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A bacteriophage cocktail targeting *Escherichia coli* reduces *E. coli* in simulated gut conditions, while preserving a non-targeted representative commensal normal microbiota

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ABSTRACT

Antibiotics offer an efficient means for managing diseases caused by bacterial pathogens. However, antibiotics are typically broad spectrum and they can indiscriminately kill beneficial microbes in body habitats such as the gut, deleteriously affecting the commensal gut microbiota. In addition, many bacteria have developed or are developing resistance to antibiotics, which complicates treatment and creates significant challenges in clinical medicine. Therefore, there is a real and urgent medical need to develop alternative antimicrobial approaches that will kill specific problem-causing bacteria without disturbing a normal, and often beneficial, gut microbiota. One such potential alternative approach is the use of lytic bacteriophages for managing bacterial infections, including those caused by multidrug-resistant pathogens. In the present study, we comparatively analysed the efficacy of a bacteriophage cocktail targeting *Escherichia coli* with that of a broad-spectrum antibiotic (ciprofloxacin) using an *in vitro* model of the small intestine. The parameters examined included (i) the impact on a specific, pre-chosen targeted *E. coli* strain, and (ii) the impact on a selected non-targeted bacterial population, which was chosen to represent a defined microbial consortium typical of a healthy small intestine. During these studies, we also examined stability of bacteriophages against various pH and bile concentrations commonly found in the intestinal tract of humans. The bacteriophage cocktail was slightly more stable in the simulated duodenum conditions compared to the simulated ileum (0.12 vs. 0.58 log decrease in phage titers, respectively). It was equally effective as ciprofloxacin in reducing *E. coli* in the simulated gut conditions (2–3 log reduction), but had much milder (none) impact on the commensal, non-targeted bacteria compared to the antibiotic.

1. Introduction

Antibiotics are a type of antimicrobial drug used for either killing (*bactericidal* antibiotics; e.g., vancomycin) or inhibiting the growth (*bacteriostatic* antibiotics; e.g., tetracycline) of microorganisms. The discovery of antibiotics in the first half of the 20th century revolutionized medicine as it allowed treatment of bacterial infections that were essentially untreatable during the pre-antibiotic era; in addition to saving countless lives, it also fostered the development of many sophisticated medical procedures (e.g., organ transplantation surgeries) that were previously not possible due to, in part, the inability to manage bacterial infections that are unavoidable during such surgical procedures. However, extensive use (and

sometimes misuse) of antibiotics since their discovery has also led to an emergence of antibiotic-resistant bacteria – bacteria that cannot be killed by commonly available antibiotics, and in some cases by any available antibiotics; e.g., multiple drug resistance (MDR), extensively drug resistant (XDR), and pan-drug-resistant (PDR) bacteria.

This emergence of drug-resistance is a very serious problem in modern medicine, which imposes a significant social and economic burden on the society, including the loss of lives.[1,2] The problem is further exacerbated by the fact that most antibiotics are broad-spectrum. In practice this means that in addition to targeting disease-causing bacteria, antibiotic therapy can cause substantial collateral damage to the

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human microbiota by killing many other, non-targeted, and often beneficial bacteria. This collateral effect can, and often does, lead to dysbiosis,[3,4] further promoting the emergence of resistant bacteria (via selective pressure), and may also facilitate horizontal transfer of resistance genes.[5,6] Moreover, growing evidence suggests that microbiota disturbance caused by antibiotics may promote various other health problems such as obesity, asthma, inflammatory bowel disease, and diabetes.[1-4] Therefore, there is an increasing interest to manage infections caused by antibiotic-resistant pathogens by selectively targeting the disease-causing bacteria, without disturbing the commensal microbiota of the human gastrointestinal (GI) tract. One intriguing concept in that regard is the use of lytic bacteriophages, to selectively and specifically kill disease-causing bacteria, including MDR, XDR, and PDR.

Bacteriophages (or "phages" for short) are bacteria-infecting viruses. Lytic phages have potent bactericidal activity against their host bacterial strains. During the lytic cycle the phage infects the cell, using the cell's replication and translation machinery to replicate and then lyses the cell releasing new phage particles into the environment. In cases where overwhelming concentrations of phage are applied "lysis from without" might occur as well.[7,8] Phages are also very specific: they only attack their targeted bacterial hosts, and they cannot infect human or other eukaryotic cells. Even within bacterial taxa, and in clear contrast to broad-spectrum antibiotics, phages usually only lyse strains or a subgroup of strains within the bacterial species, making targeted bacterial therapy possible.

Increased presence of Adherent-Invasive *Escherichia coli* in the ileum has been associated with Ileal Crohn's disease (ICD)[9-11] and ICD-associated *E. coli* has been found to manifest multidrug resistance.[12] Therefore, phages targeting these bacteria might be an alternative for antibiotics and have potential therapeutic ability in ICD management. In order to lyse their targeted bacteria in the small or large intestine, orally-administered phages must pass through the harsh environment of the human GI tract, including the low pH in the stomach, presence of pancreatic enzymes, and bile salts in the small intestine.[13-15] These factors may reduce phage viability/stability and render them less effective or ineffective; yet, despite the long history of therapeutic use of phages in humans, there is striking paucity of information on the pharmacokinetics of bacteriophages when they are administered orally.[16-18] Thus, the goal of this study was to start to elucidate the persistence of bacteriophages in the human GI tact, by testing a phage cocktail targeting *E. coli* in a simulated human small intestinal model system. During these studies, we also compared the impact of the phage cocktail vs. a commonly used broad-spectrum antibiotic, ciprofloxacin, on the levels of *E. coli*, and other selected (non-targeted, representative of the "normal" microbiota) bacteria in the simulated small intestine system.

2. Materials and methods

2.1. Small intestine model system: Consortium of microorganisms and growth conditions

To simulate a normal, healthy, small intestine microbiome, a consortium of 7 bacterial species were selected to represent a healthy ileal microbiota[19,20] (Table 1). All bacteria were acquired from the German Collection of Microorganisms and Cell cultures (DSMZ).[21] Strains were propagated in Gifu Anaerobic Medium (GAM Broth, NISSUI) in an anaerobic bench (CoyLab, USA), under strictly anaerobic conditions using an Anaerobic jar (Merck) together with the AnaeroGen system (Oxoid). Before each experiment, all strains were cultured separately in 10 ml GAM broth for the incubation time optimal for each individual strain according to.[23] After propagation, bacterial cells were centrifuged for 2 minutes at 4,000 *g*, the

Table 1. Bacterial strains, their source, culturing time and range on growth media. Taken from {Cieplak:Rpap5xgx}.

Species	Strain source	Origin	Culture time [h]	Culture media
Escherichia coli	DSM 1058	Human intestine	24	VRB, MCC, GAM
Streptococcus salivarius	DSM 20560	Blood	6	M17, GAM
Streptococcus luteinensis	DSM 15350	Human isolate	24	M17, GAM
Enterococcus faecalis	DSM 20478	Human faeces	24	MCC, GAM
Bacteroides fragilis	DSM 2151	Appendix abscess	24	GAM
Veillonella parvula	DSM 2008	Human intestine	48	GAM
Flavonifractor plautii	DSM 6740	Human faeces	48	GAM

VRB: Violet Red Bile Agar, M17: M17 Agar, MCC: MacConkey Agar and GAM: Gifu Anaerobic Agar.

supernatant was discarded, and precipitated bacteria were resuspended in volumes of PBS (pH 7.4) adequate to obtain a final concentration of ca. 10^8 CFU/ml for each strain. After adjusting concentrations, all strains were mixed in an equal ratio and either used immediately or stored in liquid nitrogen until future use.

2.2. Bacteriophage preparation

For this pilot study, we specifically formulated a bacteriophage preparation to target one strain from the representative ileal consortium: *E. coli* DSM 1058. For the phage cocktail, individual monophages were selected from Intralytix's bacteriophage collection, based on their ability to lyse the DSM 1058 strain in the classical Spot Test assay.[22] Various dilutions were used for the spot test to differentiate between lysis and inhibition (i.e., low dilutions resulting in plaques were tested as well). The resulting susceptibility data were analyzed using the PhageSelector[TM] program (proprietary program developed by Intralytix) to formulate bacteriophage cocktail Ec17B153DK1. The resulting cocktail is composed of 3 phages (ECML-363, ECML-122 and ECML-359) each having potent lytic activity against *E. coli* DSM 1058. Each component monophage was

propagated separately in their respective *E. coli* host strains at 37°C, with Multiplicity of infections (MOIs) ranging from 2×10^{-4} to 1×10^{-1}. Following propagation, each phage was harvested by filtering through a 0.2-micron filter and concentrated/buffer exchanged in a 0.85% saline solution. Following buffer exchange, the three monophages were combined in approximately equal concentrations to produce the phage cocktail Ec17B153DK1. The final cocktail was then sterile filtered through a 0.2-micron filter and stored refrigerated (2–8°C) until use.

The ability of each of the three phages included in the Ec17B153DK1 phage cocktail, and the phage cocktail itself, was also tested against an additional 607 *E. coli* strains (Fig. 1) and against the 6 non-*E. coli* strains (Table 1) used in our model system.

2.3. Small intestine in vitro simulation

To simulate passage of phages through the human small intestine (SI), we used a newly developed dynamic *in vitro* model (TSI).[23] The TSI consists of 5 reactors with working volumes of 12 ml each. Each reactor simulates the small intestine of one individual. Parameters like pH, temperature, and bile salts concentration and pancreatic juice were monitored and
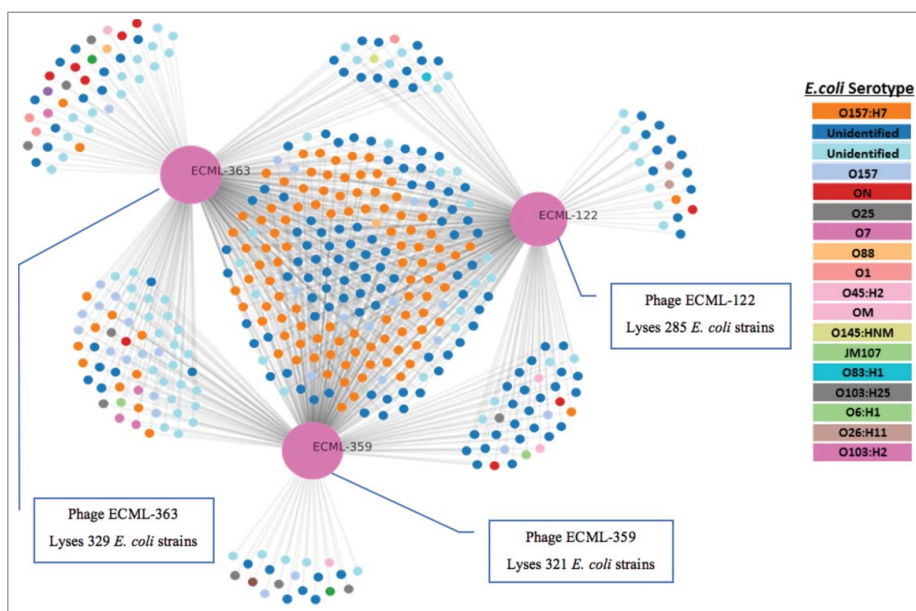
Figure 1. Host range of the Ec17B153DK1 component phages against 408 *E. coli* isolates. Host range and interaction network of the three monophages (ECML-122, ECML-363, ECML-359) included the Ec17B153DK1 phage cocktail. Phages are depicted as large pink circles. Results shown are for 408 susceptible *E. coli* strains (i.e., 199 resistant strains were not included in the chart for simplicity). Each small circle is an *E. coli* isolate. The gray lines connecting each phage to bacterial strains indicate the ability to infect and kill that given *E. coli* strain based on the Spot Test assay. Bacterial strains killed by more than one phage are grouped towards the canter of the chart. The graph was generated using proprietary PhageSelector[TM] program developed by Intralytix, Inc.

maintained at physiologically relevant levels during simulations (4–10 mM bile salts; 40 – 100 U/ml pancreatic juice). Both bile salts and pancreatic juice originated from pigs. Reactors were prepared and run according to.[23] Briefly, before addition of phages to the reactors, the pH was adjusted and confirmed to be 6.5 at which point 0.5 ml of the phage cocktail (10.81 log PFU/ml) or controls were added to the simulated small intestines. From there on the whole experiment is divided into 3 stages: duodenum, jejunum and ileum, each of them characterized by different conditions. During duodenum passage (2 hours), pH is elevated from 6.5 to 6.8 and pancreatic juice and bile salts are administrated. During the jejunum stage (4 hours), pH is raised from 6.8 to 7.2, while bile salts and small nutrients are re-absorbed. Finally, during the ileum stage the small intestine microbial consortium / E. coli suspension is added and pH is stabilized at 7.2 during 2 hours as described in detail in.[23]

2.4. Bacteriophages persistence and impact on E. coli during small intestine passage

To test the persistence of the phage cocktail during small intestinal transit, and its efficacy against the targeted E. coli strain, four TSI reactors were inoculated with 0.5 ml of the bacteriophage cocktail (10.81 log PFU/ml), in the last reactor we added saline solution (0.5 ml, 0.9% NaCl) as control. Simulated small intestinal digestion was carried out as described in.[23] Before the ileal step 1 ml of E. coli suspension (7 log CFU/ml), propagated in a similar manner as described in section 3.1. was added into each reactor. Persistence of the phage cocktail was tested before the duodenum (baseline), after duodenum and after the ilium stage by determining plaque forming units (PFU) counts[24] in an E. coli host strain grown on VRB (Violet Red Bile Agar, Sigma-Aldrich) selective agar media. Efficiency of phage cocktail against E. coli was quantified at the end of the ileal stage by comparing bacteria counts before and after the ileal stage in phage-treated vs. untreated control samples, via colony plate counts on VRB agar medium.

2.5. Impact of bacteriophage cocktail versus ciprofloxacin on the microbiota

To compare the impact of the bacteriophage cocktail vs. an antibiotic on the ileal microbiota, a mixture of 7 bacterial strains of various spp. (Table 1) was co-incubated with the phage cocktail, the antibiotic (ciprofloxacin), or water (control), and the impact of each treatment on the overall bacterial counts was determined by plating on selective and non-selective substrates. Experiments were conducted for each preparation and feeding condition in duplicate. Briefly, 0.5 ml of phage cocktail (10.81 log PFU/ml) was added in two reactors and 0.5 ml of water was added to the other three reactors, of which two were also supplemented with 1 ml of ciprofloxacin (500 mg/l in final solution). Before the ileal phase of the experiment, 1 ml of microbial consortia (see Table 1), and 3 ml of fresh SIF consisting of bile salts and pancreatic juice were added to each reactor. After the experiment, samples were taken from each reactor and plated on the following 4 media, followed by incubation at 37 °C for 24 h: (1) VRB for enumeration of E. coli, (2) M17 Agar (Oxoid) for enumeration of Streptococcus sp., (3) MacConkey Agar (MCC, Sigma-Aldrich) for enumeration of Enterococcus feacalis, and (4) Gifu Anaerobic Agar (GAM, NISSUI) where all microbes from the small intestine consortium (Table 1) can grow.

2.6. Statistical analysis

All statistical analyses were performed using the Prism 7 v 7.0b software (GraphPad). Differences between groups were calculated using one-way ANOVA with Tukey's multiple comparisons test, with a single pooled variance. Significance was determined at $P < 0.05$ level.

3. Results

3.1. Bacteriophage preparation: Target range and specificity

Bacteriophage cocktail Ec17B153DK1 was specifically formulated for this study. It was specifically designed to lyse E. coli DSM 1058, which is one of 7 strains included in the small intestine model consortia. Each of the three phages included in the Ec17B153DK1 cocktail was capable of lysing DSM 1058. We also tested monophages in the cocktail for their ability to lyse a panel of an additional 607 E. coli isolates, using the classical Spot Test assay; the cocktail lysed 408 (67%) of 607 E. coli strains examined (including DSM 1058) (Fig. 1). The cocktail was also tested against six strains of the non-targeted consortia included in our model system (Table 1), using the same Spot Test assay. None of the six non-E. coli strains included in our TSI model were susceptible to the cocktail (data not shown).

3.2. Bacteriophage stability in the small intestine in vitro model

We tested persistence of phages in the small intestine in the presence of *Escherichia coli* and in two different feeding conditions mimicking either a "fasted" small intestine (i.e., mimicking conditions before a meal; bile salts = 4mM; pancreatic Juice = 40 U/ml) or a "fed" small intestine (i.e., mimicking conditions after a meal; bile salts = 10 mM; pancreatic juice = 100 U/ml). The number of phages did not change significantly ($P > 0.05$) under the fasted conditions for either the duodenum (10.59 ± 0.06 log PFU/ml) or the ileum simulated conditions remaining at ca. 10.32 ± 0.17 log PFU/ml). In the fed state, the concentration of phages remained stable until the end of duodenum stage (10.76 ± 0.14 log PFU/ml) and then slightly decreased at the end of the ileum stage (from 10.81 log PFU/ml to 10.17 log PFU/ml (±0.35); $P = 0.048$),.

3.3. Impact of the bacteriophage cocktail vs. antibiotic on the levels of E. coli in the small intestine in vitro model

The ability of our phage cocktail to reduce the levels of the targeted *E. coli* strain in the model system was evaluated by inoculating 1 ml of *E. coli* culture into the simulated ileal section (both fed and fasted) of the small intestine, and adding either the phage cocktail or sterile water (as control). For both fasted and fed conditions, we observed a significant decrease in *E. coli* cell counts (Fig. 2 Fasted: $P < 0.01$, Fed: $P < 0.01$). Specifically, addition of bacteriophages reduced the levels of the targeted *E. coli* cells by ca. 2.5 log (99,5%) in both fed and fasted conditions. There was no reduction of *E. coli* levels in the phage-untreated, control group; in fact, the *E. coli* population slightly increased in control samples under the fed conditions (Fig. 2). The reduction in the *E. coli* population was slightly more pronounced under the fed condition compared to fasted conditions, although the difference was not statistically significant.

3.4. Impact of bacteriophage cocktail vs. antibiotic on the ileal bacterial community

The impact of a broad-spectrum antibiotic (ciprofloxacin) on the ileal bacterial consortia was compared to that of the bacteriophage cocktail (Fig. 3). The phage cocktail and ciprofloxacin showed similar efficacy in reducing *E. coli*, each yielding on average a 2.5 log (99.5%) reduction (Fig. 3). However, the phage cocktail was highly specific towards targeted *E. coli* species and did not significantly reduce any of the other species of the ileal consortium ($p > 0.05$ for both Fasted state and Fed state) (Fig. 3). In contrast, ciprofloxacin reduced
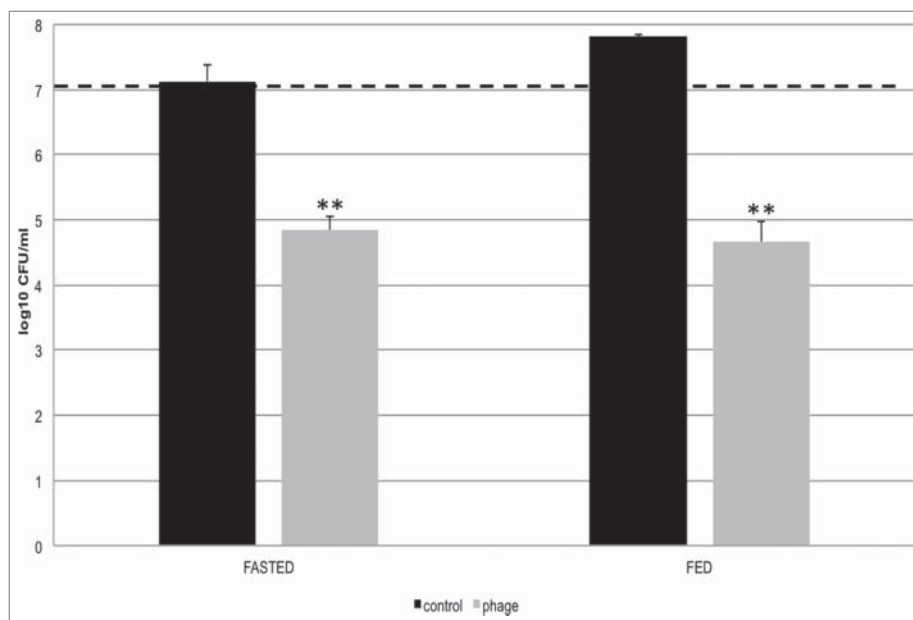

Figure 2. Lytic activity of the bacteriophage cocktail against targeted bacteria in the TSI model. Testing the lytic activity of the phage cocktail in two feeding conditions (fasted, fed). *E. coli* culture was added to the reactors at the ileum stage (ca. 7 log CFU/mL). The data shown are for the ileum compartment. The dotted line indicates the number of *E. coli* before treatment with the bacteriophage cocktail. All experiments were performed in triplicates (n = 3). *$P < 0.05$ – significant. **$P < 0.01$ – highly significant.
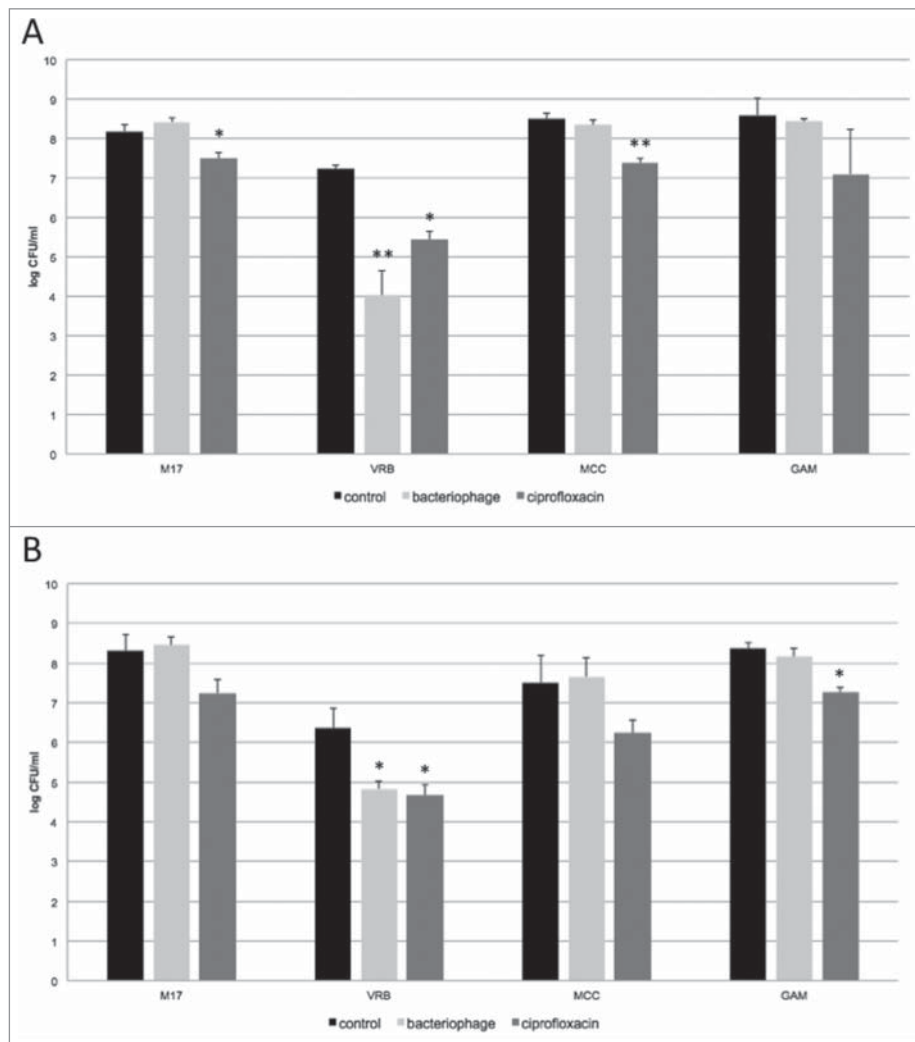
Figure 3. Impact of ciprofloxacin and bacteriophage cocktail on simulated small intestine microbiota in fasted (A) and fed (B) conditions (7 different bacterial spp., Table 1) in the TSI model. Survival of different bacteria species from the simulated small intestinal microbiota was tested on four different culturing media: Violet Red Bile Agar (VRB) for enumeration of *E. coli*, M17 Agar (M17) for enumeration of *Streptococcus* sp., MacConkey Agar (MCC) for enumeration of *E. faecalis*, and Gifu Anaerobic Agar (GAM) where all species from small intestinal consortium can be cultivated. All experiments were performed in duplicate (n = 2). *P < 0.05 – significant (difference between bacteriophage or ciprofloxacin treatment compared to control samples). **P < 0.01 – highly significant (difference between bacteriophage or ciprofloxacin treatment compared to control samples).

counts of all other bacteria on average of 1 log (90%) on all growth media for both fed and fasted states (Fig. 3).

4. Discussion

4.1. Phages retain significant infectivity after passage through simulated small intestines

Most phage treatments are administrated orally, although phages have been applied to humans therapeutically via various other routes as well, including auricular, intravesical, intrapulmonary, rectal, topical, and intravenous (reviewed in[25]). For oral administration, it is generally believed that phages

are deleteriously impacted by the low acidity of the stomach; hence, many oral applications involved administering phages together with bicarbonate water (to neutralize stomach acidity) or in a special enteric formulations (gel caps or tablets) that provide protection during passage through the acidic stomach environment (pH ranging from 1 – 2 up to 4 – 5) by dissolving and releasing phages only during less acidic sections of the intestinal track, such as the small intestine (pH 6 – 7.4), caecum (pH 5.7) or the rectum (pH 6.7).[26] However, rigorous data on the persistence of phages in various GI tract sections are not available, and it is not well established how various physiological conditions

that impact gut parameters (e.g., consumption of foods) may further influence this.

To begin addressing these questions, a TSI *in vitro* system[23] was used to determine phage stability and efficacy by simulating various sections of the small intestine (duodenum, jejunum or ileum) both in "fasted" or "fed" conditions. In general, the varying environmental conditions (pH, bile) during simulated small intestinal passage did not affect the stability of the phages. There was slight decrease in phage titers in the ileum under the fed conditions; however, the reduction was relatively minor and the phage cocktail was still able to significantly reduce *E. coli* levels in our *in vitro* system. Specifically, the phage cocktail reduced *E. coli* levels by the same ca. 2.5 log both in fed and fasted conditions. This study's results diverge from the study by Ma et al., who reported larger (1.29 and 1.67 log units, respectively) loss of *Salmonella* phage Felix O1) titers in 1% and 2% bile solution.[27] The negative impact of bile salts on phage viability has been reported by some other authors as well[28-30]; however, the topic is still not fully understood as other investigators – and this study – suggest that phages can be fairly stable and remain infectious when exposed to bile salts ranging from 4–10 mM).[31] It is possible that the phage viability will be more profoundly reduced *in vivo* due to many additional factors (e.g., intestinal peristalsis, more complex microbiota, various diets, etc.) that may deleteriously impact phage particles in the GI tract. Also, different bacteriophages can have different stability against bile salts, pH, etc. Elucidating the underlying mechanisms could prove to be a fertile area for subsequent investigations, and the results could offer important insights into the basic phage biology as well as for designing optimal phage preparations for various oral phage therapy applications.,

4.2. Bacteriophages preserve representative small intestine microbiome

In our studies, the phage preparation and ciprofloxacin reduced the *E. coli* levels in our *in vitro* model system by the same ca. 2.5 log. However, the two antimicrobials had very different impact on the non-targeted microbiota. Specifically, phage administration had no detectable impact on the six non-*E. coli* bacterial species included in our model system; whereas, ciprofloxacin reduced the levels of all bacteria in the consortium (Fig. 3). Short and long-term use of antibiotics could lead to a wide range of undesired effects, such as diarrhea, dysbioses, and other indirect deleterious effects such as obesity or colitis. These deleterious effects are usually due to the antibiotic-caused non-specific reduction of beneficial gut microbes, and the associated general perturbance in the gut microbiota. In this context, ciprofloxacin is one of the most widely used antibiotics to treat *E. coli* infections in the gut.[32,33] Unfortunately, it is also a major disruptor of the gut microbiota, and has been shown to profoundly alter the natural taxa in the GIT after only a single course of treatment, with some taxa never recolonizing.[34] Our studies provide further support of this indirect action of the antibiotic, as it led to a 1 log-reduction of the representative SI consortia in this study. In contrast, the phage preparation only impacted the *E. coli* populations, and had no impact on any of the other six "commensal" bacterial species included in our model.

The specificity of bacteriophages may offer some important medical / health benefits. For example, it can help mitigate unnecessary bacterial diversity reduction (and potential dysbiosis) in the gut. While there is a plethora of knowledge on microbial dysbiosis as it relates to antibiotic treatments, there is less understanding on how phages may affect the microbiota. Of the few studies examining phage therapy, none have reported any adverse effects on human health, or major alterations of the microbiome.[17,18,35,36] The present study corroborated those findings. Noteworthy, the bacteriophage cocktail did also lyse a large number of other *E. coli* strains *in vitro*; i.e., it was not solely lytic for one targeted *E. coli* strain. Thus, the use of such cocktail may still potentially impact some non-targeted *E. coli* in the small intestine. However, the use of a broad host range *E. coli* phage T4 ("broad host range" defined here as the ability of a given bacteriophage to lyse several strains of the same bacteria species) has been reported not to deleteriously change the normal *E. coli* microbiota of human volunteers.[16] Additional studies, including larger scale trials in human volunteers, will be required to better elucidate the impact of phage administration on the commensal microbiota. Overall, our data support the idea that properly selected phage preparations could be potentially at least equally effective as a commonly prescribed antibiotic

in reducing the *E. coli* levels in the small intestine, but may have a significantly milder impact on non-targeted, "normal microbiota" bacteria.

Disclosure of potential conflicts of interest

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