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1 **Bacteriophages targeting adherent invasive *Escherichia coli* strains as a promising new**
2 **treatment for Crohn's disease**

3

4 Matthieu Galtier,^{1,2*} Luisa De Sordi,^{1*} Adeline Sivignon,³ Amélie de Vallée,³ Damien
5 Maura,^{1,2,4} Christel Neut,^{5,6} Oumaira Rahmouni,^{5,6} Kristin Wannerberger,⁷ Arlette Darfeuille-
6 Michaud,³ Pierre Desreumaux,^{6,8} Nicolas Barnich,³ Laurent Debarbieux¹

7

8 Running title: Phage therapy for Crohn's disease

9

10 *: these authors contributed equally to this work

11 1: Institut Pasteur, Biologie Moléculaire du Gène chez les Extrêmophiles, Département de
12 Microbiologie, F-75015 Paris, France

13 2: Université Paris Diderot, Sorbonne Paris Cité, Cellule Pasteur, rue du Docteur Roux, F-
14 75015 Paris, France

15 3: Université Clermont Auvergne, UMR 1071 Inserm/Université d'Auvergne, INRA, Unité
16 Sous Contrat 2018, Clermont-Ferrand, France

17 4: Present address: Department of Surgery, Harvard Medical School and Massachusetts
18 General Hospital, Boston, 02114, Massachusetts, United States of America.

19 5: Division de Bacteriologie, Faculté des Sciences Pharmaceutiques et Biologiques,
20 Communauté d'Universités et d'Établissements Lille Nord de France, F-59006 Lille Cedex,
21 France.

22 6: Inserm U995 - LIRIC - Lille Inflammation Research International Center,
23 F-59000 Lille, France

24 7 : Ferring International Center SA, Ch. de la Vergognausaz 50, 1162 St-Prex, Suisse

25 8 : CHU Lille, Service des Maladies de l'Appareil Digestif et de la Nutrition, Hôpital Claude

26 Huriez, F-59037 Lille, France

27

28 Correspondence to

29 Laurent Debarbieux

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

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

32 e-mail: laurent.debarbieux@pasteur.fr

33

34 **Abstract**

35 Background and Aims

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

41 Methods

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

44 Results

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

53 Conclusions

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

56

57

58 **Introduction**

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

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

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

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

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

107 species, and have, therefore, a much smaller impact on the composition of the microbiota²³⁻²⁵.
108 In this study, we evaluated the potential of bacteriophages to decrease intestinal colonization
109 with AIEC bacteria, in wild-type and CEACAM6-expressing mice. Our results demonstrating
110 the high efficacy of bacteriophages in reducing intestinal colonization of AIEC strain LF82
111 strongly support their clinical evaluation in CD patients.
112

113 **Materials and Methods**

114 **Ethics statement**

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

125

126 **Bacterial strains and growth conditions**

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

133

134 **Bacteriophage isolation, preparation and characterization**

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

148

149 ***Ex-vivo* replication of individual bacteriophages in colonized gut samples**

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

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

163

164 **Bacteriophage treatment in CEABAC10 mice**

165 We assessed the colonization of the gut of CEABAC10 mice with LF82, using modified
166 version of the method described by Carvalho *et al.*¹⁰. Briefly, 8- to 10-week-old FVB/N
167 CEABAC10 transgenic mice were given dextran sulfate sodium (DSS, molecular mass =
168 36,000-50,000 daltons, MP Biomedicals) at a concentration of 0.25% in drinking water
169 (previously shown to favor gut colonization of strain LF82¹⁰), beginning three days before
170 administration of the LF82 strain. Mice received a single dose of 5 mg of streptomycin
171 (Euromedex) orally 24 h before oral challenge with 1×10^9 LF82 bacteria (day 0 of the
172 experiment). On the next day, mice received PBS or the cocktail of three bacteriophages ($3 \times$
173 10^7 pfu per mouse twice on the same day, 7 hours apart, in 200 μ l of sterile 20% sucrose and
174 2.6% sodium bicarbonate, pH 8) by oral gavage. Fresh fecal samples were collected to assess
175 the degree of colonization with strain LF82. One or four days after phage administration, the
176 mice were euthanized by cervical dislocation under isoflurane anesthesia, for assessment of
177 the numbers of LF82 bacteria associated with colonic or ileal tissues by direct and indirect
178 methods, and for the determination of bacteriophage levels by the spotting of 10-fold serial
179 dilutions onto LB agar plates covered with a lawn of strain LF82. Assessment of neutrophil
180 influx was performed on 0.5 cm-sample of proximal colon tissues (50 mg/mL), thoroughly
181 washed in PBS and homogenized in 0.5% hexadecyltrimethylammonium bromide (Sigma) 50
182 mM in PBS, (pH 6.0), freeze-thawed 3 times, sonicated, and centrifuged. Myeloperoxidase

183 (MPO) was assayed in the supernatant by adding 1 mg/mL of dianisidine dihydrochloride
184 (Sigma) and $5 \times 10^{-4}\%$ H_2O_2 , and the change in optical density measured at 450 nm. Human
185 neutrophil MPO (Sigma) was used as standard. One unit of MPO activity was defined as the
186 amount that degraded 1.0 mmole of peroxide per minute at 25°C.

187

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

189 BALB/cYJ animals were treated as described above for the *ex-vivo* experiments, including
190 LF82SK gavage (1×10^8 cfu in 200 μL). The concentration of streptomycin was then decreased
191 to 0.5 mg/ml during the entire experiment, with renewal once per week. Three days after the
192 introduction of LF82SK, DSS (2% final concentration) was added to the drinking water. This
193 concentration of DSS was maintained for 25 days (renewed weekly) and caused mild
194 symptoms of colitis, as shown by the disease activity index (DAI), which takes into account
195 weight loss, stool consistency and the presence of blood in stools (Hemocult II test (SKD
196 SARL) (see online supplementary table S3), with no effect on mouse survival. We established
197 this protocol from preliminary experiments in which we showed that i) decreasing
198 streptomycin concentration had no effect on the level of gut colonization with LF82SK
199 (assessed from feces) for up to three weeks, and ii) 1% DSS did not induce major symptoms
200 of colitis within 10 days, whereas 3% DSS had too strong an effect, resulting in the euthanasia
201 of the animal within three days. We also found that, in the presence of 2% DSS, mild colitis
202 symptoms appeared seven to nine days after gavage with LF82SK. Five or seven days after
203 the addition of 2% DSS to the drinking water, we administered the cocktail of bacteriophages
204 (3×10^7 pfu per mouse) to the mice by oral gavage. Fecal pellets were collected at the
205 indicated time points, for assessment of the levels of both LF82SK and bacteriophages. The
206 mice were euthanized 25 days after the administration of the LF82SK strain, to assess

207 LF82SK and bacteriophage levels in organs.

208

209 **Quantification of bacteria and bacteriophages in colonized mice**

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

221 For tissue homogenates and feces, we found, as previously reported, that direct counts of
222 *E. coli* colonies were not accurate, because of the abundance of bacteriophages, a problem
223 that can be overcome by using an indirect method based on qPCR³¹. Tissue homogenates
224 (200 μ l) were incubated with a mixture of DNase and RNase (0.2 mg/ml and 0.6 mg/ml final
225 concentration respectively, Sigma) for 30 minutes at 37°C, to eliminate nucleic acids from
226 lysed cells. They were then inactivated by heating at 95°C for 10 minutes. Bacterial DNA was
227 extracted with a Maxwell Tissue DNA kit and a Maxwell robot (Promega, USA). InstaGene
228 matrix (Biorad) (1/10 final volume) was added to the samples to clean up the DNA before
229 qPCR amplification. Amplification and detection were performed in 96-well plates (Abgene),
230 with iQ Eva Green Supermix (Biorad). Each amplification reaction was performed in

231 duplicate, in a final volume of 25 μ l, with a final concentration of 0.25 μ M for each primer
232 and 5 μ l of extracted DNA (95°C for 10 min, followed by 45 cycles of 15 s at 95°C, 1 min at
233 60°C and 1.5 min at 72°C, and 80 cycles, beginning at 55°C, with a 0.5°C increase in each
234 cycle, to collect melting curve data for analysis). *E. coli* 16S rDNA-specific primers and
235 bacterial 16S rDNA universal primers were used in parallel in each PCR for the samples from
236 the DSS-induced colitis model, whereas primers binding to the plasmid pMT
237 (CCATTCATGCAGCAGCTCTTT and ATCGGACAACATTAGCGGTGT) were used for
238 samples from the CEABAC10 mice ³².

239

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

241 Six frozen biopsies (obtained from the biological collection DC 2008 642) ³³ weighting 50 to
242 200 mg were homogenized in 0.5 to 2 ml of sterile PBS. An aliquot (50 μ l) of each
243 homogenate was plated on Drigalski agar plates to evaluate the presence of endogenous viable
244 Enterobacteria as well as bacteriophages infecting strain LF82SK, which both were found
245 negative. Each homogenate was split into three aliquots of 100 μ l and 25 μ l of a bacterial
246 suspension of strain LF82SK (3.5×10^6 cfu), washed three times in sterile water to eliminate
247 traces of growing medium, was added to two aliquots and incubated 60min at 37°C without
248 shaking. Then, 25 μ l of the phage cocktail (1.8×10^4 pfu) was added to half of the samples that
249 received strain LF82SK as well as the samples that did not received it. Volumes were adjusted
250 to 150 μ l with PBS. The same conditions were applied to strain LF82 growing in LB as
251 control. Bacteriophages were enumerated after 5 and 24 hours of incubation at 37°C without
252 shaking.

253

254 **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

258 **Results**

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

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

271

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

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

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

287

288 We also analyzed the adherent *E. coli* bacteria from ileal and colonic gut sections from both
289 the bacteriophage and control groups, with a semi-quantitative real-time PCR method for
290 bacterial quantification (the abundance of bacteriophages in samples has been shown to affect
291 the accuracy of direct bacterial counts³¹). Twenty-four hours post-treatment, the levels of the
292 adherent strain LF82 (figure 3A) were significantly lower in all the gut sections of the
293 bacteriophage group than in those of the control group. Four days post-treatment, the
294 differences between the two groups remained significant, other than for ileal sections (figure
295 3B). Thus, by direct and indirect methods, we showed that the administration of a
296 bacteriophage cocktail on one day progressively reduced the level of LF82 colonization of the
297 entire GIT over the following five days, despite the expression of the CEACAM6 receptor for
298 AIEC.

299

300 **Bacteriophages reduce colitis symptoms in conventional mice colonized with AIEC** 301 **strain LF82SK**

302 We moved one step closer to clinical settings, by investigating the relationship between the
303 ability of bacteriophages to decrease colonization by AIEC and the control of intestinal colitis
304 symptoms induced by DSS in conventional mice, by determining DAI score (methods). Three
305 groups of mice colonized with strain LF82SK received three independent treatments (figure
306 4A). As a preliminary study showed that DAI score increased between seven and nine days
307 after the administration of the LF82SK strain (methods), we investigated whether the

308 administration of a single dose of the bacteriophage cocktail (3×10^7 pfu) either before (on
309 day 8; preventive treatment) or after (on day 10; curative treatment) LF82SK administration
310 could affect colitis symptoms and LF82SK colonization levels. In the group receiving the
311 preventive treatment, DAI score on day 10 was lower than that in the other two groups. It
312 subsequently returned to background levels, and remained at this level for the next 15 days
313 (figure 4B). In the group receiving the curative treatment on day 10, DAI had decreased four
314 days post-treatment, but to a lesser extent than in the control group, and it remained low for
315 the next 11 days (figure 4B). Mean fecal levels of strain LF82SK from day 7 to day 25
316 remained stable in the control group (from $3.6 \times 10^9 \pm 2.2 \times 10^9$ to $3.6 \times 10^8 \pm 3.34 \times 10^8$ cfu/g) but
317 decreased by 6.000 (from $4.8 \times 10^9 \pm 1.6 \times 10^9$ to $7.7 \times 10^5 \pm 1.1 \times 10^6$ cfu/g) and 43.000 (4.0×10^9
318 $\pm 1.4 \times 10^9$ to $9.3 \times 10^4 \pm 1.1 \times 10^5$ cfu/g) times respectively in the preventive and curative
319 groups. Furthermore, these results were confirmed using molecular quantification of ileal and
320 colonic sections collected on day 25 (figure 4 C,D). A single bacteriophage application
321 therefore successfully decreased colitis symptoms and mediated the long-term decolonization
322 of the GIT with AIEC.

323

324 **Bacteriophages actively replicate on ileal biopsies from CD patients**

325 In order to provide an evaluation of the potential of this cocktail to target AIEC strains in
326 patients we measured its replication in six CD patients ileal biopsies spiked with strain
327 LF82SK. Active replication of the cocktail was measured after 5 hours from bacteriophage
328 administration (mean n-fold replication $9.43 \times 10^3 \pm 2.07 \times 10^3$) and after 24 hours (mean n-fold
329 replication $3.22 \times 10^4 \pm 6.31 \times 10^3$) confirming the killing potential of the cocktail in such
330 environment. Values were similar to those obtained with individual bacteriophages from ileal
331 samples of LF82SK-colonized mice.

332

333 **Discussion**

334 Dysbiosis of the gut microbiota is now recognized as a hallmark of IBD. It leads to the
335 overgrowth of enterobacteria, including *E. coli* strains from the AIEC group, which are
336 particularly predominant³⁴. Antibiotic treatments have been proposed but expose CD patients
337 to the risk of adverse events, such as exacerbation of dysbiosis, with detrimental effects, such
338 as promotion of the growth of pathogenic strains or an increase in inflammation. Antibiotic-
339 resistant bacteria may also be selected, decreasing the chances of a successful treatment
340 outcome. Furthermore, the long-term antibiotic treatments with ciprofloxacin and
341 metronidazole frequently used to treat CD are associated with a high risk of adverse effects
342 resulting in treatment intolerance. Phage therapy is not only a specific way to target pathogens
343 with a lower impact on microbiota than antibiotics, it can also provide a solution to the
344 problem of antibiotic-resistant strains^{24,25,35,36}. In this study, we investigated the activity of a
345 cocktail of three bacteriophages targeting AIEC strain LF82. The choice of these
346 bacteriophages was guided to obtain the largest host range towards the ECOR collection,
347 which embraces the genetic diversity of *E. coli* genus, as AIEC strains do not belong to a
348 particular genotype or serotype. An complementary strategy could consist on using a
349 collection of characterized AIEC strains to implement this initial cocktail towards CD patients
350 strains.

351

352 We showed *ex vivo* that each bacteriophage was able to replicate in the GIT. As in similar
353 experiments performed previously with six bacteriophages infecting two different strains of *E.*
354 *coli*, we found that each of the three LF82 bacteriophages replicated in gut sections^{25,31}.
355 These three LF82-bacteriophages belong to the *Myoviridae* family of viruses, whereas the
356 bacteriophages previously reported to have a lower performance belong to the *Podoviridae*
357 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 microbiota³⁷⁻⁴⁰. 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.

383

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

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400

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409

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412

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417

418

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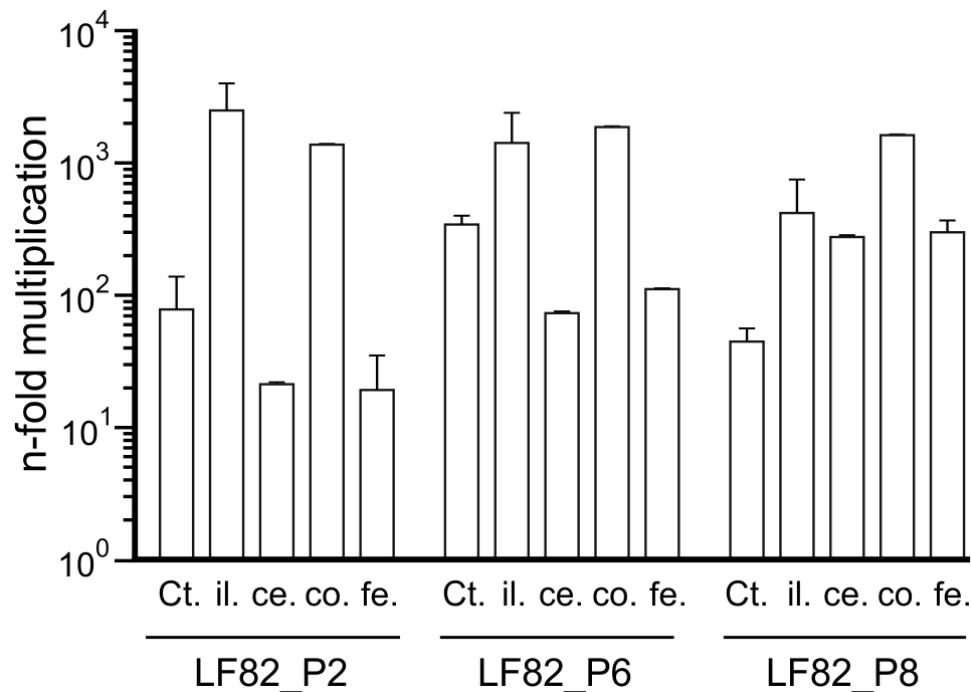
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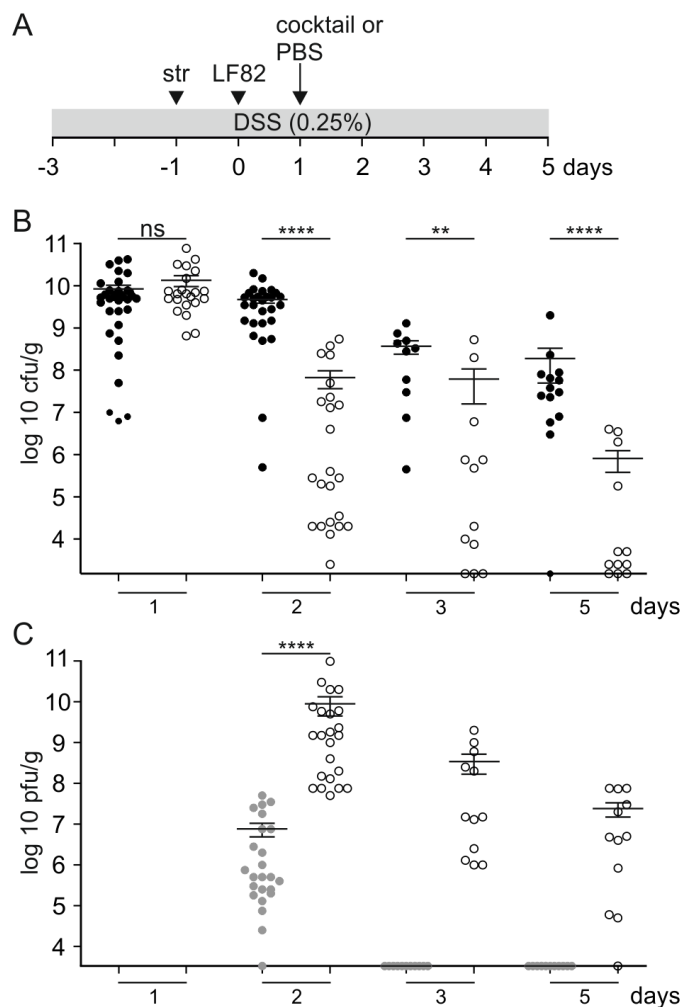


536

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

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

544



545

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

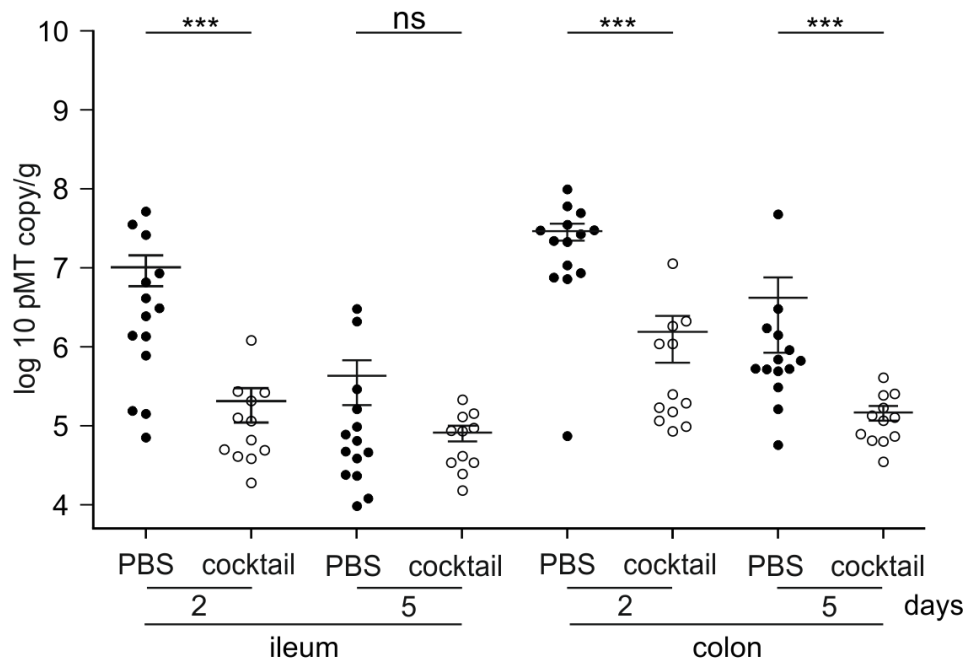
548 (A) Experimental setting, indicating the administration of streptomycin (str, 5 mg per mouse
 549 continuously exposed to 0.25% DSS) 24 hours before oral gavage with the AIEC strain LF82
 550 (1×10^9 bacteria per mouse) followed by administration of either PBS or a cocktail of the
 551 three bacteriophages (3×10^7 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 **** = $p < 0.0001$

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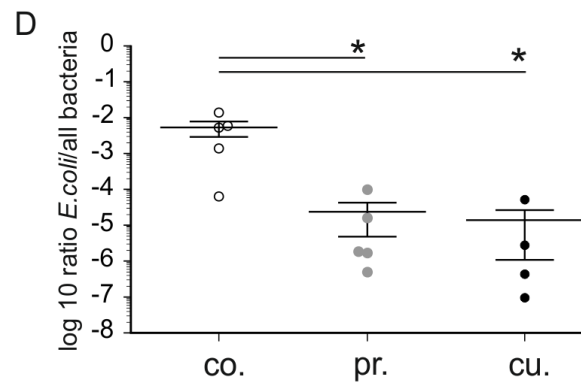
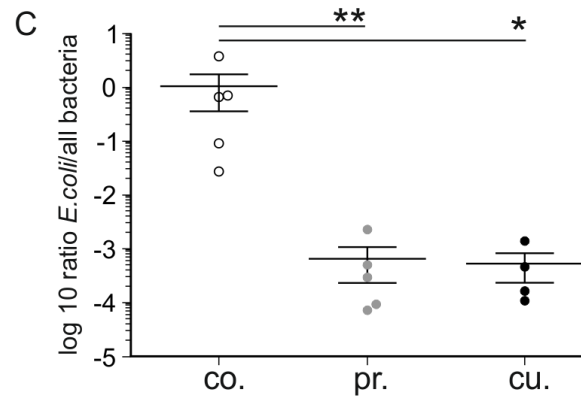
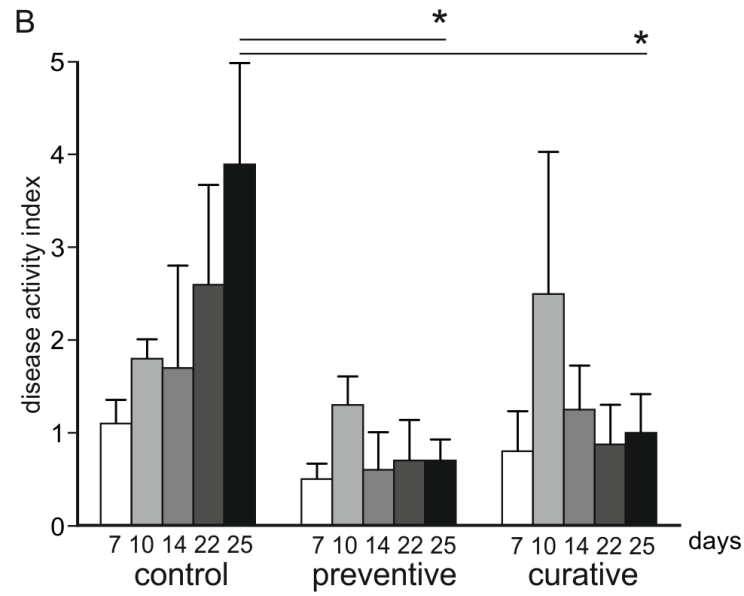
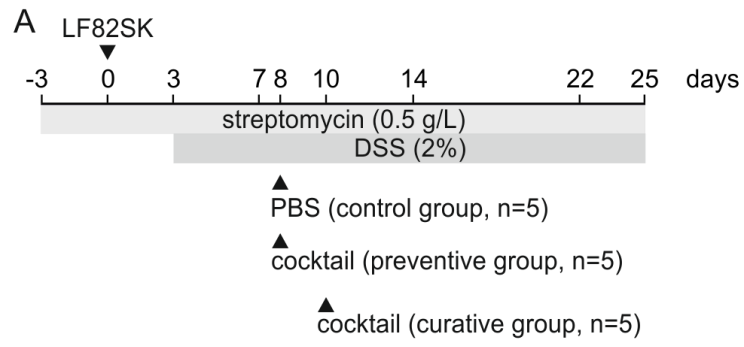


561

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

564 Analysis of the LF82 content of intestinal sections collected on days 2 and 5 from mice (n=6
 565 to 14) that received either PBS (black) or the cocktail of three bacteriophages (white; 3×10^7
 566 pfu per mouse) at the indicated time (same experimental setting that in figure 2A), assessed
 567 by qPCR with primers binding to the pMT plasmid. ns = not significant; ** = $p < 0.01$; *** =
 568 $p < 0.005$

569



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

573 (A) Schematic diagram of the experiment with three groups of mice ($n=5$) receiving either
574 PBS or a single dose of the cocktail on day 8 (3×10^7 pfu) or a single dose of the cocktail ($3 \times$
575 10^7 pfu) on day 10.

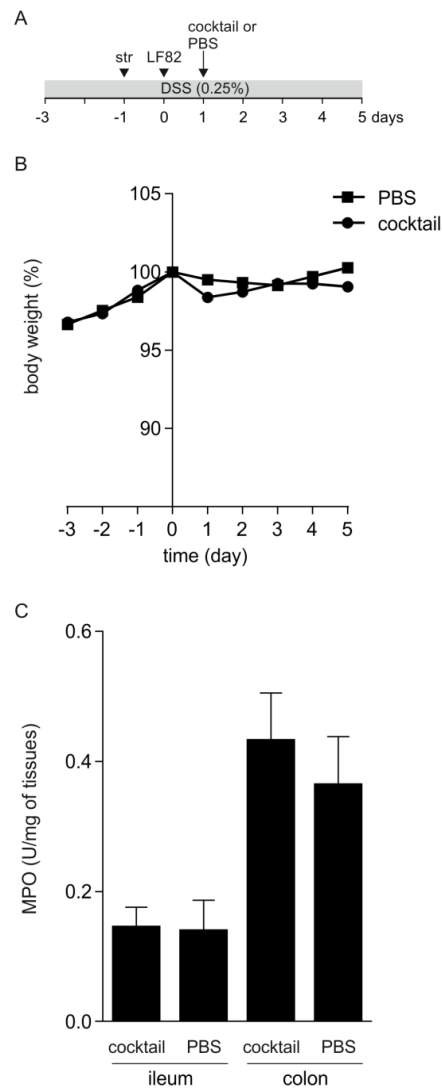
576 (B) The disease activity index was recorded at the indicated time points and plotted for each
577 group of mice (control, preventive and curative).

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

580

581

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 (1×10^9 bacteria per mouse) followed by administration of either PBS or a cocktail of the
 589 three bacteriophages (3×10^7 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 *, +, +, +: $0.1 > \text{EOP} > 1$; ++: $0.001 > \text{EOP} > 0.1$; +: $0.00001 > \text{EOP} > 0.001$; -: $\text{EOP} = 0$. The ECOR
 601 strains not indicated in this table (47 in total) were not infected by any of the three bacteriophages.

602

603 **Table S2** Bacteriophages closely related to LF82_P2, LF82_P6 and LF82_P8 (genomes with
604 more than 90% query coverage in Megablast nucleotide analysis, ranked in decreasing order
605 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
Enterobacteriophage 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
Enterobacteriophage 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

606

607

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 Hemocult test
	2	moderately positive Hemocult test
	3	highly positive Hemocult test
	4	visible blood

609

610