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Investigating the Use of Bacteriophages as New Decolonization Strategy for Intestinal Carriage of CTX-M-15-producing ST131 *Escherichia coli*: An *In Vitro* Continuous Culture System Model

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HIGHLIGHTS

- We explored the use of phages to decolonize gut carriers of CTX-M-15 ST131 *E. coli*
- An in vitro system (fermentor) was implemented with two pools of feces
- For the first pool, bacteriophages decreased the numbers of ST131 dramatically
- For the second pool, a phage-resistant mutant persisted in the continuous culture
- The individual microbiota composition may have an impact on the development of phage resistance

Investigating the Use of Bacteriophages as New Decolonization Strategy for Intestinal Carriage of CTX-M-15-producing ST131 *Escherichia coli*: An *In Vitro* Continuous Culture System Model

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Running Title: Bacteriophages to decolonize gut carriers of ESBL-*E. coli* ST131

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ABSTRACT

Objectives. We investigated the use of bacteriophages as a strategy to decolonize intestinal carriers of multidrug-resistant *Escherichia coli*.

Methods. A fermentor was used as a continuous culture system for 48 hrs. Two different pools of feces (study I and II) obtained from volunteers were spiked with a CTX-M-15-producing ST131 *E. coli* (strain 4901.28) susceptible to bacteriophages and challenged with 3 doses of *INTESTI Bacteriophage* cocktail administered at 2, 6 and 10 hrs after inoculum. Bacterial typing was performed by implementing microdilution panels, spot test, rep-PCR, and whole-genome sequencing (including cgMLST and SNV analysis) obtained using both Nanopore and Illumina platforms.

Results. In study I, bacteriophages decreased the numbers of 4901.28 dramatically ($\leq 10^1$ CFU/mL after 6 hrs). In contrast, during study II a phage-resistant mutant of 4901.28 persisted in the continuous culture (10^4 CFU/mL at 48 hrs). WGS revealed the presence of two additional plasmids in the mutant as well as 11 SNVs, including one chromosomal in a glycosyltransferase family 2 protein that is responsible for the transfer of sugars to polysaccharides and lipids. In both studies, the commensal *E. coli* population remained unchanged by the phage treatment maintaining itself at 10^8 CFU/mL.

Conclusions. Our data indicates that bacteriophage cocktails may be implemented to decolonize some intestinal carriers. However, the individual microbiota composition may have an impact on the development of phage resistance. Mechanisms underlying this phenomenon are likely to be various and complex. Further *in vivo* studies and protein expression experiments are needed to confirm our observations and hypotheses.

KEY WORDS: bacteriophages, gut, multidrug-resistant, *E. coli*, ST131, CTX-M-15

1. INTRODUCTION

Multidrug-resistant (MDR) *Escherichia coli* are spreading worldwide due to hyperepidemic high-risk clones; among them, those of sequence type (ST) 131 are of particular concern. This lineage is a major driver of antibiotic resistance and is recognized as a highly prevalent, uropathogenic and pandemic clone harboring numerous virulence factors. Clinical isolates of ST131 usually display an MDR phenotype where the extended-spectrum β-lactamases (ESBLs) are the main resistance mechanism (especially the CTX-M-15). The reasons behind the success of ESBL-producing ST131 *E. coli* expansion and dissemination on large scale are still to be elucidated. Though, main reasons are likely to be colonization at intestinal level as well as prolonged persistence [1, 2].

Notably, intestinal colonization with MDR organisms (MDROs) has four main consequences: *i*) risk to spread these pathogens in the environment [1, 3]; *ii*) cross-transmission among people and/or animals [4, 5]; *iii*) risk to sporadically developing untreatable infections (e.g., bloodstream and urinary-tract infections) [6, 7]; and *iv*) risk of a life-long carriage of MDROs with consequent potential horizontal transfer of resistance genes (e.g., *via* plasmids) to indigenous bacterial species within the gut [8, 9].

Several strategies aimed to decrease the density as well as relative abundance of MDR Gram-negatives at intestinal level have been suggested [10, 11]. For instance, it has been proposed to use selective digestive decontamination using broad-spectrum antibiotic(s) administered for short periods. However, for Gram-negatives, only a few works have examined its efficacy, especially to decolonize healthy carriers from ESBL-producing Enterobacterales [12, 13]. This strategy seems to not completely eradicate the targeted strain, but rather decrease its number, which could lead to gut re-colonization [13]. Moreover, these antibiotic-based approaches present the major disadvantage of reducing species diversity within the intestinal microbiota. This can lead to disrupted colonization resistance, increasing the risk for developing infections, as well as resistance against last-line antibiotics [14, 15].

More recently, the fecal microbiota transplantation, other than for preventing recurrent *Clostridium difficile* infections, has been implemented to lower the density of MDROs (alone or preceded by short courses of antibiotics). Although promising preliminary results have been recorded, a major drawback is patient compliance due to the difficult-to-accept nature of treatment [16]. Therefore, standardized, easy to use, and effective strategies to decolonize intestinal carriers of MDROs are still not available.

In this overall context, bacteriophages could represent a new and alternative approach. In fact, some of these bacterial viruses are highly species-specific, namely with the potential to selectively spare commensal populations unlike an antimicrobial treatment. Moreover, thanks to their self-propagating nature, in presence of the targeted bacterial species they display a self-limiting action. However, though they have been part of the standard therapy regimens in Russia, Georgia and Poland for one hundred years, yet they have received very little attention in western countries [17, 18]. As a consequence, we are facing a lack of rigorous scientific studies analyzing their efficacy for treating and preventing human infections [19].

To the best of our knowledge, bacteriophages have never been studied in the context of human intestinal decolonization of MDR *E. coli*. Therefore, we investigated the use of a commercial preparation of bacteriophages as a gut decolonization strategy against an ESBL-producing *E. coli* belonging to the pandemic ST131 lineage in a simplified *in vitro* model of intestinal colonization.

2. MATERIALS AND METHODS

2.1. Bacterial typing. *E. coli* strain 4901.28 was used as the *wild type* (WT) targeted strain. It was isolated from a urine sample of a 69 years-old woman [7]. The isolate was previously characterized by phenotypic (MICs determined using the Sensititre GNX2F and ESB1F plates; Thermo Fisher Diagnostics) and genotypic methods (characterization of *bla* genes, multilocus sequence typing, and plasmid replicon typing) [7]. In the present work, *E. coli* 4901.28 underwent whole-genome sequencing (WGS) analysis along with one representative bacteriophage-resistant mutant (see below).

2.2. Continuous culture system. A 2-liter glass fermentation vessel, operated under the control of a New Brunswick™ BioFlo®/CelliGen® 115 Unit (Eppendorf) was chosen as the *in vitro* system (chemostat). The starting volume of the vessel was one liter and the growth medium implemented was Brain Hearth Infusion (BHI) broth (Becton Dickinson). Fresh sterilized medium was added via a peristaltic pump at a constant rate of 18 mL/h and waste culture liquid was removed at the same rate. The system was operated in aerobic conditions and the temperature maintained at 37°C using circulating water in the double wall. Moderate agitation at 70 rpm was applied.

2.3. Characterization of donor stools and preparation of fecal inoculum. Fresh feces from healthy volunteers negative for extended-spectrum cephalosporin-resistant Enterobacterales (ESC-R-Ent) were chosen for the experiments. Screening to confirm negativity was performed to detect ESC-R- and carbapenem-resistant Enterobacterales as previously done [5, 8, 9, 20]. Briefly, ~20 µg of fresh stools was enriched overnight in 10 mL Luria-Bertani (LB) broth containing a 10-µg disk of cefuroxime. Then, 100 µl were plated on BLSE, ChromID ESBL (bioMérieux) and home-made SuperCarba selective plates. After overnight incubation, selected colonies were identified using the MALDI-TOF MS (Bruker).

Two different pools of feces were tested (pool A for study I and pool B for study II), each coming from three non-colonized volunteers and corresponding to a combined total of 1 g. Stools were uniformly suspended in 10 mL BHI and vigorously vortexed for 2-3 min. Homogenized feces were

equilibrated in a 37°C incubator for approximately 15 min before starting the experiment. The chemostat vessel was then inoculated through a port in the top with the fecal suspension (1 g in 10 mL); after 15 min, the first time-point sample (T_0) was taken.

2.4. Bacteriophages. *INTESTI Bacteriophage* (lot # M2-801; Eliava BioPreparation) was used as antimicrobial agent to selectively target *E. coli* 4901.28. This preparation represents a sterile-filtrate phage lysate (total of 1 x 10^{5-6} PFU/mL) of several pathogenic *E. coli*, *Shigella* spp., *Salmonella* spp., *Proteus vulgaris*/*mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus* spp., and *Enterococcus* spp. This biopreparation has been fully characterized with a metagenomic approach [21].

Susceptibility to the *INTESTI Bacteriophage* cocktail was determined by implementing the spot test with the double agar method (where "opaque lysis/++" is part of the sensible phenotype scale, and "R" stands for phage-resistant) after two passages on BHI plates [22]. Notably, *E. coli* 4901.28 was fully susceptible to the *INTESTI* cocktail [23].

2.5. Study design. In a first blank experiment (with pool A of feces), 4901.28 was added (see below) in the chemostat system 30 min after the fecal inoculum (i.e., T_0 plus 15 min) in order to evaluate the growth trend of the pathogen compared to the total *E. coli* microbial population in the chemostat system (Figure 1).

A second experiment consisted of investigating whether 4901.28 was able to maintain itself despite the introduction of *INTESTI Bacteriophage* cocktail aliquots. Specifically, three doses of 1 mL undiluted cocktail were added to the chemostat at T_2, T_6, and T_{10}. This experiment was performed in duplicate (experiment "a" and "b") and also with two different pools of feces (study I with pool A and in Study II with pool B). All experiments were conducted for 48 hrs, during which 20 time points were taken (15 time points for the first day and 5 during the second one). Graphs were generated with GraphPad Prism 7.0 (GraphPad Software).

2.6. Bacterial inoculum and population dynamics. *E. coli* 4901.28 was grown overnight on a MacConkey agar plate (Becton Dickinson). Colonies were suspended in sterile NaCl 0.9% to reach a concentration of 1.2 x 10^8 CFU/mL (corresponding to 0.4 McFarland scale), then 80µl of this

suspension was added in 10 mL BHI to reach a final concentration of 10^7 CFU in total. The 10 mL were finally poured into the 1-liter BHI contained in the chemostat vessel 15 min after T_0. After an additional 15 min ($T_{0.5}$), the second sample was taken to measure the starting number (CFU/mL) of the targeted strain.

At each time point (from T_0 to T_{48}) the cultivable microbiota was monitored by removing 5 mL of sample from the vessel; one mL was serially diluted in PBS and plated on CHROMagar™ Orientation plus vancomycin (8 µg/mL) (for the total *E. coli* count) and on CHROMagar™ Orientation plus vancomycin (8 µg/mL) and cefotaxime (2 µg/mL) (for selective ESBL-*E. coli* ST131 count). Plates were incubated overnight at 37°C and the next day only violet colonies (corresponding to *E. coli* species) were counted. Lastly, sample aliquots were prepared: one mL per each sample was stored at -80°C in 20% glycerol, while the remaining three mL were used for the viral titration (see below).

2.7. Viral population dynamics. The bacteriophage population was monitored by titration using the double-agar method on the host strain (*E. coli* 4901.28). At day one, titration was performed at T_3, T_5, T_7, T_9, T_{11} and T_{13} (for ExIa T_{10} was taken instead of T_9), while at day two it was performed at each time point (T_{24}, T_{28}, T_{32}, T_{35} and T_{48}). Briefly, 1 mL of the undiluted chemostat sample was filtrated through a 0.22 µm syringe filter (Carl Roth GmbH) and further serially diluted up to 10^{-7} times. Then, 100 µl of 4901.28 (concentration of 1.5 x 10^8 CFU/mL) were supplemented with 1 mL of the dilutions 10^{-1}, 10^{-3}, 10^{-5}, 10^{-7} and with 5 mL of BHI soft agar (0.7%). The solutions were then poured on BHI agar plates and incubated for 24 hrs at 37°C. Plaques were counted the next day in order to calculate the viral titer.

2.8. Repetitive Extragenic Palindromic PCR (rep-PCR). The clonal relatedness of *E. coli* strains recovered from samples was studied using rep-PCR. Briefly, violet colonies were picked from CHROMagar™ Orientation plates supplemented with cefotaxime, followed by DNA extraction with Chelex® 100 sodium form (Merck KGaA). Extracts were subjected to rep-PCR and resulting PCR products were run on a DNA chip (Agilent Technologies) using the Agilent 2100 Bioanalyzer (Agilent Technologies) [7, 24, 25].

2.9. Genotyping. WGS was obtained using both MinION (Oxford Nanopore) and HiSeq (Illumina) as previously done [25-28]. In brief, total DNA was extracted with the QIAamp Mini Kit (Qiagen). For MinION, the SQK-LSK108 2D ligation sequencing kit, a R9.5 SpotON flow cell and the MinION Mk1B device (Oxford Nanopore) were used for the 24 hrs run. Data acquisition, as well as base-calling, was carried out with the MinKNOW software (Oxford Nanopore). Raw reads were converted to fastq with Poretools and *de novo* assembled with the Canu pipeline. For Illumina sequencing, reads were first trimmed with Trimmomatic software and then aligned to MinION contigs using Burrows-Wheeler Alignment (BAM) and Sequence Alignment/Map (SAM) for file conversion. FASTA sequences of each corrected contig were extracted from Geneious software and interpreted with Res-, Plasmid, Virulence-Finder (https://cge.cbs.dtu.dk/services/), CRISPRCasFinder (https://crisprcas.i2bc.paris-saclay.fr/CrisprCasFinder/Index) and CRISPRone (http://omics.informatics.indiana.edu/CRISPRone/).

In addition, assemblies of the raw Illumina reads with SPAdes Software were used for core genome MLST (cgMLST) analysis by implementing cgMLSTFinder (https://cge.cbs.dtu.dk/services/). Single nucleotide variants (SNVs) analysis was implemented to compare the chromosomes of 4901.28 and phage-resistant mutant (ExIIa_T32_C2). Briefly, the core-genome alignment was performed with Parsnp v1.2 (https://github.com/marbl/parsnp). All strains were treated as curated genomes (-c parameter), and the chromosomal hybrid assembly of the mutant was used as a reference genome to fine-tune the core-genome alignment including only chromosomal sequences and excluding the plasmid ones. To maximize genome coverage across all genomes, the –c parameter was optimized to 6. Other parameters were let as default. Variant Call Format (VCF) data from Parsnp core-genome alignment were extracted from the Gingr formatted binary archive output with Harvest-Tools v1.2 (https://github.com/marbl/harvest-tools). Core-genome alignment coverage was determined with Gingr v1.2 (https://github.com/marbl/gingr). Variants with no flags (PASS) were determined as reliable [29], and used for downstream SNV analysis with a custom R v3.6.2 script (https://www.r-project.org/). The translate tool ExPASy (http://www.web.expasy.org/translate/) followed by Protein

BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) were finally used to identify and compare amino acid (AA) substitutions. Annotations of both hybrid and Illumina assemblies were conducted by the NCBI Prokaryotic Genome Annotation Pipeline.

2.10. GenBank accession numbers. Hybrid assembly (BioProject: PRJNA551948) for 4901.28: VMRI00000000 (chromosome, VMRI01000001 - plasmid A, VMRI01000002); for ExIIa_T32_C2: VMRH00000000 (chromosome, VMRH01000003-VMRH01000006 - plasmid A, VMRH01000001 - plasmid B, VMRH01000007 - plasmid C, VMRH01000002). Sole Illumina (BioProject: PRJNA605932) for 4901.28: JAAHTE000000000; for ExIIa_T32_C2: JAAHTF000000000.

3. RESULTS

3.1. E. coli dynamics without bacteriophage treatment. In the blank experiment, both monitored populations (*E. coli* 4901.28 and the total *E. coli*) exponentially increased for the first 5 hrs, and then reached a plateau from T_5 to T_{48}. In particular, *E. coli* 4901.28 reached a stationary phase at a population size of 10^6 CFU/mL, whereas the total *E. coli* microbial population stabilized itself at 10^8 CFU/mL (Figure 1).

3.2. E. coli dynamics with 3 doses of bacteriophages and pool A of feces (study I). For the first pool of feces, phage treatment resulted in an immediate decrease of the population size of 4901.28 (from 10^5 to 10^1 CFU/mL) 2 hrs after the inoculation of the first dose of phages; moreover, after stopping phage treatment, the population of the target MDR pathogen never restored itself. On the other hand, the total *E. coli* microbial community was maintained constant despite the phage treatment (i.e., increasing for the first 7 hrs and then maintaining itself at 10^8 CFU/mL). Similarly, the bacteriophage population increased during the first 3-6 hrs to 10^7 PFU/mL and then, after 12 hrs, stabilized itself at 10^6 PFU/mL (Figure 2; Table S1).

3.3. E. coli dynamics with 3 doses of bacteriophages and pool B of feces (study II). For the second pool of feces, similarly as in study I, 4901.28 increased for the first 4 hrs and dropped below the LOD 2 hrs after the inoculation of the first treatment dose (T_5) (Figure 3; Table S1). However, in contrast

to study I, a phage-resistant population started to emerge after T_5. It then continued to grow with some oscillations during the second (T_6) and third (T_{10}) dose of cocktail treatment, eventually stabilizing itself at 10^{3-4} CFU/mL. We also noted that the total *E. coli* population showed similar dynamics to study I and blank experiments (i.e., increasing for the first 8 hrs and reaching a plateau of 10^8 CFU/mL). In contrast, the bacteriophage population showed a more rapid and higher titer than observed in study I (i.e., at 5 hrs 10^9 PFU/mL that then stabilized at 10^8 PFU/mL after about 12 hrs).

3.4. Characterization of phage-resistant mutants. For study II, 6 re-growing cefotaxime-resistant *E. coli* colonies taken from the time points T_{28} and T_{32} of experiment IIa (ExIIa) and two from T_{35} and T_{48} from experiment IIb (ExIIb) were isolated and analyzed. In particular, their rep-PCR profiles were identical to each other, but slightly different compared to 4901.28 (i.e., with three less intense or absent bands; Figure S1). One of these cefotaxime-resistant isolates (strain ExIIa_T32_C2), recovered during study IIa at T_{32} and phenotypically resistant to the phage cocktail using the spot test, was randomly chosen as a representative strain for further analyses.

As shown in Table 1, the phenotype, ST, plasmid replicons, and resistance genes of ExIIa_T32_C2 were identical to those of the WT strain *E. coli* 4901.28. WGS data of ExIIa_T32_C2 revealed the presence of two additional plasmids of 4kb and 7kb (plasmids B and C, respectively), as compared to *E. coli* 4901.28 which originally only carried a 170kb $bla_{\text{CTX-M-15}}$-positive plasmid (plasmid A). Plasmid A carried several resistance genes, the virulence factor for increased serum survival, and the three replicon types FII, FIB, FIA as well as the colicinogenic marker Col156. Plasmid B carried 5 genes encoding two replication proteins and 3 that were functionally uncharacterized. Plasmid C carried 8 genes encoding proteins for mobilization, replication, conjugal transfer, and unknown function (n=2 each). Resistance genes or virulence factors were not found in both plasmids B and C (Table 1).

Large chromosomal deletions or insertions were not detected in the mutant. However, core-genome analysis revealed that ExIIa_T32_C2 possessed 11 chromosomal SNVs compared to the WT strain (Table 2). Three were located in the IS*3* family transposase gene, and the remaining in AAA family

transposase, glycosyltransferase family 2 protein (transfer of nucleotide-diphosphate sugars to polysaccharides and lipids), IS*66* family transposase, hypothetical protein, DUF945 domain-containing protein (domain of unknown function), RadC family protein (DNA repair and recombination protein), and polB (DNA polymerase β) genes, and one in a non-coding region (Table 2). Finally, CRISPR-*cas* analysis showed only the presence of questionable CRISPR spacers and the complete absence of *cas* genes (data not shown).

4. DISCUSSION

E. coli belonging to ST131 are responsible for the increasing prevalence and spread of cephalosporin resistance worldwide. Particularly worrisome is their silent carriage at intestinal level, which may translate into future difficult to treat infections [1]. Efforts to try decolonizing the gut using antibiotic treatment can cause disturbance of the normal bacterial flora leading to overgrowth of pathogenic strains (exogenous or already present in the gut) [30]. As an alternative, bacteriophages could enable to maintain colonization resistance (i.e., protection by the endogenous flora against pathogenic bacteria) at physiological level.

4.1. The in vitro model. Operated with 1-liter volume and spiked stool, our system can host both the pathogenic strain and commensal *E. coli* populations. Moreover, compared to more simplistic *in vitro* systems, this continuous culture approach allows to come a step closer in mimicking the *in vivo* conditions of the gut (e.g., through introduction of fresh nutrients and elimination of left overs in the chemostat). However, the aerobic conditions used are not able to comprehensively reflect the complex diversity of bacterial populations present in the bowel (i.e., for a total of 10^{11} CFU/g of feces) [31]. Indeed, anaerobic species could play a role in colonization resistance and could modulate the population size of the targeted ST131 *E. coli* strain, with consequent influence on success or failure of phage-treatment. Nevertheless, among the enriched facultative-anaerobe Enterobacterales we could observed a total count of *E. coli* of about 10^{8} CFU/mL, in line with concentrations recovered *in vivo* (i.e., reaching in the gut 10^{8-9} CFU/g of feces) [32]. Concerning the dosage protocol, we chose

to administer multiple doses in order to simulate a continuous treatment since the effectiveness of phage therapy is known to be correlated to the dosage and treatment time-point. Precisely, several studies have shown that early administration of multiple doses are more effective than a single dose in eradicating the targeted bacterial strain [33].

4.2. Occurrence of resistant mutants. An interesting finding in our study was the identification of phage-resistant mutants isolated only from one of the two tested fecal pools. Bacteriophage resistance is a known phenomenon in natural environments where phages outnumber bacteria 10:1, and thus exert a strong predatory pressure on them. It therefore represents a predictable evolutionary response to viral attack [34]. Already in 1943-1945, Demerec and Fano together with Luria and Delbrück described multiple resistance mechanisms that simultaneously occur in *E. coli* against different bacteriophages [35, 36].

Nowadays, various phage resistance mechanisms have been well characterized and include preventing phage adsorption (e.g., by blocking phage receptors or producing extracellular matrix), preventing phage DNA entry [e.g., superinfection exclusion (Sie) system], cutting phage nucleic acid [e.g., restriction-modification (R-M) system, CRISPR-*Cas* system], and abortive infection (Abi) systems. Other resistance strategies have been observed, yet their mechanisms are still to be unveiled; moreover, many other completely unknown phage resistance mechanisms are likely to exist [37]. In particular, the CRISPR-*Cas* system is composed by CRISPR-motifs scattered in the genome, each one containing sets of conserved inverted direct repeats intercalated by a spacer sequence originating by exogenous DNA and accompanied by *cas* genes. It represents an anti-phage and anti-plasmid adaptive immunity harbored by ~40% of all bacteria [38, 39].

In the present work, we could not find any *cas* gene indicative of a functional CRISPR system [40]. Only questionable CRISPR were detected, likely corresponding to repeated regions in the genome (data not shown). This is not surprising as some groups of *E. coli*, comprehending the phylogenetic group B2 to which our strains belong to, have been previously shown to completely lack this system [41].

We hypothesize that more than one resistance mechanism coexists in our phage-resistant mutant, when being in presence of a complex cocktail containing multiple lytic phages against the ST131 *E. coli* strain. On this regard, the chromosomal amino acid substitution that we detected in the glycosyl transferase family 2 protein domain could potentially block one or more phage receptors by over-transferring sugars to its outer-membrane substrates. However, a functional study of the mutated enzyme should be done to confirm this hypothesis. Additionally, to better understand a possible link with the resistant phenotype, a protein-expression-level approach should be implemented by comparing the mRNA profiles of mutant and WT strain. This analysis would also be essential to explore both Abi and R-M systems, which exploit several heterogeneous proteins to provide resistance [37]. Finally, several genes present in the newly acquired plasmids could not be assigned to a known function. Their implication in the acquisition of resistance could not be further confirmed with conjugation experiments. In fact, due to their living and evolving nature, it is technically unfeasible to prepare stable plates selective for any phage or phage cocktail, enabling the further selection of transconjugants.

4.3. The host microbiota may affect activity of bacteriophages. Regarding the divergence of results between study I and II, we hypothesize that the emergence of phage resistance in only one pool of feces (pool B) could be dependent on the different profiles of their bacterial populations. Particularly, some fecal bacteria may help each other by mean of quorum sensing (QS) signaling to fight against viral predators. Notably, QS are chemical signals exploited by some bacteria as well as by eukaryotic cells to communicate within or between different bacterial populations (e.g., leading to expression of biofilm or of virulence factors). They have also been recognized playing a role in the relationship between bacteria and phages, namely to communicate the presence of viruses in the environment and to further control and coordinate the expression of anti-phage defenses [34].

The ST131 *E. coli* strain 4901.28 may thus be able to sense the presence of phages thanks to signals produced by other species present in feces of specific individuals, and consequently be prepared against a possible attack [34]. This could enable bacterial populations to increase their defenses only

in presence of high viral titer, thereby sparing the energy required to maintain a constant high-level defense in case of lower danger of infection. Notably, Hoyland-Kroghsbo *et al*. found a particular pathway of QS signaling in *E. coli* that cause a temporary diminished number of phage receptors. It is activated only during high phage density and despite the consequent diminished fitness (e.g. lower absorption of specific nutrients) [34].

In our case, producers of QS signals could be individual fecal bacterial populations or alternatively eukaryotic cells (also known to exert QS towards bacterial cells in natural environments), specifically colonic epithelial cells that are part of the normal stool composition. The consequent reversible decreased expression of particular receptors may have spared *E. coli* 4901.28 from being infected by bacteriophages in the second pool of feces (study II), yet not in the first one (study I). This hypothesis is supported by the observation that in study II the viral titer resulted much higher than in study I (Figure 2 *vs*. Figure 3, respectively). It can be speculated that in pool B of feces some of the bacteriophages included in the *INTESTI* cocktail found specific bacterial host(s) were to replicate better and faster than in pool A. Then, the higher viral concentration induced QS signals able to protect bacteria under the risk of infection.

Our work suggests that a deeper and detailed knowledge on the nature of bacterial populations favoring or hampering the emergence of phage resistance is necessary for the future application of phage therapy as decolonization strategy.

5. CONCLUSION

We hypothesized that bacteriophages could represent a possible alternative strategy to decolonize intestinal carriers of MDR *E. coli*. Certainly, phage cocktails are lacking the major drawbacks presented by antibiotic regimens, as well as by other strategies aimed to decolonize intestinal carriers from MDROs. Nevertheless, phage decolonization should be performed with caution since phage resistance may emerge in certain circumstances. In fact, our data indicates that bacteriophages efficacy may be influenced by the individual microbiota composition. Moreover, the phenomenon of

resistance against bacteriophages may imply different and simultaneous mechanisms, especially in presence of complex phage cocktails. Evidently, an *in vivo* model of intestinal colonization should be developed alongside with protein expression level experiments in order to further confirm these findings.

DECLARATIONS

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Competing interests: none.

Ethical Approval: Not required.

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LEGEND TO THE FIGURES

Figure 1. Blank experiment: *E. coli* dynamics without bacteriophage treatment. Dynamics of fecal *E. coli* community and of *E. coli* 4901.28 alone in the chemostat system in the absence of bacteriophages (pool A of feces, as for Study I). Feces were inoculated into the chemostat 15 min before T_0 (that was the first sampling point). Blue line: total *E. coli* population; red line: CTX-M-15-producing *E. coli* ST131 4901.28. LOD: limit of detection (10^1 CFU/mL). Graph generated with GraphPad Prism 7 on data from one experiment.

Figure 2. Study I: *E. coli* dynamics with 3 doses of *INTESTI Bacteriophage* cocktail and pool A of feces. Influence of bacteriophage treatment on the fecal *E. coli* community and on *E. coli* 4901.28 performed in the chemostat system with the first pool (A) of feces. Blue line: total *E. coli* population; red line: CTX-M-15-producing *E. coli* ST131 4901.28.; black stars, administered bacteriophage doses. LOD: limit of detection (10^1 CFU/mL). Graph generated with GraphPad Prism 7 on data from two experiments. Appearance: median and error. Plot: range. Error bars not drowned by the software when shorter than the height of the symbol.

Figure 3. Study II: *E. coli* dynamics with 3 doses of *INTESTI Bacteriophage* cocktail and pool B of feces. Influence of bacteriophage treatment on the fecal *E. coli* community and on *E. coli* 4901.28 performed in the chemostat system with the second pool (B) of feces. Feces were inoculated into the chemostat 15 min before T_0 (that was the first sampling point). Blue line: total *E. coli* population; red line: CTX-M-15-producing *E. coli* ST131 4901.28.; black stars, administered bacteriophage doses. LOD: limit of detection (10^1 CFU/mL). Graph generated with GraphPad Prism 7 on data from one duplicate experiment. Appearance: median and error. Plot: range. Error bars not drowned by the software when shorter than the height of the symbol.

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Table 1. Molecular and phenotypic features of phage-sensitive WT strain 4901.28 and of phage-resistant mutant ExIIa_T32_C2

Characteristics	***E. coli* 4901.28**	**E. coli ExIIa_T32_C2 (mutant)**
ASTs (MICs, µg/mL) [a]	P/T4 (≤8/4), FOT (>32), TAZ (16), FEP (16), AZT (>16), ETP (≤0.25), GEN (8), AMI (16), CIP (>2), SXT (>4/76), DOX (16), TGC (1), COL (≤0.25), FOX (≤4), AMP (>16), T/C (≤0.12/4), F/C (≤0.12/4)	P/T4 (≤4/4), FOT (>32), TAZ (16), FEP (8), AZT (>16), ETP (≤0.25), GEN (≤4), AMI (>32), CIP (>2), SXT (>4/76), DOX (16), TGC (0.5), COL (≤0.25), FOX (≤4),, AMP (>16), T/C (≤0.12/4), F/C (≤0.12/4)
Spot test results [b]	++	R
ST	131	131
PlasmidFinder (replicon)		
- Plasmid A (170kb)	FII, FIB, FIA, Col156	FII, FIB, FIA, Col156
- Plasmid B (4kb)	na	Col (BS512)
- Plasmid C (7kb)	na	-
ResFinder (resistance genes) [c]		
- Chromosome	*mdf(A)*	*mdf(A)*
- Plasmid A (170kb)	*bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{OXA-1}}$, *aadA5, aacA4, aac(6')-Ib-cr, mph(A), catB3, sul1, dfrA17, tet(A)*	*bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{OXA-1}}$, *aadA5, aacA4, aac(6')-Ib-cr, mph(A), catB3, sul1, dfrA17, tet(A)*
- Plasmid B (4kb)	na	-
- Plasmid C (7kb)	na	-
VirulenceFinder (virulence genes) [d]		
- Chromosome	*gad, iha, sat, nfaE, iss*	*gad, iha, sat, nfaE, iss*
- Plasmid A (170kb)	*senB*	*senB*
- Plasmid B (4kb)	na	-
- Plasmid C (7kb)	na	-

Note. ST, sequence type; na, not applicable; -, no output (genes not previously annotated).

[a] ASTs, antimicrobial susceptibility tests (MICs interpreted according to EUCAST 2019, version 9.0, except for doxycycline for which CLSI 2019, M100-S29, was used); P/T4, piperacillin/tazobactam; FOT, cefotaxime; TAZ, ceftazidime; FEP, cefepime; AZT, aztreonam; ETP, ertapenem; GEN, gentamicin; AMI, amikacin; CIP, ciprofloxacin; SXT, trimethoprim/sulfamethoxazole; DOX, doxycycline, TGC, tigecycline; COL, colistin; FOX, cefoxitin; AMP, ampicillin; T/C, ceftazidime/clavulanic acid; F/C, cefotaxime/clavulanic acid.

[b] Spot test performed with the double agar method where "opaque lysis/++" is part of the sensible phenotype scale, and "R" stands for phage-resistant.

[c] *mdf(A)*, macrolide-associated resistance; *aadA5*, aminoglycoside resistance; *aadA4*, aminoglycoside resistance; *bla*$_{\text{CTX-M-15}}$, β-lactam resistance; *bla*$_{\text{OXA-1}}$, β-lactam resistance; *aac(6')Ib-cr*, fluoroquinolone and aminoglycoside resistance; *mph(A)*, macrolide resistance; *catB4*, phenicols resistance; *sul1*, sulphonamides resistance; *dfrA7*, trimethoprim resistance.

[d] *gad*, glutamate decarboxylase; *Iha*, adherence protein; *sat*, secreted autotransporter toxin; *nfaE*, diffuse adherence fibrillary adhesion gene; *gad*, glutamate decarboxylase; *iss*, increased serum survival; *senB*, plasmid-encoded enterotoxin

Table 2. Results of SNVs analysis comparing the chromosomes of WT strain 4901.28 and its phage-resistant mutant ExIIa_T32_C2

SNVs environment [a]	ExIIa_T32_C2 hybrid assembly [b]	ExIIa_T32_C2 sole Illumina [b]	4901.28 sole Illumina [b]	Target CDS	AA change [c]	AAs identity
GGCTTTCCAG CCCTTATTT	C	C	A	IS*3*-like element IS*1397* family transposase	Q33L E37A	99% (198/200)
ACAGGGAGCT CCGCTTTGA	G	G	T	IS*3* family transposase		
CGCTTTGAAC GTCGCTGAA	A	A	T	IS*3* family transposase		
AAATGTATAA TCATACTTT	T	T	G	Non-coding region	na	na
TAACCCCGGC TTTCGTTTC	T	T	C	AAA family ATPase	-	100% (170/170)
TACATCGGGG TAACAAAGA	G	G	T	Glycosyltransferase family 2 protein	N49T	99% (223/224)
CGATGGGCCG GAAGGCGCG	T	C	T	IS*66* family transposase	-	100% (512/512)
ACGTGCGCGC CCCGTGCCA	T	T	G	Hypothetical protein	A123S	99% (130/131)
CCCGGCGTCG GGCGTCAGA	C	T	C	DUF945 domain-containing protein	-	100% (158/158)
TGTATCTGAA AACCAGAAT	C	C	T	RadC family protein	-	100% (158/158)
AGATCTGCGT ACCAGCTCG	C	C	T	PolB	-	100% (649/649)

Note. SNVs, single nucleotide variants; AA, amino acid. na, not applicable.
[a] Space between bases in each sequence represents the nucleotide position of the mutation.
[b] Letters represent the bases contained in the sequence spaces reported in the first column. C, cytosine; G, guanine; T, thymine; A, adenine.
[c] The first AAs abbreviation belongs to 4901.28 (wild-type), while the second to the phage-resistant mutant ExIIa_T32_C2. Q, Glutamine; L, Leucine; E, Glutamic acid; A, alanine; N, asparagine; T, threonine; A, alanine; S, serine.

Figure 1-2-3

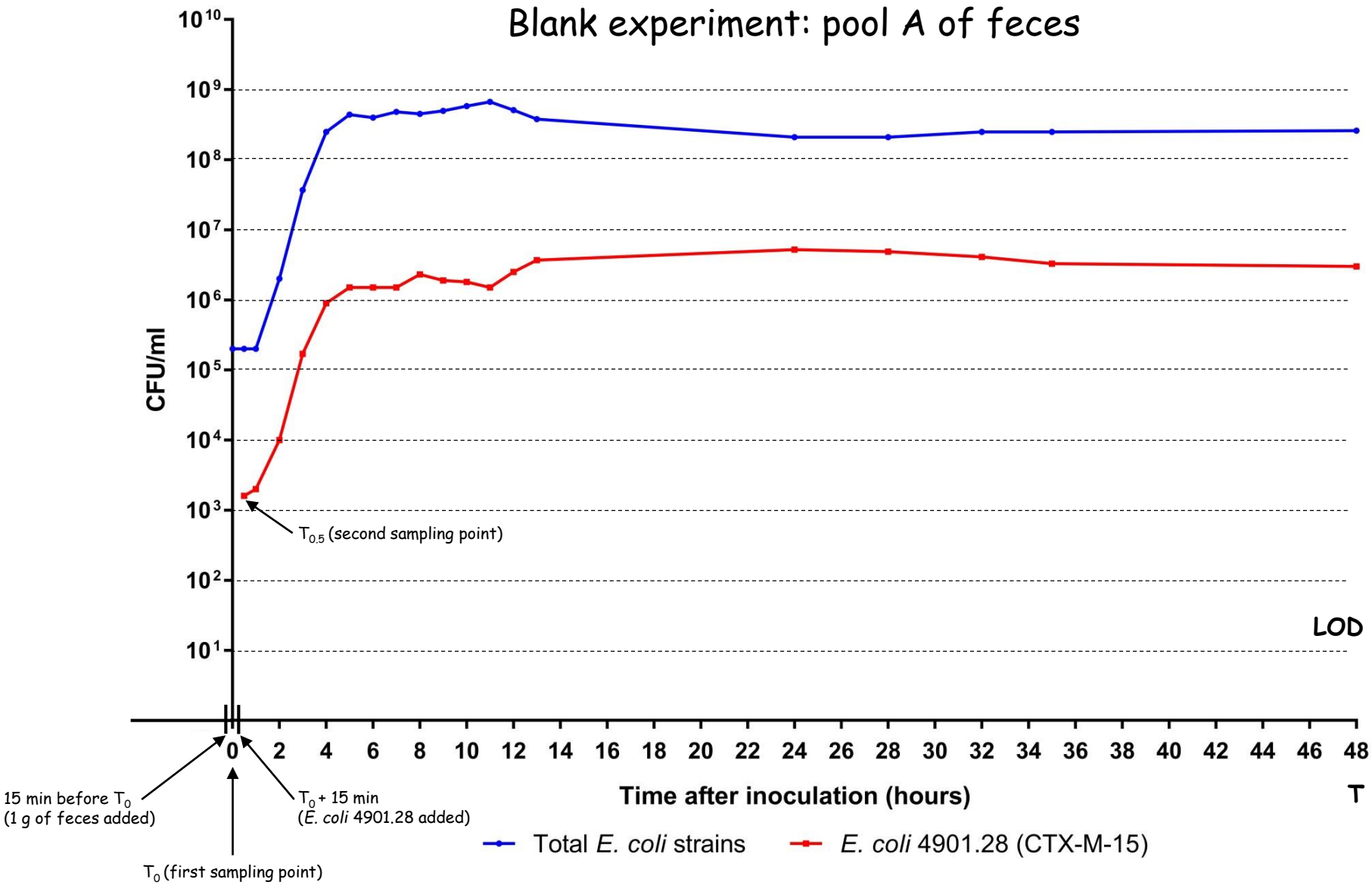

Figure 1. Blank experiment: *E. coli* dynamics without bacteriophage treatment. Dynamics of fecal *E. coli* community and of *E. coli* 4901.28 alone in the chemostat system in the absence of bacteriophages (pool A of feces, as for Study I). Feces were inoculated into the chemostat 15 min before T_0 (that was the first sampling point). Blue line: total *E. coli* population; red line: CTX-M-15-producing *E. coli* ST131 4901.28. LOD: limit of detection (10^1 CFU/mL). Graph generated with GraphPad Prism 7 on data from one experiment.

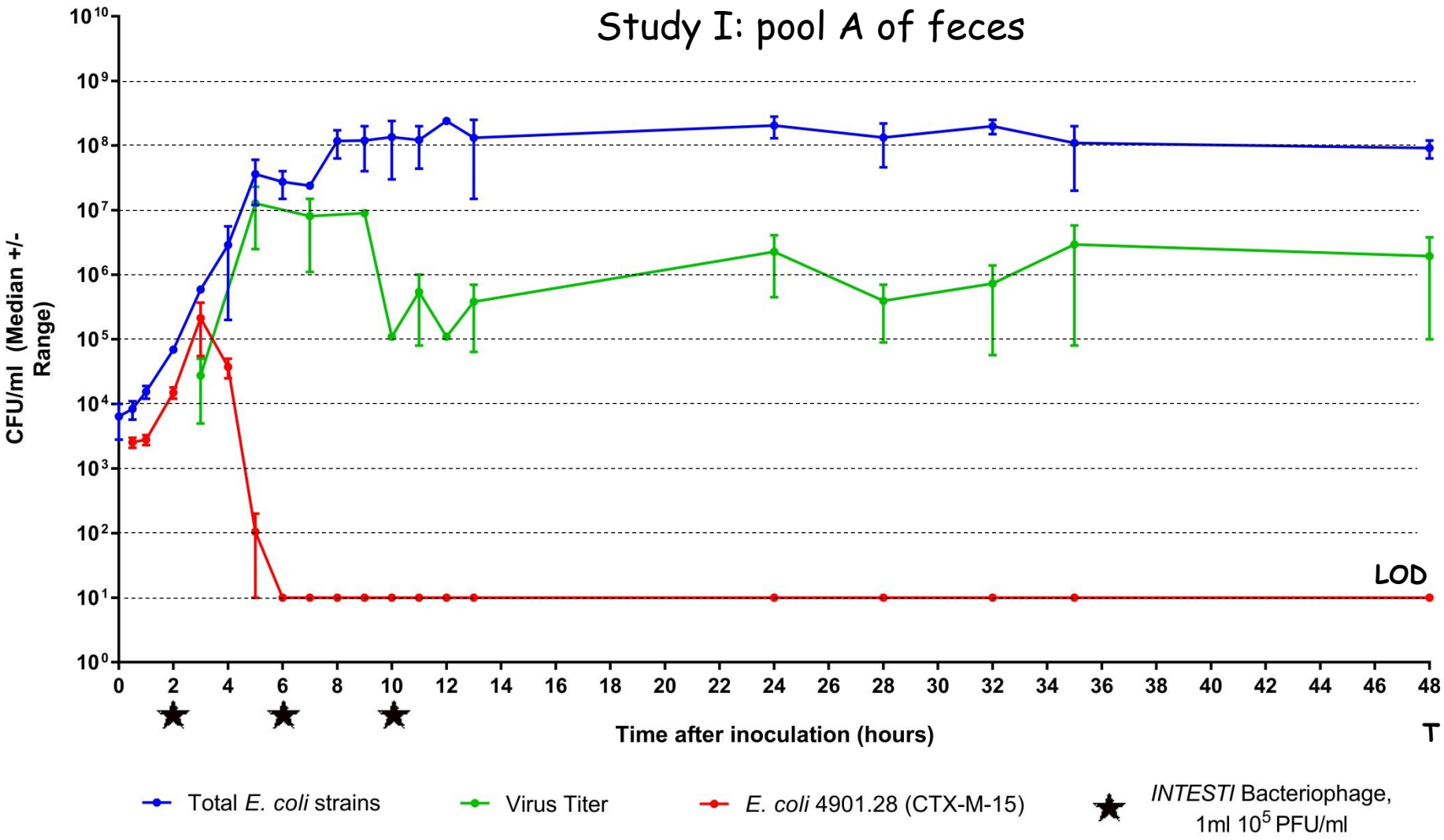

Figure 2. Study I: *E. coli* dynamics with 3 doses of *INTESTI Bacteriophage* cocktail and pool A of feces. Influence of bacteriophage treatment on the fecal *E. coli* community and on *E. coli* 4901.28 performed in the chemostat system with the first pool (A) of feces. Blue line: total *E. coli* population; red line: CTX-M-15-producing *E. coli* ST131 4901.28.; black stars, administered bacteriophage doses. LOD: limit of detection (10^1 CFU/mL). Graph generated with GraphPad Prism 7 on data from two experiments. Appearance: median and error. Plot: range. Error bars not drowned by the software when shorter than the height of the symbol.

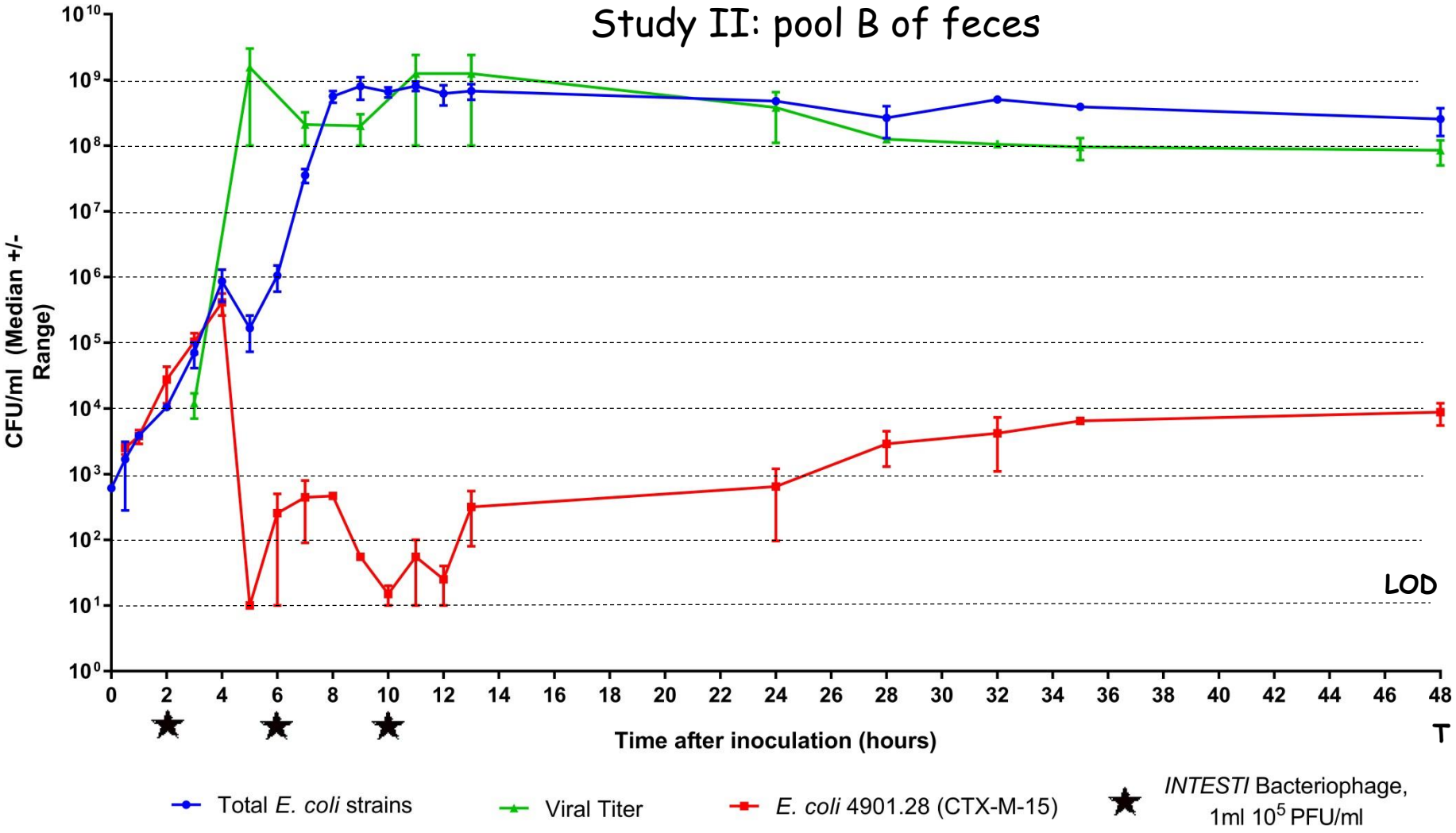

Figure 3. Study II: *E. coli* dynamics with 3 doses of *INTESTI Bacteriophage* cocktail and pool B of feces. Influence of bacteriophage treatment on the fecal *E. coli* community and on *E. coli* 4901.28 performed in the chemostat system with the second pool (B) of feces. Feces were inoculated into the chemostat 15 min before T_0 (that was the first sampling point). Blue line: total *E. coli* population; red line: CTX-M-15-producing *E. coli* ST131 4901.28.; black stars, administered bacteriophage doses. LOD: limit of detection (10^1 CFU/mL). Graph generated with GraphPad Prism 7 on data from one duplicate experiment. Appearance: median and error. Plot: range. Error bars not drowned by the software when shorter than the height of the symbol.