, raw meat, and food-contact surfaces. *L. monocytogenes* grows at refrigeration temperatures and establishes persistent biofilms in processing plants; *E. coli* O157:H7 causes severe disease at a very low infectious dose. Chemical sanitizers can be unsuitable on RTE foods, degrade sensory quality, and leave residues incompatible with clean-label and organic positioning; antibiotics are inappropriate as food antimicrobials. Lytic bacteriophages are an unusually well-matched alternative — host-specific, self-amplifying in the presence of the target, residue-free, and compatible with clean-label and organic positioning. The peer-reviewed precedent is strong and food-relevant: a *Listeria* cocktail reduced or eliminated *L. monocytogenes* on lettuce, apples, cheese, smoked salmon, and frozen foods (0.7–2.2 log; Perera et al., 2015); a six-phage cocktail reduced *Salmonella* by >1 log within 60 min in dried pet food, including naturally contaminated product (Heyse et al., 2015); and phages reduced *E. coli* O157:H7 — and, importantly, simultaneously controlled extended-spectrum β-lactamase (ESBL)–producing *E. coli* — on raw beef (Son et al., 2018; Tomat et al., 2013). This USDA SBIR project will, in **Phase I**, assemble and characterize a defined, strictly lytic multi-phage cocktail with broad strain coverage against all three pathogens and demonstrate proof-of-concept reductions on representative RTE matrices and stainless-steel surfaces; and in **Phase II**, complete matrix/biofilm validation, suppress phage-insensitive mutants by rational multi-phage design, develop a scalable low-endotoxin production process, and assemble the regulatory dossier required to commercialize a clean-label, organic-compatible antimicrobial for U.S. food producers.

Specific Aims

Foodborne *L. monocytogenes*, *Salmonella enterica*, and *E. coli* O157:H7 drive recalls and deaths and resist control on RTE foods and processing surfaces, where sanitizers and antibiotics are poorly suited. Lytic phage cocktails are host-specific, residue-free, and self-amplifying, and — uniquely among the available tools — kill by a mechanism independent of antibiotic-resistance status, so STEC O157:H7 and ESBL-producing *E. coli* are both valid targets (Son et al., 2018). Building on published cocktails and phages that reduced these pathogens on real food matrices (Perera et al., 2015; Heyse et al., 2015; Son et al., 2018; Tomat et al., 2013), we will develop and validate a defined cocktail and the package needed to commercialize it.

Aim 1 — Assemble and characterize a defined, strictly lytic phage cocktail with broad strain coverage. Compile candidate lytic phages against *L. monocytogenes*, *Salmonella enterica*, and *E. coli* O157:H7; screen host range against curated strain panels spanning relevant serovars/serotypes (including O157:H7 and ESBL-producing *E. coli*) by spot and efficiency-of-plating assays; confirm strictly lytic biology and absence of undesirable genes by whole-genome sequencing; and down-select a multi-phage cocktail balanced across distinct receptors to maximize coverage and limit cross-resistance. Define titer and stability specifications. *(Primary Phase I aim.)*

Aim 2 — Validate efficacy on representative RTE matrices and food-contact surfaces, including biofilm and BIM suppression. Quantify log reductions of each pathogen on representative matrices (e.g., leafy greens, fresh-cut produce, RTE dairy/seafood, raw meat) and on inoculated stainless-steel coupons under refrigeration and ambient conditions, benchmarked to published matrix-specific reductions (0.7–2.2 log for *Listeria*; ≤~2.3 log for *E. coli* on beef). Challenge established biofilms, and directly compare the cocktail to single phages for emergence of bacteriophage-insensitive mutants (BIMs). *(Phase I proof-of-concept; full validation in Phase II.)*

Aim 3 — Assemble the regulatory and product-readiness package for commercialization. Develop a scalable propagation/purification process yielding high-titer, low-endotoxin cocktail with release specifications (titer, endotoxin, sterility, identity); define and bench-test application formats (surface spray, carcass/produce wash, packaging coating); and compile a GRAS/food-additive regulatory dossier and FDA/USDA-FSIS engagement plan. *(Primary Phase II aim.)*

Impact: Success delivers a clean-label, organic-compatible phage antimicrobial — and the regulatory dossier and scalable process behind it — that food processors can deploy from farm to fork to reduce contamination by three of the most consequential foodborne pathogens without altering taste, texture, or organic status.

Significance

L. monocytogenes, *Salmonella enterica*, and *E. coli* O157:H7 are leading causes of foodborne illness, recalls, and death in the United States and are notoriously difficult to control across the food chain. *L. monocytogenes* uniquely grows at refrigeration temperatures and establishes persistent biofilms in processing environments, making post-process recontamination of RTE foods a recurrent problem. *E. coli* O157:H7 causes severe disease, including hemolytic-uremic syndrome, at a very low infectious dose, so even modest contamination of beef or produce is consequential. *Salmonella enterica* spans many serovars across produce, seafood, poultry, and dried products. Conventional interventions are constrained: chemical sanitizers can damage organoleptic quality, are unsuitable on many RTE foods, and leave residues incompatible with clean-label and organic positioning; antibiotics are inappropriate as food antimicrobials and increasingly compromised by resistance.

Lytic phages directly address these gaps. Their host specificity kills the target pathogen without disturbing beneficial microbiota, fermentation cultures, or sensory quality; they self-amplify while host bacteria are present; and they are residue-free and clean-label. Critically, the killing mechanism — receptor adsorption, genome injection, host takeover, and lysis — is independent of antibiotic-resistance status, so STEC O157:H7 and even ESBL-producing *E. coli* are valid targets; Son et al. (2018) demonstrated simultaneous control of O157:H7 and ESBL *E. coli* on raw beef. This is not a speculative application. The peer-reviewed record documents efficacy on real foods across all three pathogens: 0.7–2.2 log reductions of *L. monocytogenes* across lettuce, apples, cheese, smoked salmon, and frozen foods, including complete elimination on commercial smoked salmon (Perera et al., 2015); >1 log reduction of *Salmonella* within 60 min in dried pet food, including naturally contaminated product, using a six-phage cocktail (Heyse et al., 2015); and reductions of *E. coli* O157:H7 on beef — up to ~2.3 log at ambient temperature and ~0.9 log under refrigeration with a single phage (Son et al., 2018), and 0.67–1.15 log with a two-phage set, with low BIM emergence frequencies of 6.5×10^{-7}–1.8×10^{-6} (Tomat et al., 2013). A defined, GRAS-track cocktail unifying all three targets, with documented matrix and surface efficacy and engineered-in resistance management, would give U.S. producers a practical, residue-free tool that reduces recalls and illness while preserving clean-label and organic claims.

Innovation

The innovation is translational and integrative rather than a claim of unprecedented biology.

- **One program, three priority pathogens, one regulatory framework.** We unify control of *Listeria*, *Salmonella*, and *E. coli* O157:H7 within a single development and regulatory effort rather than treating them as three separate products.
- **Direct engineering against the two dominant real-world failure modes.** We pair food-

matrix efficacy with explicit, quantitative attention to (i) biofilm persistence — the niche where *Listeria* survives sanitation — and (ii) BIM emergence, which we suppress through rational multi-phage design targeting distinct receptors. The published BIM frequencies for a two-phage set (6.5×10^{-7}–1.8×10^{-6}; Tomat et al., 2013) give us a concrete, citable baseline to beat with a larger, receptor-diverse cocktail.

- **Resistance-status-independent kill.** Because lytic killing does not depend on antibiotic susceptibility, the same product addresses both STEC O157:H7 and ESBL-producing *E. coli* (Son et al., 2018) — a dual-use benefit no chemical sanitizer offers.
- **Regulatory dossier and scalable, low-endotoxin process as primary deliverables.** We treat the GRAS/food-additive package and a manufacturable process as co-equal outputs developed alongside the science, shortening the path from validated cocktail to deployable product.

We note, without overstating, that phage-associated depolymerases and endolysins can degrade biofilm matrix and that engineered/CRISPR-armed phages are an emerging research direction; neither is on the critical path for this commercialization-focused proposal, and neither is invoked as efficacy evidence here.

Approach

Aim 1 — Assemble and characterize a defined, strictly lytic phage cocktail with broad strain coverage

Rationale. Any single phage covers only a subset of strains, and the cited beef studies that used one or two phages achieved relatively modest reductions (Son et al., 2018; Tomat et al., 2013), whereas the six-phage *Salmonella* cocktail achieved >1 log within 60 min (Heyse et al., 2015). A defined, strictly lytic, receptor-diverse cocktail with documented host-range breadth is therefore the foundation for both efficacy and resistance management.

Experimental design. Compile candidate lytic phages active against *L. monocytogenes*, *Salmonella enterica*, and *E. coli* O157:H7 from in-house collections and accessible repositories. Assemble curated bacterial panels spanning relevant serovars/serotypes, including O157:H7 and ESBL-producing *E. coli*. Score host range by spot and efficiency-of-plating assays; confirm strictly lytic lifestyle and absence of integrase, known virulence, and antibiotic-resistance genes by whole-genome sequencing and annotation. Down-select a cocktail balanced across distinct host receptors to maximize panel coverage while minimizing shared escape routes. Characterize selected phages for one-step growth, optimal multiplicity of infection, and titer stability across pH and refrigerated storage.

Expected outcomes. A defined, sequenced, strictly lytic multi-phage cocktail with high in-vitro

coverage of each pathogen panel and documented titer/stability specifications suitable for formulation.

Quantitative go/no-go (Phase I). Proceed if the cocktail covers ≥80% of each curated strain panel by efficiency-of-plating and all components are confirmed strictly lytic and free of undesirable genes.

Potential pitfalls & alternatives. If coverage gaps remain for particular serotypes, broaden sourcing and add/substitute phages. If a candidate shows lysogeny markers or undesirable genes, exclude it in favor of strictly lytic alternatives, consistent with food-safety expectations.

Aim 2 — Validate efficacy on representative RTE matrices and food-contact surfaces, including biofilm and BIM suppression

Rationale. Regulatory and commercial credibility rests on reductions on the actual foods and surfaces where these pathogens occur. Published reductions are matrix-dependent — 0.7–2.2 log for *Listeria* across five matrices (Perera et al., 2015) and ≤~2.3 log for *E. coli* on beef (Son et al., 2018; Tomat et al., 2013) — and biofilms and BIMs are the principal real-world challenges.

Experimental design. Surface-inoculate representative matrices (e.g., leafy greens, fresh-cut produce, RTE dairy and seafood, raw meat) with each target pathogen; treat by spray/dip; enumerate survivors over storage at refrigeration and ambient temperatures with appropriate controls and replication. In parallel, treat and enumerate inoculated stainless-steel coupons (food-contact-surface model). Grow established biofilms on coupons and challenge with the cocktail to assess matrix penetration and viable-cell reduction. Quantify BIM emergence by recovering survivors and testing residual phage susceptibility, comparing the cocktail head-to-head with single phages against the Tomat et al. (2013) baseline frequencies.

Expected outcomes. Matrix- and temperature-specific reductions consistent with or exceeding published benchmarks, measurable biofilm reduction, and markedly lower BIM emergence with the cocktail than with single phages.

Quantitative go/no-go. Phase I proof-of-concept advances on ≥1 log reduction on at least one food matrix and one surface for each pathogen, with cocktail BIM frequency below the single-phage comparator. Phase II targets reductions at or above the matrix-specific published values and validated biofilm reduction.

Potential pitfalls & alternatives. Matrix effects (surface topology, fat, low temperature — the same factors that lowered beef and refrigerated reductions in the cited studies) may attenuate efficacy; optimize titer, contact time, and reapplication, and prioritize matrices with the strongest published precedent. If biofilm penetration is limited, evaluate higher doses and, as a research-stage adjunct

only, endolysin/depolymerase-based matrix degradation.

Aim 3 — Assemble the regulatory and product-readiness package for commercialization

Rationale. A commercialization-ready dossier plus a scalable, low-endotoxin process is what converts validated efficacy into a marketable product and de-risks Phase II partnering and sale.

Experimental design. Develop a scalable propagation and purification process yielding high-titer, low-endotoxin cocktail, with release specifications (titer, endotoxin, sterility, identity). Define and bench-test application formats: surface spray for RTE foods and produce, carcass/produce wash, and incorporation into food-packaging films/coatings. Compile a GRAS/food-additive dossier (identity, manufacturing, specifications, safety rationale, intended uses and use levels) and a regulatory engagement plan covering FDA and, as applicable for meat and poultry, USDA-FSIS.

Expected outcomes. A complete draft regulatory dossier, a documented scalable process with release specifications, and validated application formats — the package required for a Phase II commercialization plan and regulatory engagement.

Potential pitfalls & alternatives. If endotoxin or yield targets are not met, optimize host strain and purification (e.g., additional polishing steps). If a specific intended use needs additional data, scope targeted studies and pursue use-specific GRAS notifications.

Timeline

All durations [ILLUSTRATIVE]. **Phase I (months 0–9 [ILLUSTRATIVE]):** Aim 1 cocktail assembly and characterization (months 0–6 [ILLUSTRATIVE]); initial Aim 2 matrix/surface proof-of-concept and BIM assays (months 4–9 [ILLUSTRATIVE]); go/no-go on ≥80% panel coverage and ≥1 log reductions. **Phase II (months 10–33 [ILLUSTRATIVE]):** full Aim 2 matrix/surface/biofilm validation (months 10–24 [ILLUSTRATIVE]); Aim 3 scale-up, specifications, and GRAS/food-additive dossier (months 12–33 [ILLUSTRATIVE]); application-format testing and commercialization planning (months 24–33 [ILLUSTRATIVE]).

Budget Justification (modular sketch)

All figures [ILLUSTRATIVE] and structured as modular direct-cost estimates; final figures will conform to USDA SBIR Phase I/II caps and terms. **Phase I:** ~~$275,000 [ILLUSTRATIVE] total over 9 months [ILLUSTRATIVE]. Personnel ($160,000 [ILLUSTRATIVE]): PI/principal scientist, one~~

~~microbiologist, part-time regulatory consultant. Supplies (~~$70,000 [ILLUSTRATIVE]): bacterial panels, phage propagation, food matrices, stainless-steel coupons, media, sequencing. Other (~$45,000 [ILLUSTRATIVE]): endotoxin/quality assays and indirect costs per USDA SBIR terms. **Phase II:** ~$1,000,000 [ILLUSTRATIVE] over ~24 months [ILLUSTRATIVE]: expanded personnel (process/QA, food scientists), pilot-scale production and purification, full matrix/biofilm validation, packaging-format work, and regulatory dossier preparation. Equipment is requested only as needed for scale-up and itemized in Phase II. No funds are budgeted for animal or human studies in the core plan (see below).

Vertebrate Animals

Not applicable. The core program is conducted in vitro and on food matrices and food-contact surfaces and proposes no vertebrate animal experiments. (The cited *Salmonella* precedent, Heyse et al., 2015, is itself an in-vitro dried-pet-food study and likewise involved no live-animal work.) Should any in-vivo food-animal validation be added in Phase II, it would be introduced under a separate amendment with full IACUC review, justification of species and numbers, and humane endpoints.

Human Subjects / Clinical Trial

Not applicable. This is a food-biocontrol product evaluated on foods and surfaces, not a human therapeutic; no human subjects are enrolled. This USDA SBIR program proceeds under the FDA food-additive/GRAS framework (and USDA-FSIS for meat and poultry applications), not as a clinical investigation.

Team & Environment

Template roles to be completed with real names, institutions, and biosketches.

- **Principal Investigator (Small Business) — [Name], [Company]:** phage biology and food microbiology; overall scientific and commercialization lead.
- **Co-Investigator / Food Microbiologist — [Name], [Institution]:** matrix and surface challenge studies, biofilm models.
- **Process / Manufacturing Lead — [Name], [Company]:** scalable propagation, purification, endotoxin/QA specifications.
- **Regulatory Affairs Consultant — [Name], [Firm]:** GRAS/food-additive dossier, FDA engagement, and USDA-FSIS coordination as applicable.
- **Academic Collaborator(s) — [Name], [University]:** strain panels and independent

validation.

- **Environment:** small-business laboratory with BSL-2 capability for foodborne pathogens; phage production and titration; food-handling and pilot-scale processing space; and access to sequencing and analytical/QA services. Aligned non-dilutive funders for related efforts include FDA (food safety) and USDA-NIFA programs.

References

1. Perera MN, Abuladze T, Li M, Woolston J, Sulakvelidze A. Bacteriophage cocktail significantly reduces or eliminates *Listeria monocytogenes* contamination on lettuce, apples, cheese, smoked salmon and frozen foods. *Food Microbiology.* 2015;52:42–48. https://pubmed.ncbi.nlm.nih.gov/26338115/
2. Heyse S, Hanna LF, Woolston J, Sulakvelidze A, Charbonneau D. Bacteriophage Cocktail for Biocontrol of *Salmonella* in Dried Pet Food. *Journal of Food Protection.* 2015;78(1):97–103. https://pubmed.ncbi.nlm.nih.gov/25581183/
3. Son HM, Duc HM, Masuda Y, Honjoh KI, Miyamoto T. Application of bacteriophages in simultaneously controlling *Escherichia coli* O157:H7 and extended-spectrum beta-lactamase producing *Escherichia coli*. *Applied Microbiology and Biotechnology.* 2018;102(23):10259–10271. https://pubmed.ncbi.nlm.nih.gov/30267128/
4. Tomat D, Migliore L, Aquili V, Quiberoni A, Balagué C. Phage biocontrol of enteropathogenic and Shiga toxin-producing *Escherichia coli* in meat products. *Frontiers in Cellular and Infection Microbiology.* 2013;3:20. https://pubmed.ncbi.nlm.nih.gov/23761050/

PhageCocktails — “Steal This Grant.” CC0 / public domain. Figures marked [ILLUSTRATIVE] are placeholders.