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Matthieu Galtier,[1,2*] Luisa De Sordi,[1*] Adeline Sivignon,[3] Amélie de Vallée,[3] Damien Maura,[1,2,4] Christel Neut,[5,6] Oumaira Rahmouni,[5,6] Kristin Wannerberger, [7] Arlette Darfeuille-Michaud,[3] Pierre Desreumaux, [6,8] Nicolas Barnich,[3] Laurent Debarbieux[1]

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*: these authors contributed equally to this work

1: Institut Pasteur, Biologie Moléculaire du Gène chez les Extrêmophiles, Département de Microbiologie, F-75015 Paris, France

2: Université Paris Diderot, Sorbonne Paris Cité, Cellule Pasteur, rue du Docteur Roux, F-75015 Paris, France

3: Université Clermont Auvergne, UMR 1071 Inserm/Université d'Auvergne, INRA, Unité Sous Contrat 2018, Clermont-Ferrand, France

4: Present address: Department of Surgery, Harvard Medical School and Massachusetts General Hospital, Boston, 02114, Massachusetts, United States of America.

5: Division de Bacteriologie, Faculté des Sciences Pharmaceutiques et Biologiques, Communauté d'Universités et d'Etablissements Lille Nord de France, F-59006 Lille Cedex, France.

6: Inserm U995 - LIRIC - Lille Inflammation Research International Center, F-59000 Lille, France

7 : Ferring International Center SA, Ch. de la Vergognausaz 50, 1162 St-Prex, Suisse

8 : CHU Lille, Service des Maladies de l'Appareil Digestif et de la Nutrition, Hôpital Claude Huriez, F-59037 Lille, France

Correspondence to

Laurent Debarbieux

Institut Pasteur, Department of Microbiology, F-75015 Paris, France

Phone: (33) 1 44 38 92 03 - Fax: (33) 1 45 68 88 34

e-mail: laurent.debarbieux@pasteur.fr

Abstract

Background and Aims

Adherent invasive *Escherichia coli* (AIEC) are abnormally predominant on the ileal mucosa of Crohn's disease (CD) patients. They bind to the CEACAM6 receptor expressed on the surface of epithelial cells. We aimed to assess the potential of bacteriophages, viruses infecting bacteria, to decrease the levels of AIEC bacteria associated with the intestinal mucosa.

Methods

We combined *ex vivo* and *in vivo* experiments with murine and human intestinal samples to quantify the ability of virulent bacteriophages to target the prototype AIEC strain LF82.

Results

We found that three virulent bacteriophages were able to replicate in ileal, cecal and colon sections and feces homogenates from murine gut samples colonized with the prototype AIEC strain LF82. A single day of *per os* treatment with the three bacteriophages cocktail given to LF82-colonized CEABAC10 transgenic mice, expressing the human CEACAM6 receptor for AIEC, decreased significantly the number of AIEC in feces and in the adherent flora of intestinal sections. In addition, a single dose of the cocktail reduced over a two-week period DSS-induced colitis symptoms on conventional mice colonized with the strain LF82. The cocktail targeted also LF82 bacteria in homogenates of ileal biopsies taken from CD patients.

Conclusions

These findings demonstrate that bacteriophages are a new treatment option for targeting AIEC in CD patients and represent a strong basis for a clinical trial evaluation.

Introduction

Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are chronic and relapsing, life-long diseases of the gastrointestinal tract (GIT). The precise cause of IBD is unknown, but this multifactorial disease is thought to result from the complex interplay between environmental factors and genetic susceptibility, resulting in an abnormal mucosal immune response [1]. Genetic studies of CD have uncovered new mechanisms involved in the pathogenesis of this disease, including defects of a biological process called autophagy (ATG16L1 and IRGM genes), innate mucosal defense (NOD2 gene), or endoplasmic reticulum stress (Xbp1 gene) [2]. The higher incidence of IBD in industrialized countries than in developing countries, in which the incidence of IBD is nevertheless increasing, clearly suggests that environmental factors make an important contribution to disease pathogenesis [3]. However, no strong environmental factors other than smoking and appendectomy have yet been identified for IBD [4]. The effects of diet have been studied in detail, but the findings of these studies have been inconsistent. However, an altered gut microbiota has long been suspected to play an important role in IBD pathogenesis [5,6].

In this context, the frequent recovery of particular strains of *Escherichia coli* with adherent and invasive properties (AIEC) from the mucosa of CD patients has attracted considerable attention. These bacteria constitute a new pathovar of *E. coli* lacking the type III secretion system and other classical virulence factors typically associated with pathogenic intestinal *E. coli* [7]. The prototype AIEC strain LF82 was isolated in 1998 from a chronic ileal lesion from a CD patient [8]. Strain LF82 has type 1 pili that can bind to the host adhesion receptor carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6), which is more strongly expressed in the ileal tissues of CD patients than in those of controls [9]. In transgenic CEABAC10 mice with high levels of expression of human CEACAMs, LF82

efficiently binds to epithelial cells and colonizes gut mucosa in a type 1 pilus-dependent manner [10].

Several therapeutic strategies targeting AIEC colonization have been proposed, to slow, or even halt the natural course of CD. Manipulation of the patient's microbiota through antibiotic treatment, fecal transplantation, nutritional interventions or the administration of pre/probiotics could be used either alone or in combination with immunotherapy, to induce the remission of active disease or as a postoperative treatment to prevent relapse. However, the results obtained to date have been disappointing [11]. Antibiotic treatments are becoming less effective, due to the increasing prevalence of multidrug resistance among bacteria, as highlighted by the World Health Organization [12]. Over the last 10 years, *E. coli* has acquired worryingly high levels of resistance to multiple antibiotics, with rapid dissemination worldwide, as exemplified by the epidemic clonal complex *E. coli* ST131-O25b:H4 and more recently by the spread of plasmids carrying resistance to colistin (MCR-1), one of the last resort antibiotics [13,14]. Dissemination occurs mostly through the digestive tract carriage of *E. coli* strains by mammals, including humans traveling between countries [15,16]. The human digestive tract is also an environment in which bacteria can exchange genetic material, including antibiotic resistance genes, and this has led to strong recommendations that antibiotics should not be used in a prophylactic manner [17].

An evaluation of non-conventional antibacterial treatments, such as those based on bacteriophages, is therefore required to deal with this major public health issue. Bacteriophages, viruses infecting bacteria, were discovered before antibiotics and have been used for many years in some countries (Georgia, Poland, Russia), in antibacterial treatments known as phage therapy [18-20]. Bacteriophages are omnipresent in the environment, including the human GIT, in which they are at least as numerous as bacteria [21,22]. Unlike antibiotics, they are highly specific, infecting only a limited number of strains within a given bacterial

species, and have, therefore, a much smaller impact on the composition of the microbiota [23-25]. In this study, we evaluated the potential of bacteriophages to decrease intestinal colonization with AIEC bacteria, in wild-type and CEACAM6-expressing mice. Our results demonstrating the high efficacy of bacteriophages in reducing intestinal colonization of AIEC strain LF82 strongly support their clinical evaluation in CD patients.

Materials and Methods

Ethics statement

BALB/cYJ mice (seven-week-old females) were supplied by Charles River Laboratories and housed in an animal facility in accordance with Institut Pasteur guidelines and European recommendations. FVB/N (Friend Virus B NIH Jackson) wild-type mice provided by Charles Rivers Laboratories, France, and FVB/N CEABAC10 heterozygous transgenic mice [26] were crossed in the animal care facility of the University of Auvergne (Clermont-Ferrand, France), in specific pathogen-free conditions. Food and drinking water were provided *ad libitum*. Protocols were approved by the veterinary staff of the Institut Pasteur animal facility (approval ID 10.565), the National Ethics Committee (approval number 2012-0018) and the Auvergne CEMEA ("Comité d'Ethique en Matière d'Expérimentation Animale") committee for ethical issue (permit CEMEAA CE16-09).

Bacterial strains and growth conditions

AIEC strain LF82 [8] was genetically modified to generate a streptomycin-resistant strain (LF82S), through the introduction of a point mutation into the *rpsL* gene, and then to add a kanamycin resistance gene to obtain strain LF82SK. The techniques used have been described in detail elsewhere [27,28]. Strains were routinely cultured in lysogeny broth (LB), or on LB agar or Drigalski agar plates, at 37°C. When required, streptomycin (100 µg/ml), kanamycin (50 µg/ml), ampicillin (50 µg/ml) or erythromycin (20 µg/ml) (Sigma, St. Louis, MO) was added.

Bacteriophage isolation, preparation and characterization

We isolated and purified several bacteriophages infecting strain LF82 from wastewater, with an enrichment technique [29]. Since AIEC bacteria do not belong to a specific phylogenetic group or serotype, we performed host-range determination on the *E. coli* Collection of Reference (ECOR), and selected three bacteriophages, LF82_P2, LF82_P6 and LF82_P8 showing complementary host range, and prepared large-scale solutions, as previously described (see online supplementary table S1) [29,30]. Bacteriophage titers were determined by spotting 4 µL drops of 10-fold serial dilutions onto LB agar plates covered with a lawn of strain LF82 or LF82SK. The three-bacteriophage cocktail contained equal numbers of each of the three bacteriophages, as indicated. These three bacteriophages are equally effective against strains LF82 and LF82SK and their genomes were sequenced with Illumina technology. For each genome, a single large contig was generated by reads assembly, and this contig was used to identify closely related bacteriophage genomes with the NCBI blastn tool (see online supplementary table S2).

Ex-vivo replication of individual bacteriophages in colonized gut samples

BALB/cYJ animals were kept in the animal facility for one week before experiments. Streptomycin sulfate (5 mg/ml) was added to the drinking water for three days before LF82SK administration, to disrupt the normal resident bacterial flora in the intestinal tract. Before introducing strain LF82SK, we collected feces from the mice and plated them on Drigalski agar plates, which were then incubated at 37°C for 24 hours. No bacterial colonies were observed. Mice were force-fed strain LF82SK (1×10^8 cfu) in 200 µl of sterile 20% sucrose and 2.6% sodium bicarbonate, pH 8. At the same time, the drinking water was replaced with an aqueous 0.5 mg/ml streptomycin sulfate solution. Three days later, intestinal sections from two mice were collected in aseptic conditions from LF82SK-colonized mice,

weighed (0.2 to 1 g/sample) and homogenized in 5 ml of PBS and pooled (Ultra Turrax T25, S25N-8G, IKA). Each bacteriophage (1 x 10^6 pfu) was added to homogenates (200 µl), which were then incubated for 5 h at 37°C. We then spotted 10-fold serial dilutions of samples onto LB agar plates covered with a lawn of strain LF82SK for the counting of bacteriophages.

Bacteriophage treatment in CEABAC10 mice

We assessed the colonization of the gut of CEABAC10 mice with LF82, using modified version of the method described by Carvalho *et al*. [10]. Briefly, 8- to 10-week-old FVB/N CEABAC10 transgenic mice were given dextran sulfate sodium (DSS, molecular mass = 36,000-50,000 daltons, MP Biomedicals) at a concentration of 0.25% in drinking water (previously shown to favor gut colonization of strain LF82 [10]), beginning three days before administration of the LF82 strain. Mice received a single dose of 5 mg of streptomycin (Euromedex) orally 24 h before oral challenge with 1 x 10^9 LF82 bacteria (day 0 of the experiment). On the next day, mice received PBS or the cocktail of three bacteriophages (3 x 10^7 pfu per mouse twice on the same day, 7 hours apart, in 200 µl of sterile 20% sucrose and 2.6% sodium bicarbonate, pH 8) by oral gavage. Fresh fecal samples were collected to assess the degree of colonization with strain LF82. One or four days after phage administration, the mice were euthanized by cervical dislocation under isoflurane anesthesia, for assessment of the numbers of LF82 bacteria associated with colonic or ileal tissues by direct and indirect methods, and for the determination of bacteriophage levels by the spotting of 10-fold serial dilutions onto LB agar plates covered with a lawn of strain LF82. Assessment of neutrophil influx was performed on 0.5 cm-sample of proximal colon tissues (50 mg/mL), thoroughly washed in PBS and homogenized in 0.5% hexadecyltrimethylammonium bromide (Sigma) 50 mM in PBS, (pH 6.0), freeze-thawed 3 times, sonicated, and centrifuged. Myeloperoxidase

(MPO) was assayed in the supernatant by adding 1 mg/mL of dianisidine dihydrochloride (Sigma) and $5x10^{-4}$% H_2O_2, and the change in optical density measured at 450 nm. Human neutrophil MPO (Sigma) was used as standard. One unit of MPO activity was defined as the amount that degraded 1.0 mmole of peroxide per minute at 25°C.

Bacteriophage treatment of DSS-induced colitis in wild-type mice

BALB/cYJ animals were treated as described above for the *ex-vivo* experiments, including LF82SK gavage ($1x10^8$ cfu in 200 µL). The concentration of streptomycin was then decreased to 0.5 mg/ml during the entire experiment, with renewal once per week. Three days after the introduction of LF82SK, DSS (2% final concentration) was added to the drinking water. This concentration of DSS was maintained for 25 days (renewed weekly) and caused mild symptoms of colitis, as shown by the disease activity index (DAI), which takes into account weight loss, stool consistency and the presence of blood in stools (Hemocult II test (SKD SARL) (see online supplementary table S3), with no effect on mouse survival. We established this protocol from preliminary experiments in which we showed that i) decreasing streptomycin concentration had no effect on the level of gut colonization with LF82SK (assessed from feces) for up to three weeks, and ii) 1% DSS did not induce major symptoms of colitis within 10 days, whereas 3% DSS had too strong an effect, resulting in the euthanasia of the animal within three days. We also found that, in the presence of 2% DSS, mild colitis symptoms appeared seven to nine days after gavage with LF82SK. Five or seven days after the addition of 2% DSS to the drinking water, we administered the cocktail of bacteriophages (3×10^7 pfu per mouse) to the mice by oral gavage. Fecal pellets were collected at the indicated time points, for assessment of the levels of both LF82SK and bacteriophages. The mice were euthanized 25 days after the administration of the LF82SK strain, to assess

LF82SK and bacteriophage levels in organs.

Quantification of bacteria and bacteriophages in colonized mice

Freshly collected fecal samples were weighed and homogenized in PBS (0.08 g/ml final concentration), serially diluted in PBS and 4 µl of each dilution was spotted onto Drigalski agar supplemented with streptomycin and kanamycin for the quantification of strain LF82SK, or LB agar supplemented with ampicillin and erythromycin for the quantification of strain LF82. The plates were incubated for 18 h at 37°C. When necessary, ileal or colonic sections of intestines were collected in aseptic conditions, weighed, homogenized in PBS and subjected to 10-fold serial dilution and plating, as described above. When indicated, the mice received 200 µl of the cocktail of bacteriophages containing a total of 3×10^7 pfu of bacteriophages. Bacteriophages were quantified by spotting 10-fold serial dilutions onto LB agar plates covered with a lawn of strain LF82 or LF82SK. The detection limit was 5×10^2 cfu/g or pfu/g of tissue or feces.

For tissue homogenates and feces, we found, as previously reported, that direct counts of *E. coli* colonies were not accurate, because of the abundance of bacteriophages, a problem that can be overcome by using an indirect method based on qPCR [31]. Tissue homogenates (200 µl) were incubated with a mixture of DNAse and RNAse (0.2 mg/ml and 0.6 mg/ml final concentration respectively, Sigma) for 30 minutes at 37°C, to eliminate nucleic acids from lysed cells. They were then inactivated by heating at 95°C for 10 minutes. Bacterial DNA was extracted with a Maxwell Tissue DNA kit and a Maxwell robot (Promega, USA). InstaGene matrix (Biorad) (1/10 final volume) was added to the samples to clean up the DNA before qPCR amplification. Amplification and detection were performed in 96-well plates (Abgene), with iQ Eva Green Supermix (Biorad). Each amplification reaction was performed in

duplicate, in a final volume of 25 µl, with a final concentration of 0.25 µM for each primer and 5 µl of extracted DNA (95°C for 10 min, followed by 45 cycles of 15 s at 95°C, 1 min at 60°C and 1.5 min at 72°C, and 80 cycles, beginning at 55°C, with a 0.5°C increase in each cycle, to collect melting curve data for analysis). *E. coli* 16S rDNA-specific primers and bacterial 16S rDNA universal primers were used in parallel in each PCR for the samples from the DSS-induced colitis model, whereas primers binding to the plasmid pMT (CCATTCATGCAGCAGCTCTTT and ATCGGACAACATTAGCGGTGT) were used for samples from the CEABAC10 mice [32].

Ex-vivo replication of the bacteriophage cocktail on ileal biopsies from CD patients

Six frozen biopsies (obtained from the biological collection DC 2008 642) [33] weighting 50 to 200 mg were homogenized in 0.5 to 2 ml of sterile PBS. An aliquot (50µl) of each homogenate was plated on Drigalski agar plates to evaluate the presence of endogenous viable Enterobacteria as well as bacteriophages infecting strain LF82SK, which both were found negative. Each homogenate was split into three aliquots of 100µl and 25µl of a bacterial suspension of strain LF82SK ($3.5x10^6$ cfu), washed three times in sterile water to eliminate traces of growing medium, was added to two aliquots and incubated 60min at 37°C without shaking. Then, 25µl of the phage cocktail ($1.8x10^4$ pfu) was added to half of the samples that received strain LF82SK as well as the samples that did not received it. Volumes were adjusted to 150µl with PBS. The same conditions were applied to strain LF82 growing in LB as control. Bacteriophages were enumerated after 5 and 24 hours of incubation at 37°C without shaking.

Statistical analysis

255 Data are expressed as means and SEM, and Mann–Whitney tests were carried out with Prism
256 6 software (Graphpad software, La Jolla, USA).

257

Results

Bacteriophages infect AIEC *in vitro* and *ex vivo* in colonized sections of mouse gut

Bacteriophages were isolated from wastewater and characterized *in vitro* (methods; see online supplementary table S1). We selected three bacteriophages (LF82_P2, LF82_P6 and LF82_P8) that infected both AIEC strains LF82 and LF82SK (an antibiotic-resistant derivative of strain LF82) and sequenced them. Their sequences showed that they are different to each other and revealed their similarity to previously described virulent bacteriophages (see online supplementary table S2). The ability of each bacteriophage to replicate in an intestinal environment was studied *ex vivo* in gut homogenates from the small and large intestines collected from LF82SK-colonized mice (figure 1) [31]. Bacteriophages LF82_P2 and LF82_P6 displayed similar profiles, with higher levels of amplification from ileal and colonic sections than from the cecum and feces, whereas amplification levels for LF82_P8 were high and similar for all gut sections.

Bacteriophages reduce the colonization of the gut by AIEC strain LF82 in transgenic mice expressing the human CEACAM6 glycoprotein

We then investigated the ability of a cocktail of these three bacteriophages (equal amounts of each bacteriophage) to infect adherent LF82 bacteria, in the CEABAC10 transgenic mouse model, which expresses the human CEACAM6 receptor for AIEC bacteria [10]. LF82-colonized mice received two 3 x 10^7 pfu doses of the cocktail on the same day, administered *per os* (bacteriophage group), or two doses of PBS (control group) (figure 2A). Twenty-four hours post-treatment, the concentration of strain LF82 in feces had fallen significantly, by two orders of magnitude, in the bacteriophage group. It remained significantly lower than that in the control group four days after treatment, without the administration of additional bacteriophages (figure 2B). Concomitantly we observed that the number of bacteriophages in

feces in the bacteriophage group decreases accordingly to the concentration of strain LF82, while in the group of non-colonized mice it had fallen below the detection threshold within 24 hours (figure 2C). These variations did not affect the weight of animals neither MPO levels in ileum and colon sections collected at day 5 (see online supplementary figure S1).

We also analyzed the adherent *E. coli* bacteria from ileal and colonic gut sections from both the bacteriophage and control groups, with a semi-quantitative real-time PCR method for bacterial quantification (the abundance of bacteriophages in samples has been shown to affect the accuracy of direct bacterial counts [31]). Twenty-four hours post-treatment, the levels of the adherent strain LF82 (figure 3A) were significantly lower in all the gut sections of the bacteriophage group than in those of the control group. Four days post-treatment, the differences between the two groups remained significant, other than for ileal sections (figure 3B). Thus, by direct and indirect methods, we showed that the administration of a bacteriophage cocktail on one day progressively reduced the level of LF82 colonization of the entire GIT over the following five days, despite the expression of the CEACAM6 receptor for AIEC.

Bacteriophages reduce colitis symptoms in conventional mice colonized with AIEC strain LF82SK

We moved one step closer to clinical settings, by investigating the relationship between the ability of bacteriophages to decrease colonization by AIEC and the control of intestinal colitis symptoms induced by DSS in conventional mice, by determining DAI score (methods). Three groups of mice colonized with strain LF82SK received three independent treatments (figure 4A). As a preliminary study showed that DAI score increased between seven and nine days after the administration of the LF82SK strain (methods), we investigated whether the

administration of a single dose of the bacteriophage cocktail (3 x 10^7 pfu) either before (on day 8; preventive treatment) or after (on day 10; curative treatment) LF82SK administration could affect colitis symptoms and LF82SK colonization levels. In the group receiving the preventive treatment, DAI score on day 10 was lower than that in the other two groups. It subsequently returned to background levels, and remained at this level for the next 15 days (figure 4B). In the group receiving the curative treatment on day 10, DAI had decreased four days post-treatment, but to a lesser extent than in the control group, and it remained low for the next 11 days (figure 4B). Mean fecal levels of strain LF82SK from day 7 to day 25 remained stable in the control group (from $3.6x10^9 \pm 2.2x10^9$ to $3.6x10^8 \pm 3.34x10^8$ cfu/g) but decreased by 6.000 (from $4.8x10^9 \pm 1.6x10^9$ to $7.7x10^5 \pm 1.1x10^6$ cfu/g) and 43.000 ($4.0x10^9 \pm 1.4x10^9$ to $9.3x10^4 \pm 1.1x10^5$ cfu/g) times respectively in the preventive and curative groups. Furthermore, these results were confirmed using molecular quantification of ileal and colonic sections collected on day 25 (figure 4 C,D). A single bacteriophage application therefore successfully decreased colitis symptoms and mediated the long-term decolonization of the GIT with AIEC.

Bacteriophages actively replicate on ileal biopsies from CD patients

In order to provide an evaluation of the potential of this cocktail to target AIEC strains in patients we measured its replication in six CD patients ileal biopsies spiked with strain LF82SK. Active replication of the cocktail was measured after 5 hours from bacteriophage administration (mean n-fold replication $9.43x10^3 \pm 2.07\ x10^3$) and after 24 hours (mean n-fold replication $3.22x10^4 \pm 6.31x10^3$) confirming the killing potential of the cocktail in such environment. Values were similar to those obtained with individual bacteriophages from ileal samples of LF82SK-colonized mice.

Discussion

Dysbiosis of the gut microbiota is now recognized as a hallmark of IBD. It leads to the overgrowth of enterobacteria, including *E. coli* strains from the AIEC group, which are particularly predominant [34]. Antibiotic treatments have been proposed but expose CD patients to the risk of adverse events, such as exacerbation of dysbiosis, with detrimental effects, such as promotion of the growth of pathogenic strains or an increase in inflammation. Antibiotic-resistant bacteria may also be selected, decreasing the chances of a successful treatment outcome. Furthermore, the long-term antibiotic treatments with ciprofloxacin and metronidazole frequently used to treat CD are associated with a high risk of adverse effects resulting in treatment intolerance. Phage therapy is not only a specific way to target pathogens with a lower impact on microbiota than antibiotics, it can also provide a solution to the problem of antibiotic-resistant strains [24,25,35,36]. In this study, we investigated the activity of a cocktail of three bacteriophages targeting AIEC strain LF82. The choice of these bacteriophages was guided to obtain the largest host range towards the ECOR collection, which embraces the genetic diversity of *E. coli* genus, as AIEC strains do not belong to a particular genotype or serotype. An complementary strategy could consist on using a collection of characterized AIEC strains to implement this initial cocktail towards CD patients strains.

We showed *ex vivo* that each bacteriophage was able to replicate in the GIT. As in similar experiments performed previously with six bacteriophages infecting two different strains of *E. coli*, we found that each of the three LF82 bacteriophages replicated in gut sections [25,31]. These three LF82-bacteriophages belong to the *Myoviridae* family of viruses, whereas the bacteriophages previously reported to have a lower performance belong to the *Podoviridae* and *Siphoviridae* families. However, we found that all nine bacteriophages tested *ex vivo* to

358 date replicated strongly in the ileal section of the GIT, suggesting that this compartment does
359 not restrict bacterial permissivity to bacteriophage infection as also supported by the high
360 level of bacteriophage cocktail replication in ileal biopsies of CD patients. On the other hand,
361 in other mouse gut sections, bacteriophage replication was highly variable. Therefore, the
362 biogeography of the GIT probably affects bacteriophage replication, possibly due to
363 differences in bacterial physiology between different GIT sections, which in addition display
364 different mucus layers that are associated with specific microbiota [37-40]. Also, recent
365 evidences showed that some bacteriophages bind mucus which could affect their persistence
366 in the GIT [41,42].
367
368 Using CEABAC10 mice, we showed that the cocktail of bacteriophages strongly decreased
369 intestinal colonization by strain LF82, suggesting that bacteriophages were able to reach the
370 epithelial surface bearing the CEACAM6 receptor of strain LF82. This strong decrease was
371 observed with both CEABAC10 and conventional mice in the absence and presence of
372 antibiotic pressure, respectively. These reduced colonization levels, obtained with a single
373 application of the cocktail, were highly significant (relative to the control groups) and they
374 persisted for at least two weeks, corresponding to the highest efficacy ever reported in
375 experimental models of intestinal colonization, perhaps because we used a cocktail of three
376 *Myoviridae* [25,31,43,44]. Thus, one of the key characteristics and advantages of bacteriophages
377 over antibiotics, their ability to self-amplify and increase their own concentrations locally,
378 translated directly into a decrease in the concentration of the target bacteria in the GIT. Self-
379 limitation was also observed, as the number of bacteriophages decreased over time, with
380 decreasing levels of their target. In addition, we found that the decrease in AIEC levels
381 mediated by bacteriophage treatment was accompanied by a decrease in colitis symptoms, a
382 promising result for possible future clinical applications.

The safety of bacteriophages targeting intestinal pathogens is well documented for adults and infants, based on data for a clinical trial on diarrheal diseases, in which no adverse events were reported [23,24,45]. This is not surprising, as bacteriophages are the most abundant viruses present in the human GIT, being part of our natural microbiota, and are far more abundant than viruses infecting eukaryotic cells [46,47]. Recent virome studies on samples from IBD patients showed that the microbiota of these patients displayed an altered bacteriophage composition, raising questions about the possible role of these viruses in the disease [48]. Most of the bacteriophages detected were temperate (as opposed to virulent), suggesting a possible role in dynamic interactions between bacteria, possibly leading to genetic exchanges [49]. The bacteriophages recommended for therapeutic applications, such as those reported here, are virulent, and our experimental data support their use for targeting AIEC strains as part of a gentle approach to reestablishing eubiosis in CD patients, possibly extending the intervals between relapses.

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Conflict of interest statement

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Figures and Legends

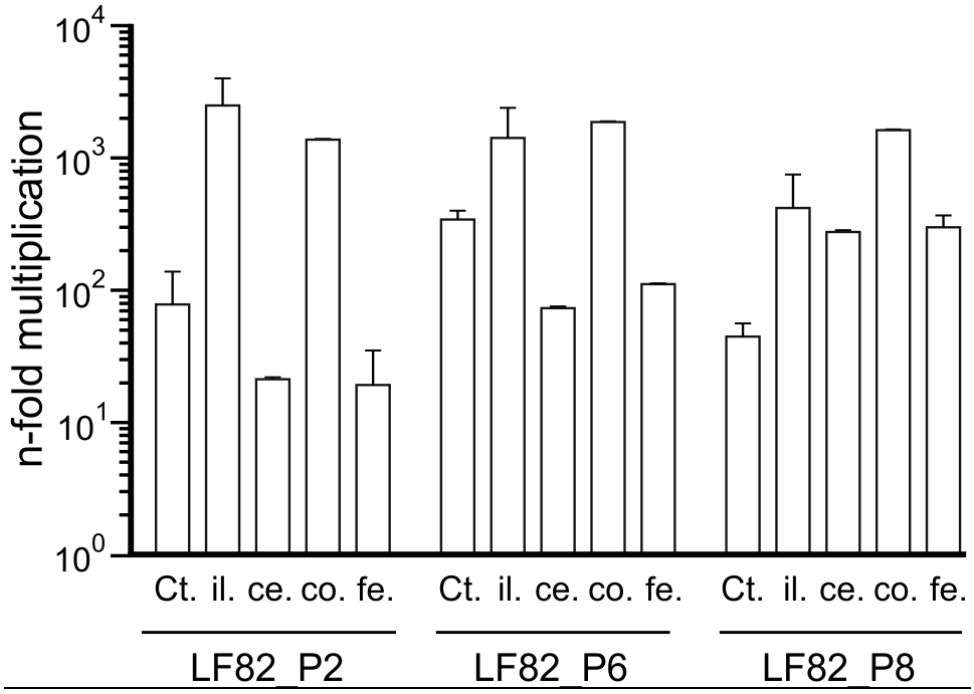
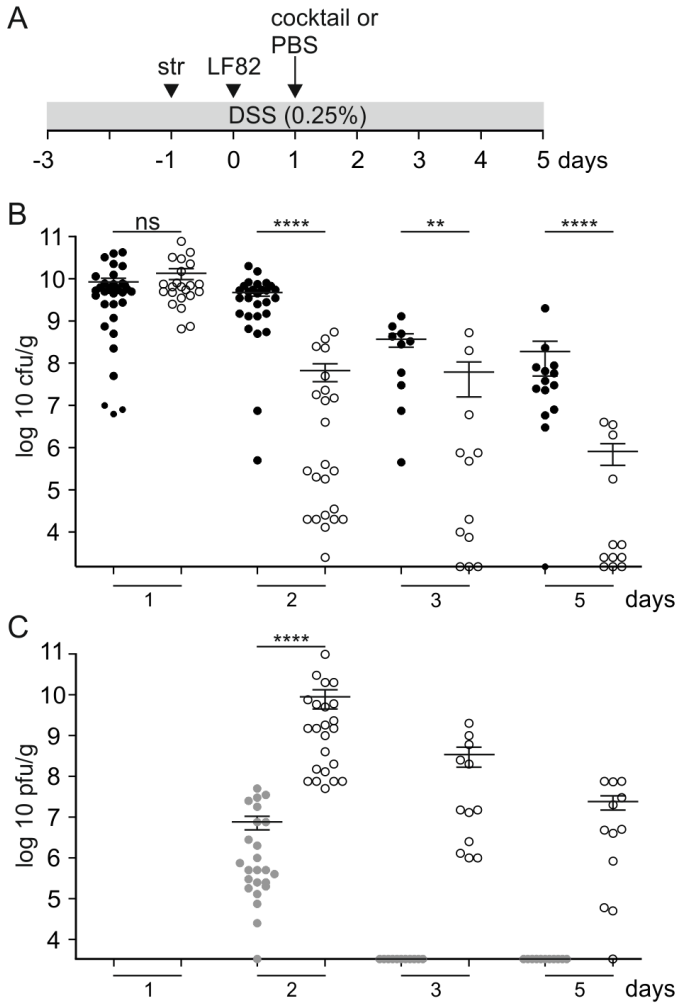


Figure 1 The three bacteriophages replicate *ex vivo* in all gut sections

Ileal (il.), cecal (ce.), colonic (co.) and fecal (fe.) gut sections collected from two LF82SK-colonized mice over a three-day period, and LB medium containing exponentially growing LF82SK (Ct), were mixed with each individual bacteriophages at an MOI of 0.01 in three replicates. Number of plaques formed on LF82SK lawns from the corresponding supernatants, following 5 hours of incubation at 37°C, plotted as n-fold multiplication relative to the initial number of bacteriophages added.



545

546 **Figure 2 Levels of the AIEC strain LF82 in feces are reduced by bacteriophage**
547 **treatment**

548 (A) Experimental setting, indicating the administration of streptomycin (str, 5 mg per mouse
549 continuously exposed to 0.25% DSS) 24 hours before oral gavage with the AIEC strain LF82
550 (1 x 10^9 bacteria per mouse) followed by administration of either PBS or a cocktail of the
551 three bacteriophages (3 x 10^7 pfu per mouse, administered twice on the same day, 7 hours
552 apart).

553 (B) Levels of the AIEC strain LF82 (CFU) recovered from the feces of LF82-colonized mice
554 receiving either PBS (black; *n*=13 to 30) or the bacteriophage cocktail (white; *n*=12 to 23), at
555 various times post-infection.

556 (C) Levels of the bacteriophage cocktail (PFU) in the feces of LF82-free (gray; *n*=24 to 12)
557 and LF82-colonized (white; *n*=12 to 23) mice.

558 ** = $p<0.01$ and **** = $p<0.0001$

559

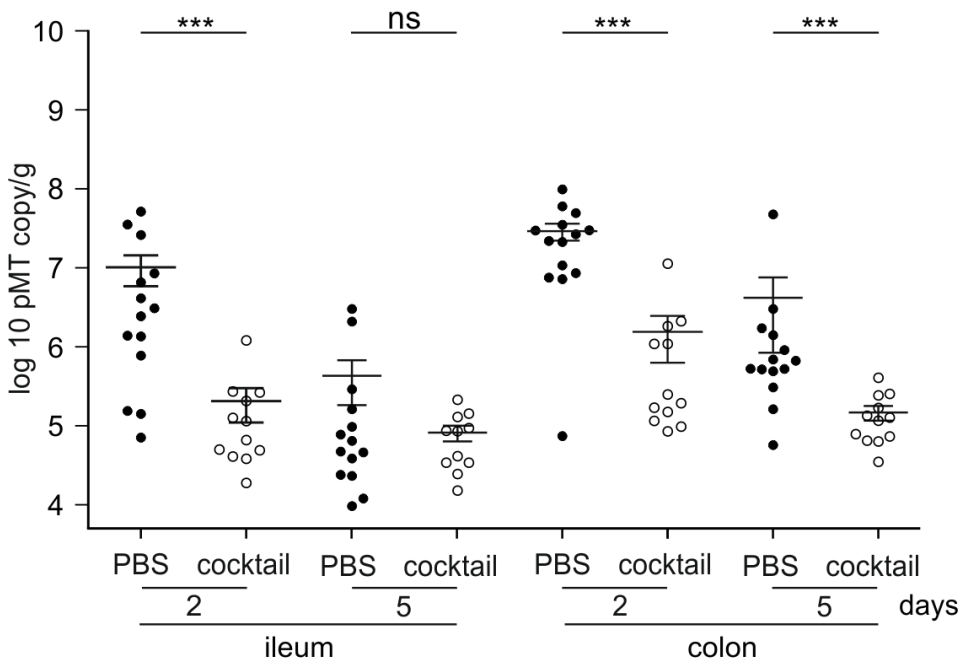
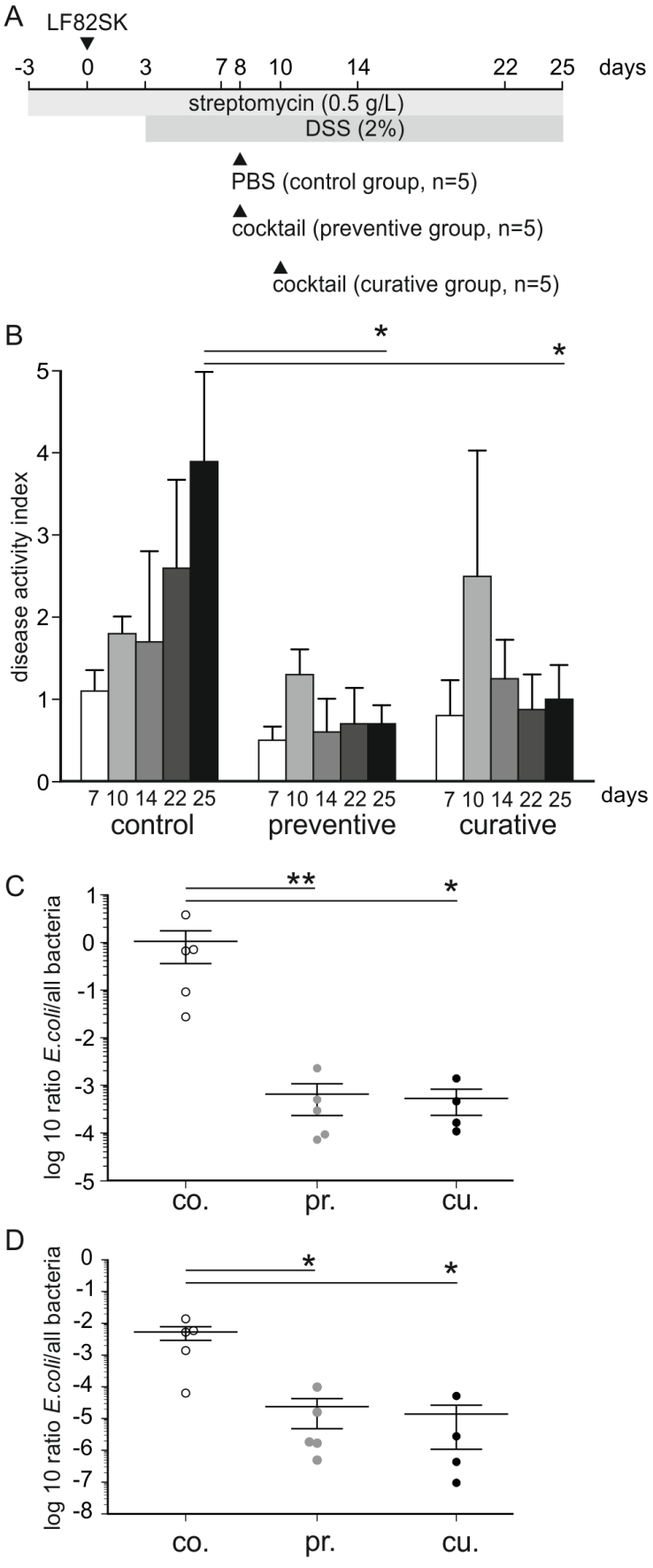


Figure 3 The bacteriophage cocktail strongly reduces the colonization of the entire gut with the AIEC strain LF82

Analysis of the LF82 content of intestinal sections collected on days 2 and 5 from mice (n=6 to 14) that received either PBS (black) or the cocktail of three bacteriophages (white; 3 x 10^7 pfu per mouse) at the indicated time (same experimental setting that in figure 2A), assessed by qPCR with primers binding to the pMT plasmid. ns = not significant; ** = $p<0.01$; *** = $p<0.005$


A
LF82SK
-3
0
3
7 8
10
14
22
25
days
streptomycin (0.5 g/L)
DSS (2%)
PBS (control group, n=5)
cocktail (preventive group, n=5)
cocktail (curative group, n=5)
B
*
*
disease activity index
0
1
2
3
4
5
7 10 14 22 25
control
7 10 14 22 25
preventive
7 10 14 22 25
curative
days
C
**
*
log 10 ratio E.coli/all bacteria
1
0
-1
-2
-3
-4
-5
co.
pr.
cu.
D
*
*
log 10 ratio E.coli/all bacteria
0
-1
-2
-3
-4
-5
-6
-7
-8
co.
pr.
cu.

570

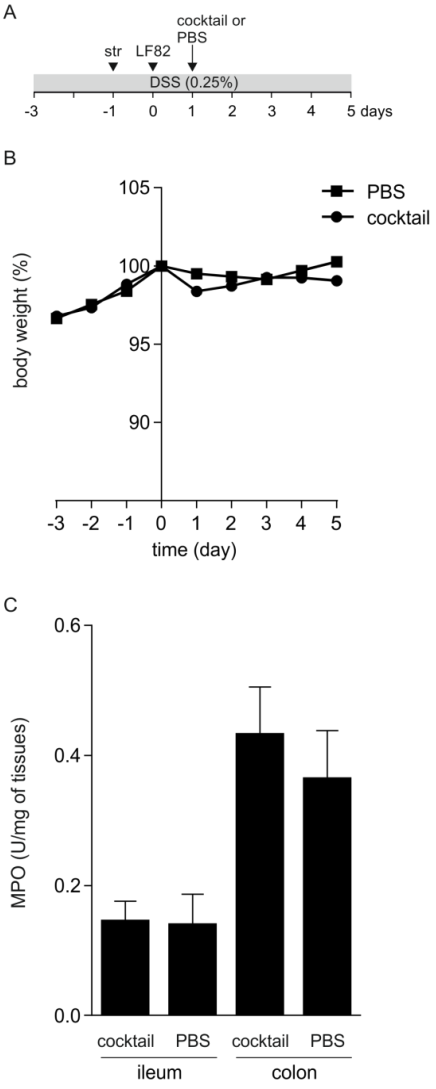
Figure 4 Preventive and curative single administrations of bacteriophages reduce both DSS-induced colitis symptoms and AIEC colonization

(A) Schematic diagram of the experiment with three groups of mice (*n*=5) receiving either PBS or a single dose of the cocktail on day 8 (3 x 10^7 pfu) or a single dose of the cocktail (3 x 10^7 pfu) on day 10.

(B) The disease activity index was recorded at the indicated time points and plotted for each group of mice (control, preventive and curative).

(C, D) On day 25, ileal (C) and colonic (D) gut sections were analyzed by qPCR to quantify *E. coli* relative to the total number of bacteria for each mouse. * = $p<0.05$; ** = $p<0.01$.

582 **Supplementary data**



583

584 **Figure S1** Evolution of body weight and level of MPO of CEABAC10 mice colonized with
585 strain LF82 and receiving either PBS or a the cocktail of bacteriophages

586 (A) Experimental setting, indicating the administration of streptomycin (str, 5 mg per mouse
587 continuously exposed to 0.25% DSS) 24 hours before oral gavage with the AIEC strain LF82
588 (1 x 10^9 bacteria per mouse) followed by administration of either PBS or a cocktail of the
589 three bacteriophages (3 x 10^7 pfu per mouse, administered twice on the same day, 7 hours
590 apart).

591 (B) Evolution of body weight of the two groups of CEABAC10 mice colonized with strain
592 LF82 and receiving either PBS (n=13) or the cocktail of bacteriophages (n=12). For clarity
593 SEM are not indicated but were all below 4%.

594 (C) MPO levels measured at day 5 from ileum and colon sections taken from CEABAC10
595 mice receiving either PBS (n=13) or the cocktail (n=12)

596 **Table S1** Host range of the three LF82-specific bacteriophages on the *Escherichia coli* Collection of

597 Reference (ECOR)

598

LF82_	**P2**	**P6**	**P8**
LF82SK	+++*	+++	+++
ECOR 1	+++	+++	-
ECOR 9	-	-	+++
ECOR 16	+++	+++	+++
ECOR 17	-	-	+++
ECOR 24	++	-	-
ECOR 25	-	-	+++
ECOR 26	-	-	+++
ECOR 27	-	-	++
ECOR 28	-	-	+++
ECOR 29	-	-	+++
ECOR 30	-	-	+++
ECOR 34	-	-	+++
ECOR 35	-	-	+++
ECOR 36	-	-	+++
ECOR 39	++	-	+++
ECOR 42	+++	+++	-
ECOR 43	-	-	+++
ECOR 44	-	-	+++
ECOR 46	-	-	+++
ECOR 48	+++	-	-
ECOR 55	-	-	++
ECOR 56	+++	-	-
ECOR 63	+++	+++	+++
ECOR 70	-	-	+++
ECOR 71	+++	+++	-

599

600 *, +++: 0.1 > EOP > 1; ++: 0.001 > EOP > 0.1; + 0.00001 > EOP > 0.001; - EOP = 0. The ECOR

601 strains not indicated in this table (47 in total) were not infected by any of the three bacteriophages.

602

Table S2 Bacteriophages closely related to LF82_P2, LF82_P6 and LF82_P8 (genomes with more than 90% query coverage in Megablast nucleotide analysis, ranked in decreasing order of query cover, are indicated).

Homologs to LF82_P2						
Description	**Max score**	**Total score**	**Query cover**	**E value**	**Identity**	**Accession**
Escherichia phage vB_EcoM-VpaE1	37362	1.304e+05	93%	0.0	97%	KM657822.1
Escherichia phage JH2	26005	1.257e+05	93%	0.0	97%	KF055347.1
Escherichia phage vB_EcoM_AYO145A	37742	1.325e+05	92%	0.0	96%	KR014248.1
Enterobacteriaphage UAB_Phi87	36509	1.312e+05	92%	0.0	97%	JN225449.1
Escherichia coli O157 typing phage 12	34743	1.319e+05	92%	0.0	95%	KP869110.1
Escherichia coli O157 typing phage 11	34743	1.319e+05	92%	0.0	95%	KP869109.1
Escherichia coli O157 typing phage 8	34743	1.316e+05	92%	0.0	95%	KP869106.1
Enterobacteria phage WV8	34736	1.316e+05	92%	0.0	95%	EU877232.1
Escherichia coli O157 typing phage 15	34322	1.321e+05	92%	0.0	95%	KP869113.1
Escherichia coli O157 typing phage 1	20473	1.316e+05	92%	0.0	97%	KP869100.1
Bacteriophage Felix 01	36784	1.268e+05	90%	0.0	96%	AF320576.1
Salmonella phage Mushroom	36714	1.291e+05	90%	0.0	96%	KP143762.1
Escherichia phage HY02	35336	1.277e+05	90%	0.0	94%	KM092515.1
Homologs to LF82_P6						
Description	**Max score**	**Total score**	**Query cover**	**E value**	**Identity**	**Accession**
Escherichia phage vB_EcoM-VpaE1	37877	1.330e+05	94%	0.0	97%	KM657822.1
Escherichia coli O157 typing phage 12	34553	1.354e+05	94%	0.0	94%	KP869110.1
Escherichia coli O157 typing phage 11	34553	1.358e+05	94%	0.0	94%	KP869109.1
Escherichia coli O157 typing phage 8	34553	1.353e+05	94%	0.0	94%	KP869106.1
Enterobacteria phage WV8	34551	1.353e+05	94%	0.0	94%	EU877232.1
Escherichia coli O157 typing phage 15	34188	1.359e+05	94%	0.0	94%	KP869113.1
Escherichia coli O157 typing phage 1	20255	1.341e+05	94%	0.0	97%	KP869100.1
Escherichia phage vB_EcoM_AYO145A	38753	1.329e+05	93%	0.0	96%	KR014248.1
Escherichia phage HY02	36963	1.291e+05	93%	0.0	94%	KM092515.1
Enterobacteriaphage UAB_Phi87	36954	1.320e+05	93%	0.0	97%	JN225449.1
Salmonella phage Mushroom	36937	1.284e+05	92%	0.0	97%	KP143762.1
Escherichia phage JH2	27069	1.248e+05	92%	0.0	97%	KF055347.1
Bacteriophage Felix 01	36996	1.256e+05	91%	0.0	97%	AF320576.1
Salmonella phage HB-2014	25418	1.238e+05	91%	0.0	97%	KP010413.1
Homologs to LF82_P8						
Description	**Max score**	**Total score**	**Query cover**	**E value**	**Identity**	**Accession**
Escherichia phage vB_EcoM_JS09	69335	2.754e+05	97%	0.0	97%	KF582788.1
Escherichia coli O157 typing phage 3	40680	2.760e+05	97%	0.0	97%	KP869101.1
Enterobacteria phage HX01	34452	2.661e+05	95%	0.0	97%	JX536493.1
Enterobacteria phage RB69	36212	2.406e+05	93%	0.0	97%	AY303349.1
Escherichia phage vB_EcoM_PhAPEC2	32398	2.453e+05	93%	0.0	97%	KF562341.1
Escherichia coli O157 typing phage 13	31852	2.647e+05	93%	0.0	98%	KP869111.1
Shigella phage Shf125875	30204	2.442e+05	93%	0.0	96%	KM407600.1
Escherichia coli O157 typing phage 6	40686	2.638e+05	92%	0.0	97%	KP869104.1
Escherichia phage EC6	25281	1.224e+05	90%	0.0	97%	JX560968.1

608 **Table S3** Disease activity index

	Score	Criteria
Body weight loss	0	no loss
	1	1 to 5%
	2	5 to 10 %
	3	10 to 20%
	4	>20%
Stool consistency	0	normal feces
	1	loose stools
	2	watery diarrhea
	3	slimy diarrhea, little diarrhea
	4	severe watery diarrhea with blood
Blood in stools	0	no blood
	1	weakly positive Hemoccult test
	2	moderately positive Hemoccult test
	3	highly positive Hemoccult test
	4	visible blood

609

610