Supplementary Materials for

Systemic 5-fluorouracil drives competitive release of multidrug-resistant, virulent *Enterococcus faecalis* lineages in the gut

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The PDF file includes:

Figs. S1 to S6

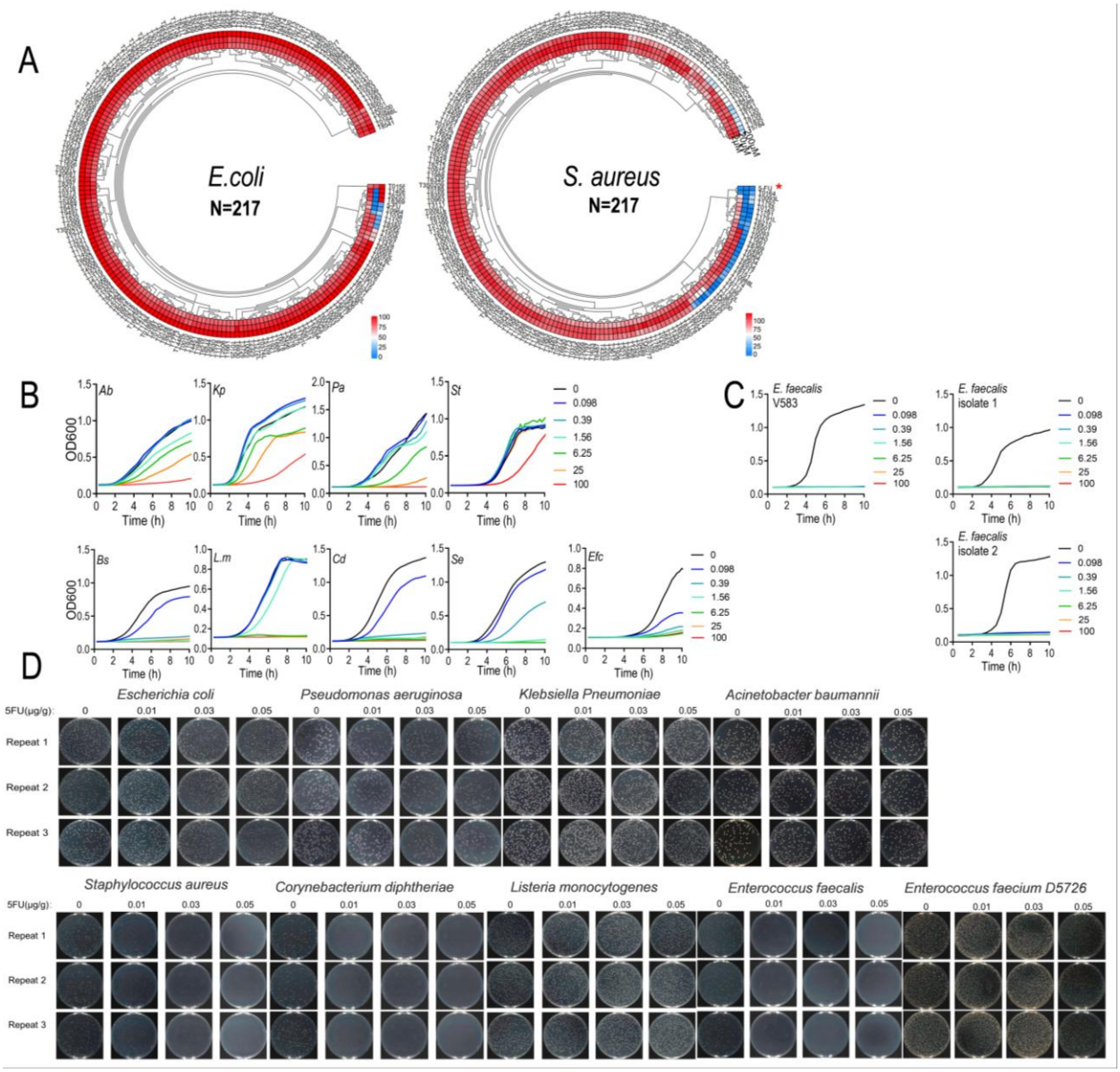
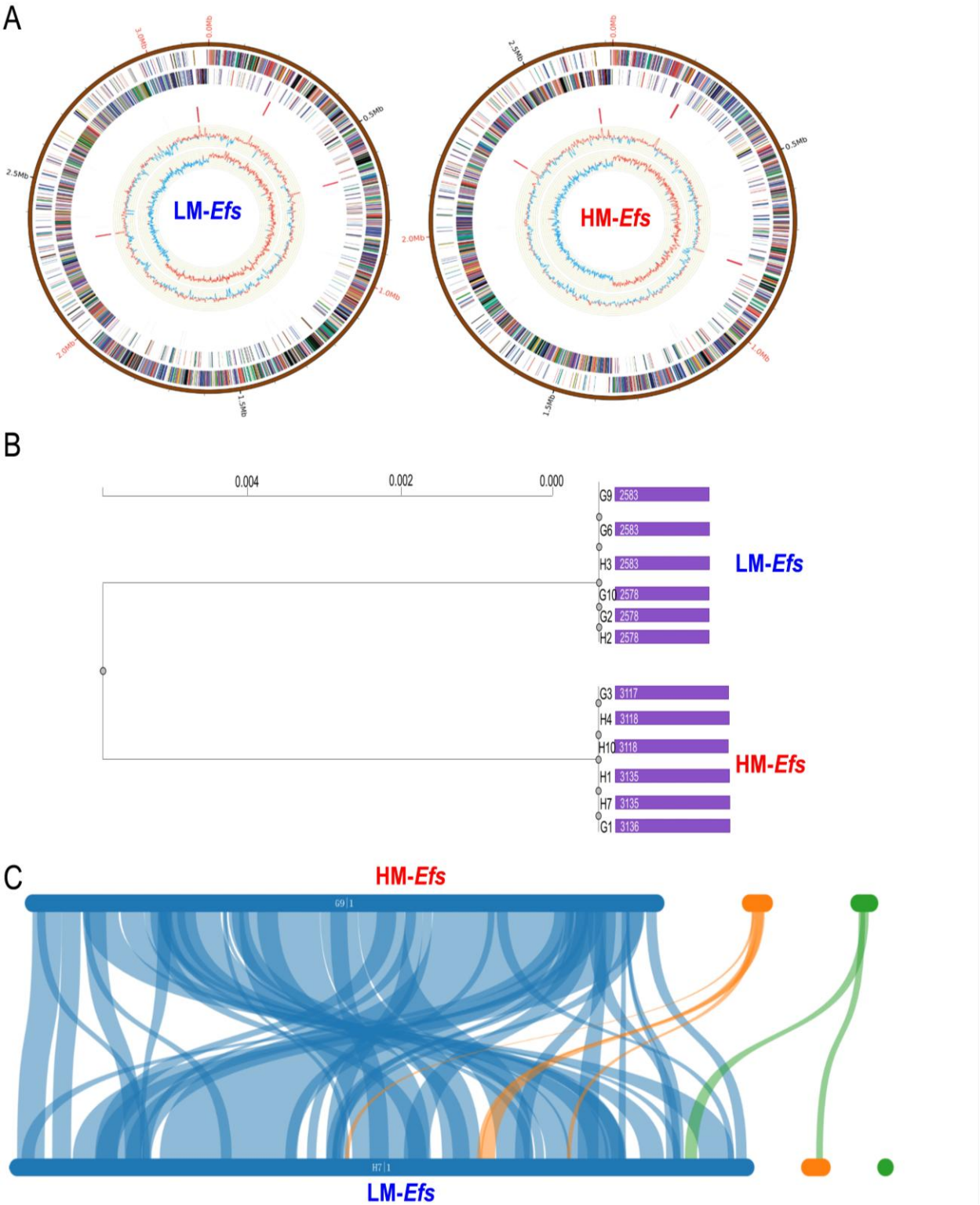


Fig. S1.

5FU exhibits antibacterial activity across diverse bacterial species and induces dose-dependent growth inhibition. (A) Screening of an FDA-approved anticancer compound library (217 compounds) against *E. coli* and *S. aureus*, showing growth inhibition profiles. **(B)** Dose-dependent growth inhibition by 5FU across multiple bacterial species. **(C)** 5FU induces complete growth inhibition across diverse *E. faecalis* clinical isolates at all concentrations tested. **(D)** Antibacterial activity of 5FU at fecal-relevant concentrations. Representative plate images showing growth inhibition of indicated bacterial species, including *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *A. baumannii*, *S. aureus*, *C. diphtheriae*, *L. monocytogenes*, *E. faecalis*, and *E. faecium*.


A
LM-Efs
HM-Efs
B
0.004
0.002
0.000
G9 2583
G6 2583
H3 2583
G10 2578
G2 2578
H2 2578
G3 3117
H4 3118
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G1 3136
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C
HM-Efs
G9 | 1
H7 | 1
LM-Efs

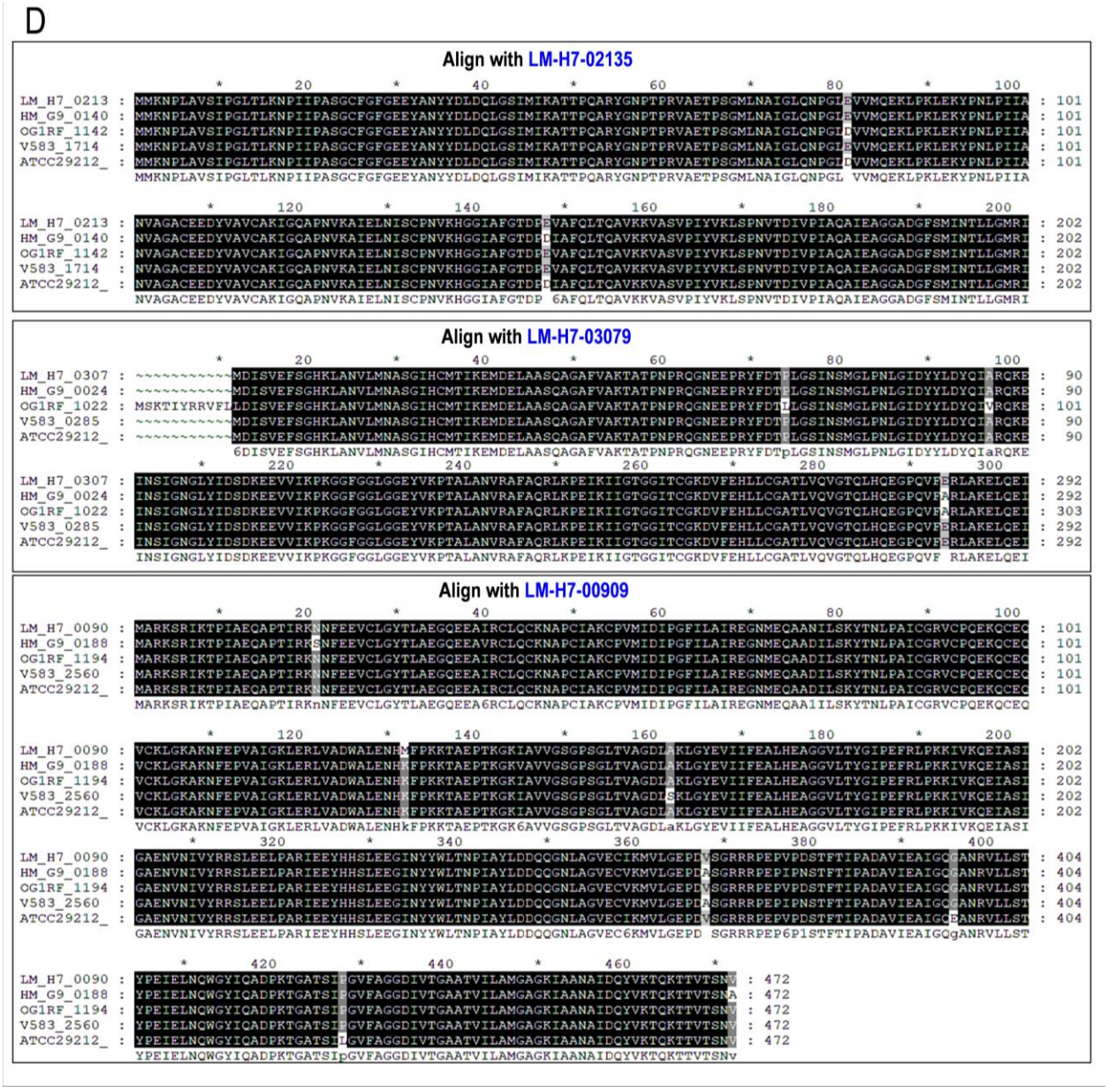

Fig. S2.

Genomic and phenotypic characterization of LM-*Efs* and HM-*Efs*. (A) Circular genome maps of LM-*Efs* and HM-*Efs* strains, showing gene annotations, GC content (inner tracks; blue/red), and orthologous gene clusters (outer track), highlighting conserved and divergent genomic features. **(B)** Phylogenetic relationships between LM-*Efs* and HM-*Efs* strains based on whole-genome analysis. **(C)** Genome-wide synteny analysis between LM-*Efs* (bottom) and HM-*Efs* (top) strains, illustrating conserved genomic organization. **(D)** Sequence alignment of PreA and PreT homologs identified in LM-*Efs*, HM-*Efs*, and reference strains (V583, OG1RF, and ATCC29212).

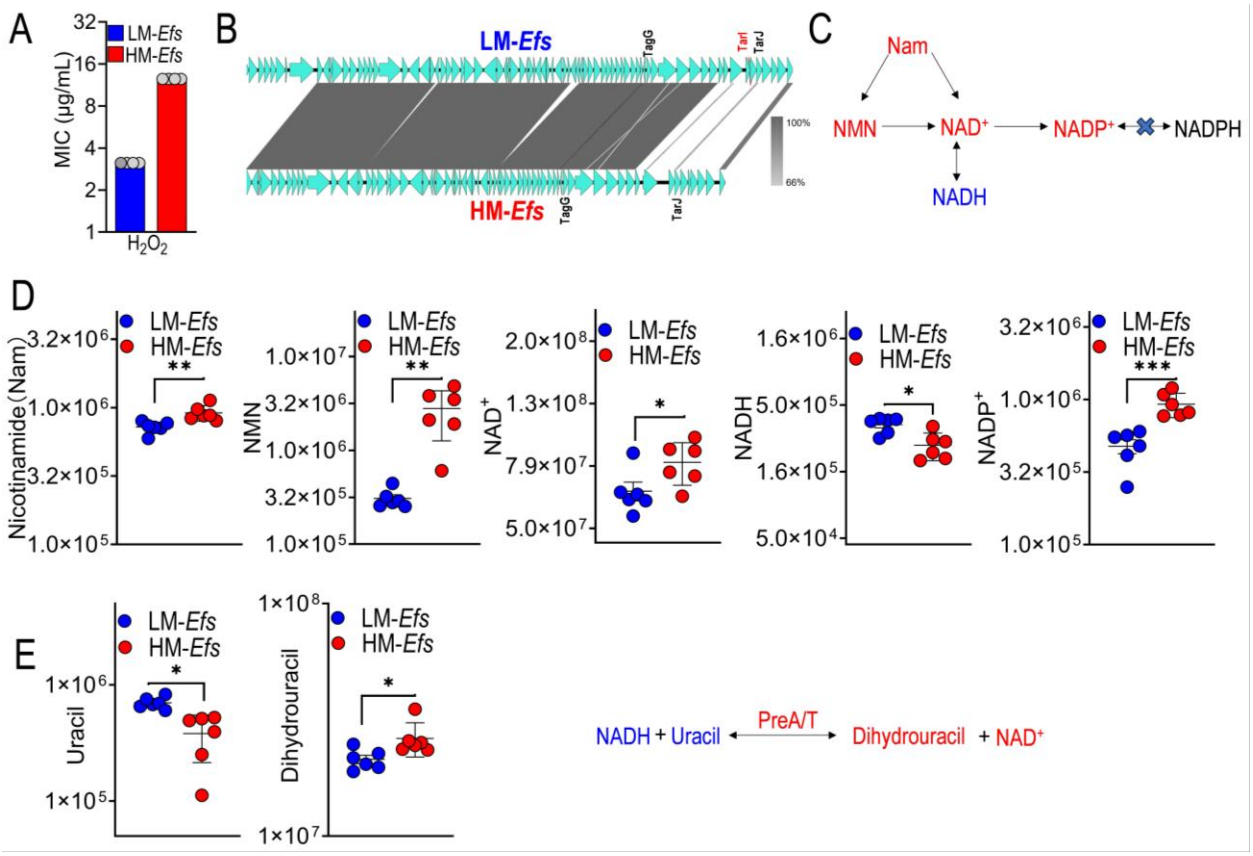

Fig. S3.

HM-*Efs* strains exhibit reduced oxidative stress sensitivity and altered redox metabolism. (A) Susceptibility to oxidative stress. MICs of hydrogen peroxide (H_2O_2) were determined for LM-*Efs* and HM-*Efs* strains. **(B)** Comparative genomic analysis identifies loss of the *tarI* gene in HM-*Efs*. **(C)** Schematic representation of the NAD^+ salvage pathway. **(D)** Abundances of nicotinamide (Nam), nicotinamide mononucleotide (NMN), NAD^+, NADH, and $NADP^+$ in LM-*Efs* and HM-*Efs* strains. **(E)** Abundances of two pyrimidine metabolites, dihydrouracil and uracil, in LM-*Efs* and HM-*Efs*. Data are presented as mean ± SEM. Significant differences (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$) were identified in D and E by two-tailed Student's *t* test.

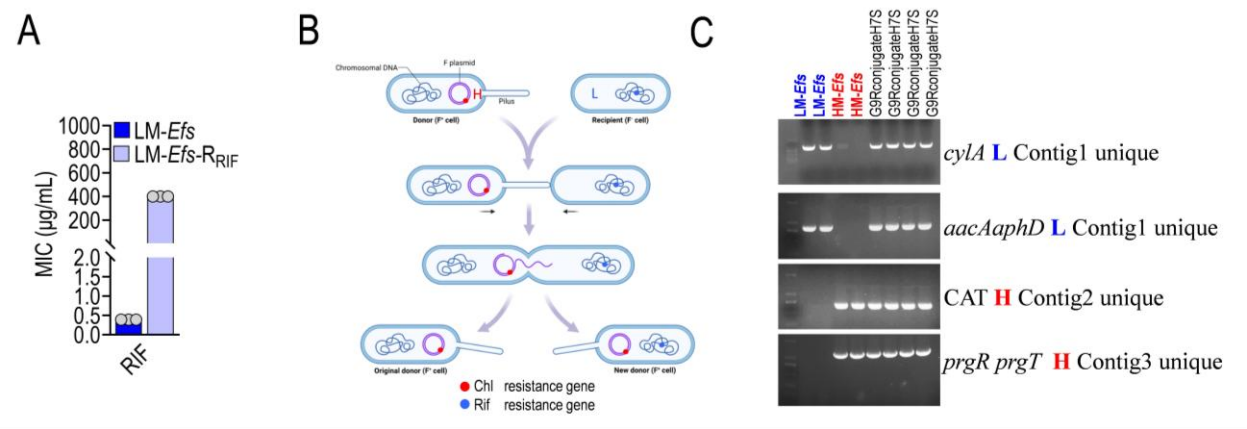

Fig. S4.

Validation of plasmid-mediated transfer. (A) Rifampicin susceptibility of recipient *E. faecalis* (LM-Efs) before and after serial passage. MICs (µg/mL) show a marked increase in rifampicin resistance following passage. **(B)** Schematic illustration of plasmid-mediated conjugation. **(C)** PCR-based validation of transconjugants.

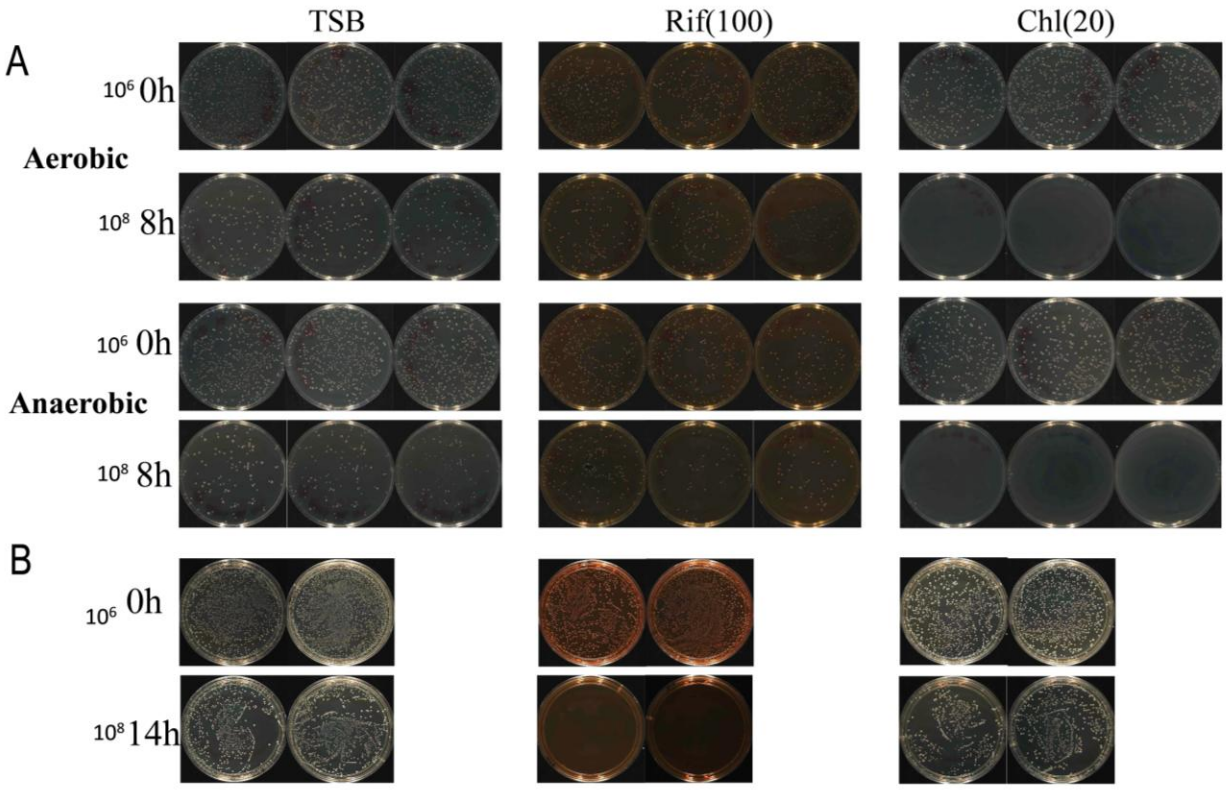

Fig. S5.

Colony counting assays validate competitive dynamics between LM-*Efs* and HM-*Efs*. (A) Representative CFU plates corresponding to the competition assays shown in Fig. 5D. **(B)** CFU plates corresponding to population dynamics analyses presented in Fig. 5G.

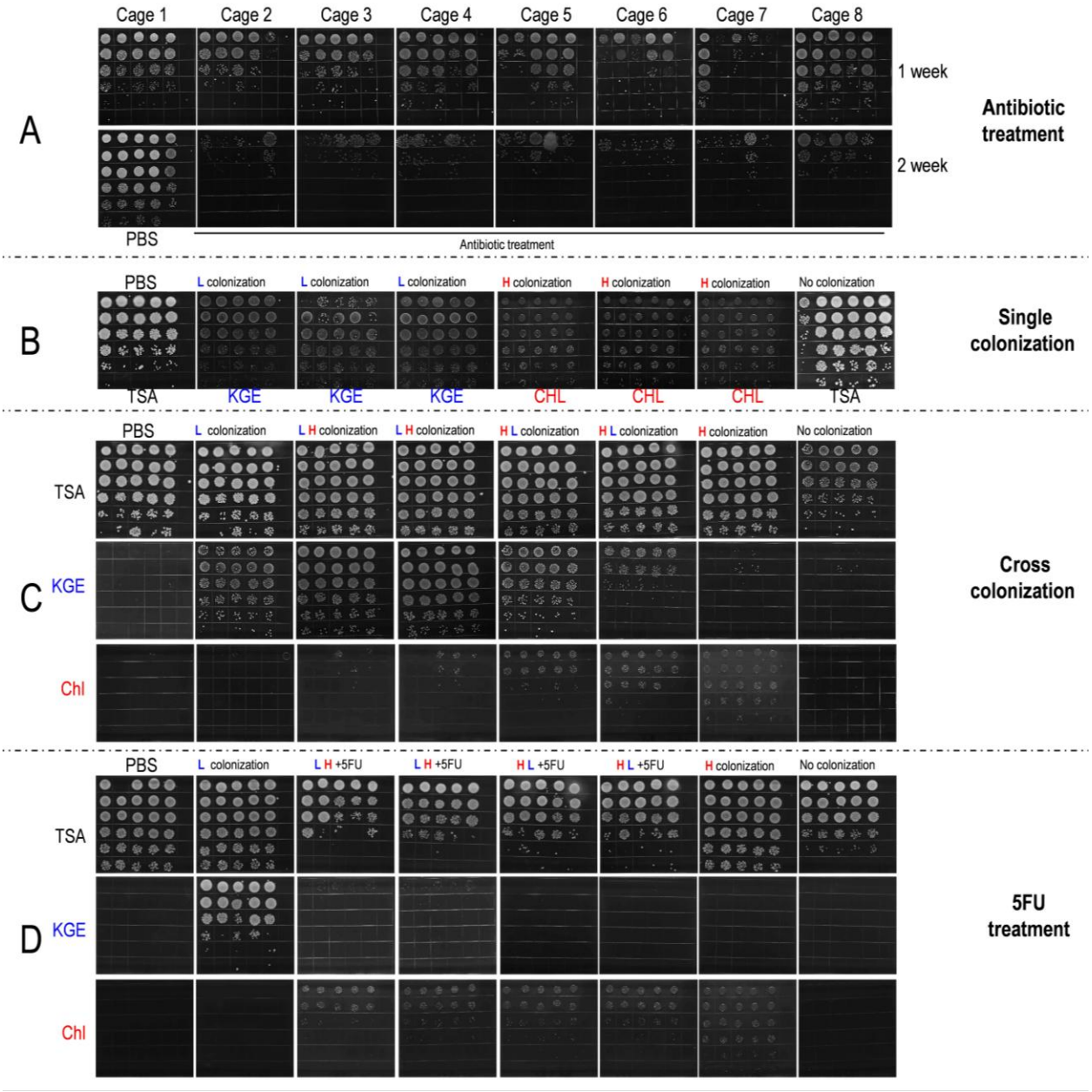

Fig. S6.

Bacterial colonization dynamics in murine fecal samples following antibiotic perturbation and 5FU exposure. A total of eight cages (five mice per cage) were used. One cage served as a PBS control without antibiotic treatment and bacterial colonization. The remaining seven cages received antibiotic pretreatment; among these, one cage was not colonized, while six cages were divided into two groups for single-strain colonization (LM-*Efs* or HM-*Efs*; three cages per group). Within each group, two cages underwent sequential colonization by gavage with the reciprocal strain, whereas one cage remained singly colonized. CFUs recovered from fecal samples indicate competitive establishment between resident and subsequently introduced strains. **(A)** Depletion of gut bacteria following antibiotic pretreatment. CFU (per g feces) measured after 2 weeks of antibiotic treatment demonstrate effective reduction of culturable bacteria compared to PBS-

treated controls. **(B)** Colonization following single-strain inoculation. CFUs recovered from mice colonized with LM-*Efs* or HM-*Efs* inocula, plated on non-selective (TSA) or selective media (KGE, containing kanamycin, gentamicin, and erythromycin, for LM-*Efs*; and CHL, chloramphenicol, for HM-*Efs*). **(C)** Sequential colonization model. Mice were first gavaged with one strain (LM-*Efs* or HM-*Efs*), followed by a second gavage with the reciprocal strain. CFUs recovered from fecal samples indicate competitive establishment between pre-colonized and subsequently introduced strains. **(D)** Impact of 5FU treatment on colonization dynamics. CFUs recovered from fecal samples following 5FU administration demonstrate shifts in population composition and colonization levels.