

1 **Studies on the interaction of three lytic bacteriophages**
2 **with a wide collection of *Escherichia coli* strains**
3 **implicated in swine enteric colibacillosis**

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46 ABSTRACT

47 The misuse of antibiotics in the swine industry and their on-going restriction requires
48 alternatives to control enterotoxigenic and shiga toxin-producing *Escherichia coli* (ETEC
49 and STEC, respectively). This study evaluates the potential of three coliphages,
50 vB_EcoM_FJ1, vB_EcoM_FN and vB_EcoM_SP1 against 104 ETEC, STEC and
51 ETEC/STEC strains isolated from pig colibacillosis in Portuguese (2018-2020) and Spanish
52 farms (2006-2016), encompassing 71.2% *mcr*-positive strains (33.7% with *mcr-1*, 1.9% *mcr-*
53 *2*, 35.6% *mcr-4* and 2.9% *mcr-5*) and 18.3% positive strains for TEM (1%), SHV (6.7%),
54 and CTX-M (11.5%) extended-spectrum beta-lactamase-encoding genes. In general, all
55 bacteriophages presented a narrow lytic spectrum (up to 2.9%) against the 104 ETEC, STEC
56 and ETEC/STEC. Bacteriophages shared >80% overall nucleotide identity with *E. coli* phage
57 T4 (*Tevenvirinae* subfamily), but a particular look at the distal part of the long tail fiber
58 (gp38) revealed no homology. All bacteriophages recognize lipopolysaccharides as
59 receptors, and additionally, FN binds to an outer membrane protein A. Bacteriophage-
60 insensitive mutants of vB_EcoM_FJ1 (90%) and vB_EcoM_FN (100%) were shown to be
61 more susceptible to pig serum inactivation comparatively to the parental strain and
62 furthermore, their adhesion capacity to porcine intestinal cells was diminished by,
63 approximately, 90%. Contrariwise, vB_EcoM_SP1 insensitive variants did not display
64 phenotypic differences comparing to the wild-type strain. This study demonstrates that
65 besides being T4-like, these bacteriophages revealed a narrow lytic spectrum against
66 diarrhoeagenic *E. coli* strains and that the acquisition of novel bacteriophage-encoded
67 adhesins (gp38) seems to be determinant for such results.

68 **Keywords:** swine colibacillosis, bacteriophages, BIM, host specificity, *mcr*, extended-
69 spectrum beta-lactamase

70 INTRODUCTION

71 Intestinal *Escherichia coli* associated infections are recurrent in pig farms worldwide
72 and often originate from environmental contamination (i.e., wastewater and animal faeces).
73 Enterotoxigenic *Escherichia coli* (ETEC) is the most prevalent pathotype involved in enteric
74 colibacillosis outbreaks of neonatal and post-weaning diarrhoea, causing high rates of
75 morbidity and mortality and requiring expensive control measures [1]. Animals with a fragile
76 immune system, particularly in the neonatal and PW periods are more susceptible to the
77 disease [2]. ETEC strains exhibit different colonization fimbriae that enable bacterial
78 adhesion by recognition of specific receptors present in enterocytes. F4 and F18 are the most
79 prevalent in the PW phase while F5, F6 and F41, whose recognition sites in enterocytes
80 decrease with the age of the pig, are less frequent [1]. ETEC also produce heat stable (ST)
81 and labile (LT) enterotoxins, responsible for overproduction of electrolytes and fluids, and
82 reduction of water adsorption, causing acute diarrhoea, dehydration, slow growth and even
83 death in pigs. Two types of ST enterotoxins, STa and STb were so far reported [3]. Shiga
84 toxin-producing *E. coli* (STEC) also implicated in enteric colibacillosis, carries the shiga
85 toxin type 2e (Stx2e) and adheres to enterocytes mainly through F18 fimbriae [1]. Hybrid
86 strains (ETEC/STEC) are also observed [4].

87 The massive use in swine of last resort antibiotics used to treat humans (e.g extended-
88 spectrum cephalosporins and colistin) has led to the presence of residues of extended-
89 spectrum beta-lactamase-producing and multidrug resistant (MDR) *E. coli* in farms [5]. The
90 European Medicine Agency has therefore restricted the use of antibiotics in farms, to mitigate
91 the potential cross-contamination risks of resistant strains along the food chain [6].
92 Consequently, the reduction of available antibacterial options turns urgent the development
93 of effective and sustainable alternatives. Some preventive measures are used to limit the

94 impact of PW diarrhoea. The effectiveness of hygienic measures and strict biosecurity rules,
95 such as vaccination of sows, use of prebiotics and probiotics, or genetic breeding for ETEC-
96 resistant herds, although important, fail to avoid the use of antibiotics [1]. Bacteriophages
97 (phages) are specific and obligatory bacterial parasites with a genome confined in a protein
98 capsid. They vary on lifestyle (virulent and lysogenic), genome type (single and double
99 stranded DNA or RNA) and morphology (mostly, tailed viruses are from *Myoviridae*,
100 *Siphoviridae* or *Podoviridae* families). Their self-replicating, self-dosing capability and
101 innocuity nature towards animal cells as well as their high specificity towards the target
102 bacterium (not affecting the commensal microbiota) are valuable traits encouraging its use
103 [7]. Virulent phages that replicate within the bacterial host, releasing their progeny after cell
104 lysis, have been of particular interest to use against bacterial pathogens, including ETEC and
105 STEC. Despite the proof of concept of phage efficacy in veterinary medicine is being
106 reported [8], studies in pigs are still few. Yet, three studies have reported successful results
107 in using phages both prophylactic and therapeutically to fight against few ETEC serotypes
108 causing infections (O149:H10:F4 [9,10] and F4 carrying strains [11]).

109 This study brings new data and promote discussion about current bottlenecks on the
110 successful use of phages to control swine colibacillosis. Here, we characterize three
111 coliphages against a collection of Portuguese and Spanish *E. coli* strains implicated in swine
112 enteric colibacillosis.

113

114 **MATERIALS AND METHODS**

115 **Bacterial strains and culture conditions**

116 In this study, 156 *E. coli* strains were isolated from pig farms in the North-Central region
117 of Portugal pig farms between 2018 and 2020. The strains were collected from fecal samples
118 and rectal swabs of pigs with diarrhoea aged between two days and one month old, or from
119 intestinal contents of dead infected animals. The Portuguese collection was tested to detect
120 the presence of ETEC and/or STEC pathotypes, as described below. Besides, a collection of
121 68 Spanish strains fully typed comprising 57 ETEC, five STEC and six ETEC/STEC
122 representative of different and prevalent seropathotypes implicated in enteric colibacillosis,
123 was included here [5]. As control, 36 Avian pathogenic *E. coli* (APEC) previously isolated
124 from organs of infected chickens recovered in Portuguese avian farms, were used to
125 comparatively assess the efficacy of the phages among diarrhoeagenic versus extraintestinal
126 pathogenic *E. coli* (DEC/ExPEC, respectively). All bacterial strains were cultivated in
127 MacConkey agar (50 g.L⁻¹, Biokar Diagnostics) for isolation, grown in Lysogeny Broth (LB,
128 NZYTech) agar (12 g.L⁻¹, VWR) at 37 °C for the subsequent studies, and stored at -80 °C.

129

130 **IPEC-1 cells maintenance**

131 For tissue cultures, the neonatal intestinal porcine cell line IPEC-1 (CVCL_2245) was
132 used. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Biochrom)
133 and Ham's F-12 (Biochrom) (1:1) supplemented with 10% fetal bovine serum (FBS,
134 Biochrom) and 1x ZellShield (Biochrom) at 37 °C in a humidified atmosphere at 5% CO₂
135 (HERAcell 150). IPEC-1 cells were subcultured every three days at 80% confluence in T-
136 flasks (Starstedt) in 10 mL complete cell culture medium. Cells used in this study were
137 subcultured from passage 12 to 17.

138

139 **Genotypic characterization of DEC**

140 The *E. coli* pathotypes and virulence factors associated with enteric colibacillosis were
141 investigated among the Portuguese swine strains by PCR of specific genes encoding for
142 toxins (STa, STb, LT, Stx2e) and adhesins (F4, F5, F6, F18 and F41). Primer pairs and PCR
143 conditions were previously reported by García-Meniño and colleagues [5] (S1 Table).

144 Those DEC strains conforming ETEC and/or STEC pathotypes were further analysed for
145 the presence of colistin resistance associated to *mcr* genes (*mcr-1*, 2, 3, 4 and 5), using
146 reported PCR conditions [5] (S2 Table). Then, the *mcr+* strains were also screened for the
147 detection of TEM, SHV, and CTX-M beta-lactamase-encoding genes [12] (S2 Table).

148

149 **Phylogroups, Sequence types (STs) and Clonotypes**

150 The main phylogenetic groups of *E. coli* (A, B1, B2, C, D, E, and F) were determined
151 for the *mcr+* strains using the quadruplex PCR method described by Clermont et al. (2013)
152 [13], based on the presence/absence of the four genetic targets *arpA*, *chuA*, *yjaA*, and
153 *TspE4.C2* (S3 Table). The STs of the strains were assigned by multilocus sequence typing
154 (MLST) following the Achtman seven-locus scheme [14] (S3 Table), and the allelic profile
155 for each isolate was retrieved through the Enterobase website
156 (https://enterobase.warwick.ac.uk/species/ecoli/allele_st_search). The clonotyping was
157 based on the internal 469-nucleotide (nt) and 489-nt sequence of the *fumC* and *fimH* genes,
158 respectively. Allele assignments for *fimH* were determined using the fimTyper database
159 available at the Center for Genomic Epidemiology website

160 (<http://www.genomicepidemiology.org/>). The combination of *fumC* (allele obtained from
161 MLST) and *fimH* allele designations was used as the CH “type” [15] (S3 Table).

162

163 **O Typing**

164 The most prevalent serogroups implicated in enteric colibacillosis of swine were
165 investigated for the *mcr+* strains by microagglutination, following the method described by
166 Guinée et al. (1981) [16] and using the specific O45, O101, O108, O138, O139, O141, O149
167 and O157 antisera at the Laboratorio de Referencia de *E. coli* (LREC-USC). Strains that did
168 not react with any of those O antisera were classified as non-assigned (NA) serogroup.

169

170 **Haemolysis type**

171 The haemolytic capacity of ETEC and STEC strains was evaluated by observing the
172 colony phenotype after cultivation in Columbia blood agar (BioMérieux) and incubated at
173 37 °C, overnight (O/N). The presence and type of lysis halos around the bacterial colonies
174 identified alpha (α) (green discoloration around the colonies), beta (β) (clear zone or
175 transparency in the surrounding medium) or gamma (γ) (absence of reaction, non-
176 haemolytic) haemolysis.

177

178 **Antibiotic susceptibility**

179 Antibiotic susceptibility was determined by microdilution assays and diffusion disks.
180 Microdilution assays were performed to assess the phenotypic resistance to colistin. Briefly,

181 O/N cultures were 100-fold diluted in fresh LB and grown at 37 °C, 120 rpm until mid-log
182 phase. Bacterial suspension of an $OD_{600nm} = 0.1$ (1×10^8 CFU.mL⁻¹) were 100-fold diluted in
183 colistin solution (final concentration 2 mg.L⁻¹) in a 96-well polystyrene microplate (SPL Life
184 Sciences). Next, the turbidity (OD_{600nm}) was measured in a spectrophotometer (Heales, MB-
185 580) after a 22 h incubation period at 37 °C. A bacterial suspension without colistin was used
186 as a control. A *mcr* strain was used as positive control. The experiments were conducted in
187 triplicate. Results were interpreted following the 2022 EUCAST breaking point
188 (<http://www.eucast.org>).

189 Additionally, the strains were subjected to disc diffusion tests (Bio-Rad) containing
190 gentamicin (10 µg), ceftiofur (30 µg), imipenem (10 µg), aztreonam (30 µg), amoxicillin +
191 clavulanic acid (20 µg + 10 µg), ampicillin (10 µg), ceftiofur (30 µg), cefepime (30 µg),
192 doxycycline (30 µg), minomycin (30 µg), colistin (10 µg), tigecycline (15 µg), marbofloxacin
193 (5 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), enrofloxacin (5 µg), trimethoprim-
194 sulfamethoxazole (75 µg) and fosfomicin (200 µg). An isolate was considered either
195 susceptible, intermediate susceptible or resistant following the manufacturer guidelines based
196 on CLSI breakpoints (M100 30th Edition, 2020).

197 When resistant to at least one agent in three or more antimicrobial categories, strains
198 were classified as multidrug-resistant as proposed by Magiorakos et al. (2012) [17].

199

200 **Phage propagation**

201 A panel containing three previously isolated phages were used. Phages vB_EcoM_FJ1
202 (FJ1) and vB_EcoM_FN (FN) were isolated from chicken litter (unpublished data). Phage
203 vB_EcoM_SP1 (SP1) was isolated from pig sewage and previously reported [18].

204 For phage propagation the host cells, O/N grown host cultures - HFJ1, HFN and SP16,
205 respectively for FJ1, FN and SP1 - were incorporated into LB soft agar overlays plates, before
206 spreading phage suspensions ($\sim 1 \times 10^7$ PFU.mL⁻¹) with a sterile strip of paper to produce
207 confluent plates. Plates were then O/N incubated at 37°C, and after that, 3 mL SM buffer
208 were added. Plates were again incubated (4 °C for 16 h), and then the liquid phase was
209 recovered, centrifuged, treated with chloroform (10% v/v), filtered (0.2 µm) and stored at 4
210 °C. Phage concentration was assessed by plaque counting (PFU.mL⁻¹) after serial diluting the
211 phage stock in SM Buffer, plating and incubation.

212

213 **Electron microscopy analysis**

214 Phages FJ1 and FN were visualized by Transmission electron microscopy (TEM).
215 Phage particles ($> 1 \times 10^8$ PFU.mL⁻¹) were collected by centrifugation (1 h, 25,000 × g, 4 °C)
216 and washed twice with water. Next, phages were deposited on copper grids with a carbon
217 coated Formvar film grid and stained with 2% uranyl acetate (pH 4.0). The visualization was
218 performed on a Jeol JEM 1400 (Tokyo, Japan).

219

220 **DNA isolation, genome sequencing and annotation**

221 Genomic DNA of phages FJ1 and FN was isolated using the phenol-chloroform-
222 isoamyl alcohol method as previously described [19]. The DNA sample was used for whole
223 genome library construction using TruSeq® Nano DNA Library Prep Kit. DNA fragments
224 were sequenced in Illumina MiSeq, using 300bp paired-end sequencing reads. After
225 removing low quality bases, reads were *de novo* assembled using Geneious Prime. The

226 assembled genomes were scan through MyRAST for open reading frames [20] and
227 tRNAscan-SE for tRNAs [21]. Protein functions were search using BLASTP against NCBI
228 nonredundant protein database and using HHpred against Protein Data Bank database, in all
229 using a E-value 1×10^{-5} threshold. Comparative genomic analysis was performed with
230 BLASTN. Phages FJ1 and FN sequenced genomes were deposited in NCBI database under
231 the accession numbers MZ170040.1 and MZ170041.1, respectively.

232

233 **Lytic spectra determination and efficiency of plaquing**

234 The host range was evaluated against a wide panel of 104 DEC strains from pigs: 31
235 ETEC and five STEC isolated within the scope of the present work, and 68 ETEC, STEC
236 and ETEC/STEC previously isolated in Spain [5]. Moreover, to assess phage activity against
237 other *E. coli* pathogenic strains, 36 APEC strains, previously isolated from chickens were
238 also included. Two parameters were then analysed. First, strains were subjected to phage spot
239 test to assess the host recognition rate: 10 μ L of each phage (1×10^8 PFU.mL⁻¹) were dropped
240 onto each bacterial lawn (prepared as previously described) and checked for clear zones after
241 incubation. Then, the range of plaque formation was evaluated in sensitive strains, by
242 measuring phage efficiency of plaquing (EOP): serial dilutions of phage suspensions (starting
243 from 1×10^8 PFU.mL⁻¹) were spotted on bacterial lawns. The relative EOP was calculated by
244 dividing the titre (PFU.mL⁻¹) of each susceptible strain by the titre of the relevant
245 propagating host, and scored as 0 (no lysis), 1 ($\leq 50\%$), 2 ($>50\% - 100\%$), 3 ($>100\%$) and
246 lysis from without (LFW) if no single plaques were observed.

247

248 **One-step growth curve**

249 One-step growth curves were performed for all phages (FJ1, FN and SP1). Shortly,
250 O/N-grown cultures were 100-fold diluted in 20 mL of fresh LB and incubated until an
251 OD_{600nm} of 0.3 was reached. Resultant cultures were then centrifuged (7,000 $\times g$, 5 min, 4
252 $^{\circ}C$), resuspended in 5 mL fresh LB medium, and mixed with 5 mL of phage suspension to
253 reach a multiplicity of infection of 0.01 (for FN) or 0.001 (for FJ and SP1). A subsequent
254 incubation (37 $^{\circ}C$ for 10 min) allowed phage adsorption to bacterial cells and then a
255 centrifugation (7,000 $\times g$, 5 min, 4 $^{\circ}C$) produced a pellet that was resuspended in 10 mL of
256 fresh LB medium. To analyse one-step growth curves, samples were taken every 5 or 10 min
257 and plated immediately over a period of 35 min, 40 min or 50 min for FJ1, FN and SP1,
258 respectively.

259

260 **Identification of phage receptors**

261 The type of phage receptors (carbohydrate or protein-based) on bacterial surface was
262 identified following the protocol proposed by Kiljunen et al. (2011) [22]. Phage host cultures
263 were treated with 1) sodium acetate (50 mM, pH 5.2) (control), 2) sodium acetate containing
264 100 mM periodate (IO_4^-) at room temperature for 2 h (to inactivate carbohydrates) or 3)
265 proteinase K (0.2 $mg \cdot mL^{-1}$) at 37 $^{\circ}C$ for 3 h (to inactive outer membrane proteins).
266 Afterwards, the phage was incubated with the treated host cells during 5 or 10 minutes at 37
267 $^{\circ}C$, and the adsorption measured by plaque counting ($PFU \cdot mL^{-1}$) after serial diluting in SM
268 Buffer. The phage adsorption rate (%) was obtained by subtracting the concentration of non-
269 adsorbed phage divided by the total phage titre. Each assay was performed at least 3 times.

270 Complementary studies were performed with phages FJ1, FN and SP1 to identify
271 specific receptor-encoding genes, using the Keio Collection composed of *E. coli* K-12
272 mutants carrying single-gene deletions [23], performing drop tests.

273

274 **Bacteriophage-insensitive mutants' survival in pig serum**

275 The vulnerability of bacteriophage-insensitive mutants (BIMs) generated by phages
276 to the pig complement system was assessed using porcine serum. For inducing the formation
277 of BIMs, mid-log phase grown cultures of host strain EC43 were challenged with FJ1, FN
278 and SP1, and incubated for 24 h. After incubation, the cultures were plated in LB agar and
279 incubated O/N. Afterwards, 10 colonies obtained from each culture were streaked at least
280 three times into new plates to guaranty purity. The confirmation of BIMs production was
281 performed by EOP. Whenever 24 h of incubation were not enough to obtain insensitive
282 mutants, the procedure was extended to 72 h.

283 Next, EC43 wild-type (WT) and respective BIMs mid-log phase cultures ($OD_{600nm} =$
284 0.3) were diluted to obtain a 5×10^5 CFU.mL⁻¹ and mixed with porcine serum (3:1 (v/v)). The
285 mixture was incubated for 1 h at 37 °C, followed by quantification of bacterial cells. Heat-
286 inactivated serum (56 °C for 30 min) was used as negative control.

287

288 **Phage-induced mutants' adhesion to epithelial cells**

289 The virulence of five BIMs of each phage (FJ1 - 1.1, 1.4, 1.6, 1.9 and 1.10, FN - 2.1,
290 2.2, 2.3, 2.7 and 2.9 and SP1 - 3.1, 3.5, 3.6, 3.7 and 3.9) in swine intestinal cells was assessed
291 by comparing the adhesion capacity (CFU.cm²) caused by the BIM and by the originating
292 strain. Briefly, IPEC-1 cells were seeded in 96-well plates and let growth for 24 h to
293 confluence confirmed by microscopy. Afterwards, cells were washed once with 10 mM PBS
294 and exposed to bacterial suspensions (MOI=100) resuspended in DMEM/Ham's F-12
295 supplemented with 10% FBS and incubated during 2 h at 37 °C, 5% CO₂ to allow adhesion.
296 After incubation, the culture medium was removed, the cells were carefully washed twice
297 with 10 mM PBS, 30 µL of trypsin/EDTA (Biochrom) was added to each well and plates
298 were re-incubated at 37 °C, 5% CO₂, for 15 min. The effect of trypsin was quenched by
299 adding 70 µL of assay medium. CFU were quantified by 10-fold serial dilutions in 0.9%
300 (w/v).

301

302 **Statistical analysis**

303 The statistical analysis of the results was performed using GraphPad Prism 6. Results
304 were compared using t-test or One-way ANOVA using Bonferroni test. All tests were
305 performed with a confidence level of 95%. Differences were considered statistically different
306 if $p\text{-value} \leq 0.05$.

307

308 **RESULTS**

309 Virulence factors, serogroups, *mcr*-types and beta-lactamase 310 encoding genes

311 A total of 156 *E. coli* strains were isolated from faecal samples or rectal swabs during
312 diarrhoea outbreaks in Portuguese pig farms between 2018 and 2020. While most strains
313 (76.9%) tested negative for all the virulent-related traits analysed by PCR, 36 (23.1%) of the
314 strains could be encompassed in two pathotypes (ETEC and STEC). Of the 36 pathogenic
315 strains, 86.1% carried enterotoxin genes (EPEC), of which 90.3%, 48.4% and 29.0% of the
316 ETEC strains showed carriage of STb, STa or LT genes, respectively. The remaining 13.9%
317 strains carried the shiga-like toxin gene *stx2e* (STEC) (Table 1). Regarding the intestinal
318 colonization factors, the most prevalent fimbriae among ETEC was F18 (41.9%) followed
319 by F4 (16.1%). Most ETEC strains carried both fimbriae and toxin-encoding genes (58.1%).
320 Fimbriae F18 was also present in 80% of the Shiga-like toxin-bearing strains. Fimbriae F5,
321 F6 and F41 were not detected within the collection. Additionally, 54.8% and 80% of ETEC
322 and STEC strains respectively, displayed β haemolytic activity.

323

324 **Table 1. Virulence factors (fimbriae, toxins) attributes of the 36 Portuguese DEC isolates and**
325 **haemolysis type.**

Pathotype	Fimbriae No. isolates (%)		Toxins No. isolates (%)				Haemolysis type No. isolates (%)	
	F4	F18	STa	STb	LT	Stx2e	β	γ
ETEC (n=31)	5 (16.1)	13 (41.9)	15 (48.4)	28 (90.3)	9 (29.0)	-	17 (54.8)	14 (45.2)
STEC (n=5)	-	4 (80)	-	-	-	5 (100)	4 (80)	1 (20)

326

327

328 The screening of plasmid-mediated colistin resistance genes (*mcr-1* to *5*) on the 36
329 ETEC and STEC identified 36.1%, 5.6% and 25% strains with *mcr-1*, *mcr-2* and *mcr-4*,
330 respectively. Among the *mcr+* strains, three different serogroups were identified.
331 Predominantly, ETEC strains belonged to serogroups O108 (31.6%) and O157 (15.8%) while
332 all STEC strains belonged to O139 (S4 Table).

333 The screening of the *bla*_{CTX-M}, *bla*_{SHV}, and *bla*_{TEM} genes within the 24 *mcr+* strains
334 bearing genes indicated that all but one strain (*bla*_{CTX-M} carrying) displayed *bla*_{TEM} in their
335 genomes. One strain encompassed both *bla*_{TEM} and *bla*_{CTX-M} genes. No *bla*_{SHV} gene was
336 identified (S4 Table).

337

338 **Phylogroups, STs, clonotypes**

339 By PCR, the 24 *mcr+* ETEC and STEC strains were assigned to four distinct
340 phylogroups: A (15 strains), B1 (three strains), E (five strains) and F (one strain). MLST
341 determined six different STs, but 15 strains of 24 belonged to CC10 (S4 Table). Among the
342 seven phylogroup-ST-clonotype (CH) combinations determined within the 24 strains, three
343 of them accounted for 79% of them: A-ST10 (CH11-24), A-ST5786 (CH11-24) and D-ST1
344 (CH2-54). Interestingly, all 9 *mcr-4* strains belonged to the clonal group A-ST10 (CH11-24),
345 mostly exhibiting serogroup O108. Besides, the five STEC strains showed the clonal group
346 D-ST1 (CH2-54), serogroup O139 and carried *mcr-1*.

347

348 **Antimicrobial resistances**

349 The inhibition assay confirmed that the *mcr* carrying strains were resistant to colistin
350 at 2 mg.L⁻¹ (EUCAST breakpoint). Additionally, the antibiotic resistance profile (Fig 1)
351 indicated high resistance rates to ampicillin (100.0%), trimethoprim-sulfamethoxazole
352 (83.3%), doxycycline (75%), gentamicin (70.8%), nalidixic acid and ciprofloxacin (62.5%)
353 and enrofloxacin (50.0%). Most strains (75% and 50%) displayed an intermediate
354 susceptibility to colistin and amoxicillin + clavulanic acid, respectively. The active
355 ingredients with higher effectiveness were fosfomycin (100.0%), cefoxitin (95.8%),
356 imipenem (91.7%), cefepime and tigecycline (79.2%), aztreonam (75%), marbofloxacin
357 (62.5%) and ceftiofur (54.2%) Also, based on the antibiotic resistance pattern, all 24 strains
358 were considered MDR.

359

360 **Fig 1. Antimicrobial susceptibility of the 24 *mcr*⁺ DEC Portuguese strains.** The strains
361 were assessed for their susceptibility towards a wide range of antibiotics used in the swine
362 industry. Results were interpreted according to the CLSI, 2020. Grey colour stands for
363 susceptible; light grey colour means intermediate susceptibility; dark grey colour stands for
364 resistance.

365

366 **Bacteriophage isolation, host recognition and plaque formation** 367 **efficiency**

368 The lytic spectra and EOP of the three phages (FJ1, FN and SP1) were firstly tested
369 against 88 ETEC, 10 STEC, six ETEC/STEC (S5a Table). Overall, all phages demonstrated
370 a narrow lytic spectrum: FJ1, FN and SP1 were able to lyse and propagate, respectively, in
371 1.0%, 2.9% and 1.0% of the 104 ETEC, STEC and ETEC/STEC (from which only FJ1 -

372 100% - score an EOP greater than 50%). Additionally, phages recognized and lysed without
373 propagation (LFW) 4.8%, 19.2% and 9.6% of the same strains.

374 The EOP was further performed in 36 APEC (S5b Table) to compare phage activity
375 with different *E. coli* pathogenic strains. Overall, comparatively to ETEC, STEC and
376 ETEC/STEC strains, FJ1, FN and SP1 were able to infect and propagate in a higher number
377 of strains, respectively, 13.9%, 25.0% and 13.9% from which 100% (FJ1), 55.6% (FN) and
378 60% (SP1) had an EOP greater than 50% (LFW in 22.2%, 38.9% and 38.9%) of the APEC.
379

380 **Phage morphology and genome**

381 TEM images showed that all phages are tailed (*Caudovirales* order) and belong to the
382 *Myoviridae* family (Fig 2a). They have highly similar genomes ranging from 165 to 170 kb
383 (269 to 280 coding sequences), sharing 87% overall nucleotide identity with *E. coli* phage
384 T4, a prototype (NC_000866) of the subfamily *Tevenvirinae* (Fig 2b). Major phage proteins
385 such as those related to DNA packing and structural proteins, DNA replication,
386 recombination and modification proteins and cells lysis were identified in all genomes,
387 however, >50% of the proteome has no assigned function. As expected, there was a high
388 homology between the long tail fiber (LTF) of the three phages but less between them and
389 the T4 phage (Fig 2c). A closer look at the distal part of the LTF indicated that such
390 differences are mainly due to gp38 sequence (no homology found).

391

392 **Fig 2. Microscopy observation and genomic comparison.** A) Transmission electron
393 micrographs of phage's FJ1 (left) and FN (right). Black scale bar represents 100 nm. B)

394 Comparison between the genomes of phage's FJ1, FN and SP1. Coloured arrows indicate
395 open reading frames according to the putative function. Similarity is indicated in grayscale.
396 Image was created using the EasyFig program. C) Comparison between the putative coding
397 sequences of the LTF of phage's T4, FJ1, FN and SP1.

398

399 **Phage infection parameters**

400 All three phages were evaluated in terms of one-step-growth curves (S1 Fig). Phages
401 FJ1 and FN displayed the shortest latency periods of - 10 and 15 minutes, respectively -
402 followed by SP1 that required 20 minutes to burst. Phages FN, FJ1 and SP1 produced 71, 96
403 and 150 phages per cell, respectively.

404

405 **Phage receptors**

406 Preliminary assays aimed to infer about the nature of the phage receptors. Host cells
407 were treated with periodate (to remove carbohydrates) or proteinase K (to remove outer
408 membrane proteins) (Fig 3). When cells were treated with periodate, the adsorption rate
409 significantly decreased for all phages (p -value<0.001) - $9.8\% \pm 4.1\%$ for FJ1, $45.8\% \pm 6.7$
410 $\%$ for FN and $28.1\% \pm 9.1\%$ for SP1 comparatively to untreated cells - $57.8\% \pm 7.3\%$ for
411 FJ1, $87.5\% \pm 3.5\%$ for FN and $97.3\% \pm 1.8\%$ for SP1. Adsorption of most phages was not
412 affected when cells were treated with proteinase K, except for FN, with a reduction of 12%.

413

414 **Fig 3. Adsorption assays of phages FJ1 (A), FN (B) and SP1 (C).** Effect of proteinase K
415 ($0.2 \text{ mg}\cdot\text{mL}^{-1}$) and periodate (50 mM sodium acetate, pH 5.2, 100 mM IO_4^-) on phage host

416 treated cells adsorption shown in residual PFU.mL⁻¹ percentage. Controls were performed
417 using distilled water instead of proteinase K or 50 mM sodium acetate, pH 5.2 only. Dark
418 gray refers to control and light gray for treated cells. Errors bars represent standard deviation
419 for an average of three repeated experiments. Significance was determined with t test when
420 the treated and untreated groups were compared. *** p -value<0.001; **** p -value<0.001

421

422 Additional studies were conducted to detect the specific host receptor using a library
423 of *E. coli* K-12 mutants. Results were in line with previous findings. Phage FN recognized
424 proteins involved in the lipopolysaccharide (LPS) layer biosynthesis (RfaY, RfaG, RfaH and
425 ADP-heptose--LPS heptosyltransferase 2 proteins) (either by moving sugar moieties and
426 rearrange the structure of LPS or by enhancing the expression of operons involved in LPS
427 synthesis) and binds to outer membrane proteins A (OmpA) as well. The specific receptor of
428 phages FJ1 and SP1 was not possible to unveiled using the mutants tested.

429

430 **BIMs survival to serum antimicrobial activity**

431 For this assay, 10 BIMs were confirmed and used for phages FJ1 and SP1, while only
432 nine were obtained to FN (there was difficulty in obtaining any other, even after 72 h
433 incubation).

434 BIMs of the strain EC43 displayed different susceptibility towards the swine serum
435 batches, depending on the originating phage (Fig 4). Overall, 90% of the FJ1 BIMs (1.1, 1.2,
436 1.3, 1.6, 1.8 and 1.9 (p -value<0.001), 1.4 (p -value=0.0021), 1.5 (p -value=0.0018) and 1.7 (p -
437 value=0.0042)) and 100% of FN BIMs (2.1 (p -value=0.0060), 2.2 and 2.8 (p -value<0.001),
438 2.3 (p -value=0.0054), 2.4 (p -value=0.0027), 2.5 (p -value=0.0469), 2.6 (p -value=0.0024), 2.7

439 (p -value=0.0066), and 2.9 (p -value=0.0377)) were reduced, in average 1.2 ± 0.2 Log
440 CFU.mL⁻¹ comparatively to EC43 WT. Conversely, mutants generated after SP1 challenge
441 did not show a higher susceptibility to serum killing activity when compared with the
442 originating strain (p -value>0.05). The control tests performed with the inactivated serum did
443 not influence the bacterial load concentration (data not shown), confirming that the reduction
444 observed was only due to the bactericidal action of the serum.

445

446 **Fig 4. Serum complements effect against WT ETEC EC43 and respective BIMs.** Mutants
447 derived from interaction with phage's FJ1 (A - 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 1.10),
448 FN (B - 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8 and 2.9) and SP1 (C - 3.1, 3.2, 3.3, 3.4, 3.5, 3.6,
449 3.7, 3.8, 3.9, 3.10). Porcine serum was challenged with EC43 WT and respective BIMs
450 emerged from contact with each phage in a 3:1 ratio and incubated for 1 h at 37 °C. Results
451 are shown in logarithm reduction of CFU.mL⁻¹. Control was performed with inactivated
452 serum (data not shown). Black refers to WT and dark gray for BIMs. Errors bars represent
453 standard deviation for an average of three repeated experiments. Significance was determined
454 with One-way ANOVA when the BIMs were compared with WT. * p -value<0.05; ** p -
455 value<0.01; *** p -value<0.001; **** p -value<0.0001

456

457 **BIMs adhesion to intestinal porcine cell line**

458 The mutants displayed different effect in the adhesion capacity to mammalian cells
459 accordingly to the phage used to generated them (Fig 5). Most BIMs of FJ1 (80% - 1.1, 1.6
460 and 1.9 (p -value<0.001) and 1.4 (p -value=0.0102)) and FN (100% - 2.1 (p -value=0.0080),
461 2.2 and 2.9 (p -value<0.001), 2.3 (p -value=0.0022), 2.7 (p -value=0.0016)) demonstrated a

462 reduced adhesion to culture cells, on average, of (5.4 ± 0.2) Log CFU.cm⁻² and (5.2 ± 0.2)
463 Log CFU.cm⁻², correspondingly, comparing with the parental strain (6.3 ± 0.2) Log CFU.cm⁻².
464 ². In opposition, SP1 generated BIMs did not show any difference regarding the adhesion
465 capacity comparing with WT EC43 (p -value>0.05).

466

467 **Fig 5. Adhesion capacity of WT ETEC EC43 and respective BIMs to mammalian cells.**

468 Mutants derived from interaction with phage's FJ1 (**A** - 1.1, 1.4, 1.6, 1.9 and 1.10), FN (**B** -
469 2.1, 2.2, 2.3, 2.7 and 2.9) and SP1 (**C** - 3.1, 3.5, 3.6, 3.7 and 3.9). IPEC-1 cells were
470 challenged with EC43 WT and five BIMs emerged from contact with each phage using a
471 MOI of 100 and incubated for 2 h at 37 °C, 5% CO₂. Results are shown in logarithm of
472 CFU.cm⁻². Black refers to WT and dark gray for BIMs. Errors bars represent standard
473 deviation for at least three repeated experiments. Significance was determined with One-way
474 ANOVA when the BIMs were compared with WT. * p -value<0.05; ** p -value<0.01; *** p -
475 value<0.001; **** p -value<0.0001

476

477 **DISCUSSION**

478 The proliferation of pathogenic *E. coli* in the intestine of pigs during the nursing and
479 PW period has a great cost for the swine industry. The overuse of antibiotics in recent decades
480 has triggered serious problems associated with antibiotic resistance events, compromising
481 the therapeutic solutions available to fight against multidrug resistant swine colibacillosis.
482 This study intended to assess the potential of three phages that were here fully characterized

483 - FJ1, FN and SP1- to tackle DEC strains, using a panel of ETEC, STEC and ETEC/STEC
484 isolated from pigs.

485 Phage taxonomic and genomic characterization indicated that all are T4-like phages
486 of the *Tevenvirinae* subfamily within the *Myoviridae* family, which, as the other T-even
487 phages, are known to infect a wide variety of Gram-negative hosts [24,25]. When host
488 recognition and plaque formation assays were performed for each phage, all revealed an
489 unexpected narrow host spectrum. As noted earlier [26], the number of infected phage-
490 propagating strains was reduced compared to those which, despite being recognized and
491 lysed, cannot propagate them: mostly low EOP scores (<50%) were recorded, and only in
492 few strains: 1.0% for FJ1 and SP1 and 2.9% for FN, compared to the occurrence rates of
493 LFW events, 4.8% for FJ1, 19.2% for FN and 9.6% for SP1. The wide variety of strains used
494 - not only the herein isolated but also the previously reported and characterized, isolated from
495 Spanish farms [5] - contributed to the robustness and heterogenicity of this analysis. The
496 panel comprised mostly fimbriae carriers' strains (75%) belonging to 15 different serogroups
497 (including the most prevalent within swine colibacillosis), with 94 ETEC and ETEC/STEC
498 strains harbouring mostly toxin STb (76.6%), followed by STa (55.3%) and LT (42.6%) and
499 10 STEC. Moreover, the fact that 71.2% were *mcr+*, >40% carry beta-lactamase-encoding
500 genes (including 18.3% extended-spectrum beta-lactamase producers) and 82.7% MDR
501 strains reinforce the relevance of this work. It has been suggested that phages infecting but
502 not being able to propagate in their hosts may be targeted by bacterial anti-phage defense
503 systems, such as Restriction Modification (RM), or Abortive Infection (Abi) systems [26]. If
504 the same report proposed that specifically T4-like phages might exhibit broad resistance to
505 RM systems, it also indicated that they may be susceptible to some Abi systems, as typically
506 their hosts encode genes able to sense phage specific proteins, triggering cell destruction and

507 preventing subsequent infections. Analyzing now the low EOP observed, the comparison
508 with APEC scores (higher EOP efficacy and wide host recognition, between 13.9% and 25%)
509 seems to indicate (regardless of the phages isolation origin) that particularly ETEC may be
510 strengthening its immunity against these viral predators. It can be speculated that this was
511 possible, for example through host specialization in anti-phage defense systems such as
512 CRISPR-CAS or Superinfection exclusion (SE) [27]. In an extensive *in silico* study, Wang
513 et al. (2020) [28] demonstrated that ETEC can include 8.4 prophages/genome, a high average
514 number, increasing the likelihood of occurring SE events.

515 T-even phages are known to infect hosts through an initial and reversible binding to
516 primary receptors, - usually surface proteins like OmpC for T4, but when not available, sugar
517 motifs in the LPS - with its LTF, followed but second and irreversible binding by its short
518 tail fiber [29]. However, it is the first step that defines the host range. In phages such as T4
519 and S16, the LTF is encoded by gp34 to gp37 which form the tail proximal to distal segments
520 [30,31] known to bind to both LPS moieties and OMP proteins. It is also known that T4 binds
521 to hosts via gp37 but needs to be co-expressed with gp38 that functions as a chaperone [32],
522 while S16 binds to hosts using gp37 bond with the gp38 which acts as an adhesin and
523 mediates host specificity [31]. Given the fact that our *E. coli* phages share similar proximal
524 end of the LTFs but have different distal segments (from the C-terminal gp37 to gp38), this
525 can also explain their different and unexpected narrow host range. Regarding their host
526 receptors, all the three phages are expected to be behave similarly to reported T-even phages.
527 Accordingly, assays revealed that FN binds to proteins involved in LPS biosynthesis and
528 OmpA. FJ1 and SP1 seems to recognize only one receptor (carbohydrates), nonetheless, we
529 were not able to identify them using K-12 mutants. The repeated *in vitro* exposure of bacterial
530 hosts to our phages led to the emergence of BIMs. This new phenotype brought fitness cost

531 to some of the mutants, depending on the inducing phage. In fact, 90% of the BIMs from
532 FJ1 and 100% from FN were more vulnerable to the pig complement system when compared
533 to the originating strain, suggesting that changes at the level of the cell wall made the host
534 more reactive to serum immunogenic proteins [33]. Conversely, pig serum has no increased
535 bactericidal effect against BIMs from SP1, recognizing only LPS. Differences regarding
536 serum sensitivity suggest that the site of the mutations at least at the LPS level will influence
537 the reaction of the immune system. Mizoguchi and colleagues had already reported the
538 formation of phage resistant strains associated with LPS alterations or OMP deficiency [34].
539 Particularly the surface protein OmpA (targeted by FN) and also carbohydrates (targeted by
540 FJ1 and FN), especially those that are part of the LPS have been implicated in resistance to
541 serum [35]. Furthermore, the bacterial structure re-conformation due to loss or alteration of
542 the FJ1 and FN binding sites in ETEC EC43 influenced the adhesion capacity to porcine
543 mammalian cells (in about 90%), even though the target of both phages is not related to their
544 adhesin structures. This seems of relevant importance since the first step in the colonization
545 of diarrhoeagenic pathotypes such as ETEC is their attachment to the host cells that promotes
546 the transferring of enterotoxins more efficiently to the target cells.

547

548 **CONCLUSIONS**

549 In summary, this study reflects the diversity of ETEC and STEC in Portuguese swine
550 farms associated with high resistance towards several class of antibiotics, including last resort
551 antimicrobials. Additionally, it demonstrated that the host range of three phages appears to
552 be conditioned by the presence of a unique region in the phage's LTFs. Overall, it suggests

553 the importance of improving knowledge about ETEC and phage interaction, enhancing the
554 importance of an extensive study of phage for a potential veterinary use.

555

556

557 **REFERENCES**

- 558 1. Luppi A. Swine enteric colibacillosis: Diagnosis, therapy and antimicrobial resistance.
559 Porc Heal Manag. 2017;3:1–18.
- 560 2. Mellor DJ, Stafford KJ. Animal welfare implications of neonatal mortality and
561 morbidity in farm animals. Vet J. 2004;168(2):118–33.
- 562 3. Nagy B, Fekete PZ. Enterotoxigenic *Escherichia coli* in veterinary medicine. Int J
563 Med Microbiol. 2005;295(6–7):443–54.
- 564 4. Luppi A, Gibellini M, Gin T, Vangroenweghe F, Vandebroucke V, Bauerfeind R, et
565 al. Prevalence of virulence factors in enterotoxigenic *Escherichia coli* isolated from
566 pigs with post-weaning diarrhoea in Europe. Porc Heal Manag . 2016;2:1–6.
- 567 5. García-Meniño I, García V, Mora A, Díaz-Jiménez D, Flament-Simon SC, Alonso
568 MP, et al. Swine enteric colibacillosis in Spain: Pathogenic potential of mcr-1 ST10
569 and ST131 *E. Coli* Isolates. Front Microbiol. 2018;9(11):1–15.
- 570 6. EMA. Categorisation of antibiotics in the European Union. European Medicines
571 Agency (EMA/CVMP/CHMP/682198/2017). 2019. Available from:
572 [https://www.ema.europa.eu/en/documents/report/categorisation-antibiotics-](https://www.ema.europa.eu/en/documents/report/categorisation-antibiotics-european-union-answer-request-european-commission-updating-scientific_en.pdf)
573 [european-union-answer-request-european-commission-updating-scientific_en.pdf](https://www.ema.europa.eu/en/documents/report/categorisation-antibiotics-european-union-answer-request-european-commission-updating-scientific_en.pdf)
574 Accessed 10 November 2021.

- 575 7. Salmond GPC, Fineran PC. A century of the phage: past, present and future. *Nat Rev*
576 *Microbiol.* 2015;13(12):777–86.
- 577 8. Zhang J, Li Z, Cao Z, Wang L, Li X, Li S, et al. Bacteriophages as antimicrobial agents
578 against major pathogens in swine: A review. *J Anim Sci Biotechnol.* 2015;6(1):1–7
- 579 9. Cha S Bin, Yoo AN, Lee WJ, Shin MK, Jung MH, Shin SW, et al. Effect of
580 bacteriophage in enterotoxigenic *Escherichia coli* (ETEC) infected pigs. *J Vet Med*
581 *Sci.* 2012;74(8):1037–9.
- 582 10. Jamalludeen N, Johnson RP, Shewen PE, Gyles CL. Evaluation of bacteriophages for
583 prevention and treatment of diarrhea due to experimental enterotoxigenic *Escherichia*
584 *coli* O149 infection of pigs. *Vet Microbiol.* 2009;136(1–2):135–41.
- 585 11. Lee CY, Kim SJ, Park BC, Han JH. Effects of dietary supplementation of
586 bacteriophages against enterotoxigenic *Escherichia coli* (ETEC) K88 on clinical
587 symptoms of post-weaning pigs challenged with the ETEC pathogen. *J Anim Physiol*
588 *Anim Nutr (Berl).* 2017;101(1):88–95.
- 589 12. Mora A, Viso S, López C, Alonso MP, García-Garrote F, Dabhi G, et al. Poultry as
590 reservoir for extraintestinal pathogenic *Escherichia coli* O45: K1: H7-B2-ST95 in
591 humans. *Vet Microbiol.* 2013;167(3–4):506–12.
- 592 13. Clermont O, Christenson JK, Denamur E, Gordon DM. The Clermont *Escherichia*
593 *coli* phylo-typing method revisited: Improvement of specificity and detection of new
594 phylo-groups. *Environ Microbiol Rep.* 2013;5(1):58-65.
- 595 14. Wirth T, Falush D, Lan R, Colles F, Mensa P, Wieler LH, et al. Sex and virulence in
596 *Escherichia coli*: An evolutionary perspective. *Mol Microbiol.* 2006;60(5):1136–51.
- 597 15. Weissman SJ, Johnson JR, Tchesnokova V, Billig M, Dykhuizen D, Riddell K, et al.
598 High-resolution two-locus clonal typing of extraintestinal pathogenic *Escherichia*

- 599 *coli*. Appl Environ Microbiol. 2012;78(5):1353–60.
- 600 16. Guinée, P. A. M., Jansen, W. H., and Wasdröm TRS. Laboratory Diagnosis in
601 Neonatal Calf and Pigs Diarrhoea: Current Topics in Veterinary and Animal Science.
602 1981;13 p. Netherlands: Springer.
- 603 17. Magiorakos A, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al.
604 Bacteria : an International Expert Proposal for Interim Standard Definitions for
605 Acquired Resistance. 2011;18(3):268-81.
- 606 18. Ferreira A, Oliveira H, Silva D, Almeida C, Burgan J, Azeredo J, et al. Complete
607 Genome Sequences of Eight Phages Infecting Enterotoxigenic *Escherichia coli* in
608 Swine. Microbiol Resour Announc. 2020;9(36):8–11.
- 609 19. Melo LDR, Sillankorva S, Ackermann HW, Kropinski AM, Azeredo J, Cerca N.
610 Isolation and characterization of a new *Staphylococcus epidermidis* broad-spectrum
611 bacteriophage. J Gen Virol. 2014;95(Pt2):506–15.
- 612 20. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, et al. The RAST
613 Server: Rapid Annotations using Subsystems Technology. BMC Genomics.
614 2008;9(1):75.
- 615 21. Lowe TM, Eddy SR. TRNAscan-SE: A program for improved detection of transfer
616 RNA genes in genomic sequence. Nucleic Acids Res. 1996;25(5):955–64.
- 617 22. Kiljunen S, Datta N, Dentovskaya S V., Anisimov AP, Knirel YA, Bengoechea JA, et
618 al. Identification of the lipopolysaccharide core of *Yersinia pestis* and *Yersinia*
619 *pseudotuberculosis* as the receptor for bacteriophage ϕ A1122. J Bacteriol.
620 2011;193(18):4963–72.
- 621 23. Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, et al. Construction of
622 *Escherichia coli* K-12 in-frame, single-gene knockout mutants: The Keio collection.

- 623 Mol Syst Biol. 2006;2:2006.0008.
- 624 24. Marti R, Zurfluh K, Hagens S, Pianezzi J, Klumpp J, Loessner MJ. Long tail fibres of
625 the novel broad-host-range T-even bacteriophage S16 specifically recognize
626 *Salmonella* OmpC. Mol Microbiol. 2013;87(4):818–34.
- 627 25. Xu J, Chen M, He L, Zhang S, Ding T, Yao H, et al. Isolation and characterization of
628 a T4-like phage with a relatively wide host range within *Escherichia coli*. J Basic
629 Microbiol. 2016;56(4):405–21.
- 630 26. Maffei E, Shaidullina A, Burkolter M, Heyer Y, Estermann F, Druelle V, et al.
631 Systematic exploration of *Escherichia coli* phage–host interactions with the BASEL
632 phage collection. PLOS Biology. 2021;19(11): e3001424.
- 633 27. Hampton HG, Watson BNJ, Fineran PC. The arms race between bacteria and their
634 phage foes. Nature. 2020;577(7790):327–36.
- 635 28. Wang M, Zeng Z, Jiang F, Zheng Y, Shen H, Macedo N, et al. Role of enterotoxigenic
636 *Escherichia coli* prophage in spreading antibiotic resistance in a porcine-derived
637 environment. Environ Microbiol. 2020;22(12):4974–84.
- 638 29. Trojet SN, Caumont-sarcos A, Perrody E, Comeau AM, Krisch HM. The gp38
639 adhesins of the T4 Superfamily: a complex modular determinant of the phage's host
640 specificity. Genome Biol Evol. 2011;3:674–86.
- 641 30. King J, Laemmli UK. Polypeptides of the tail fibres of bacteriophage T4. J Mol Biol.
642 1971;62(3):465–77.
- 643 31. Dunne M, Denyes JM, Arndt H, Loessner MJ, Leiman PG, Klumpp J. *Salmonella*
644 Phage S16 Tail Fiber Adhesin Features a Rare Polyglycine Rich Domain for Host
645 Recognition. Structure. 2018;26(12):1573-1582.e4.
- 646 32. Hashemolhosseini S, Stierhof YD, Hindennach I, Henning U. Characterization of the

- 647 helper proteins for the assembly of tail fibers of coliphages T4 and λ . J Bacteriol.
648 1996;178(21):6258–65.
- 649 33. Sumrall ET, Shen Y, Keller AP, Rismondo J, Pavlou M, Eugster MR, et al. Phage
650 resistance at the cost of virulence: *Listeria monocytogenes* serovar 4b requires
651 galactosylated teichoic acids for InlB-mediated invasion. PLoS Pathog.
652 2019;15(10):1–29.
- 653 34. Mizoguchi K, Morita M, Fischer CR, Yoichi M, Tanji Y, Unno H. Coevolution of
654 bacteriophage PP01 and *Escherichia coli* O157:H7 in continuous culture. Appl
655 Environ Microbiol. 2003;69(1):170–6.
- 656 35. Miajlovic H, Smith SG. Bacterial self-defence: How *Escherichia coli* evades serum
657 killing. FEMS Microbiol Lett. 2014;354(1):1–9.

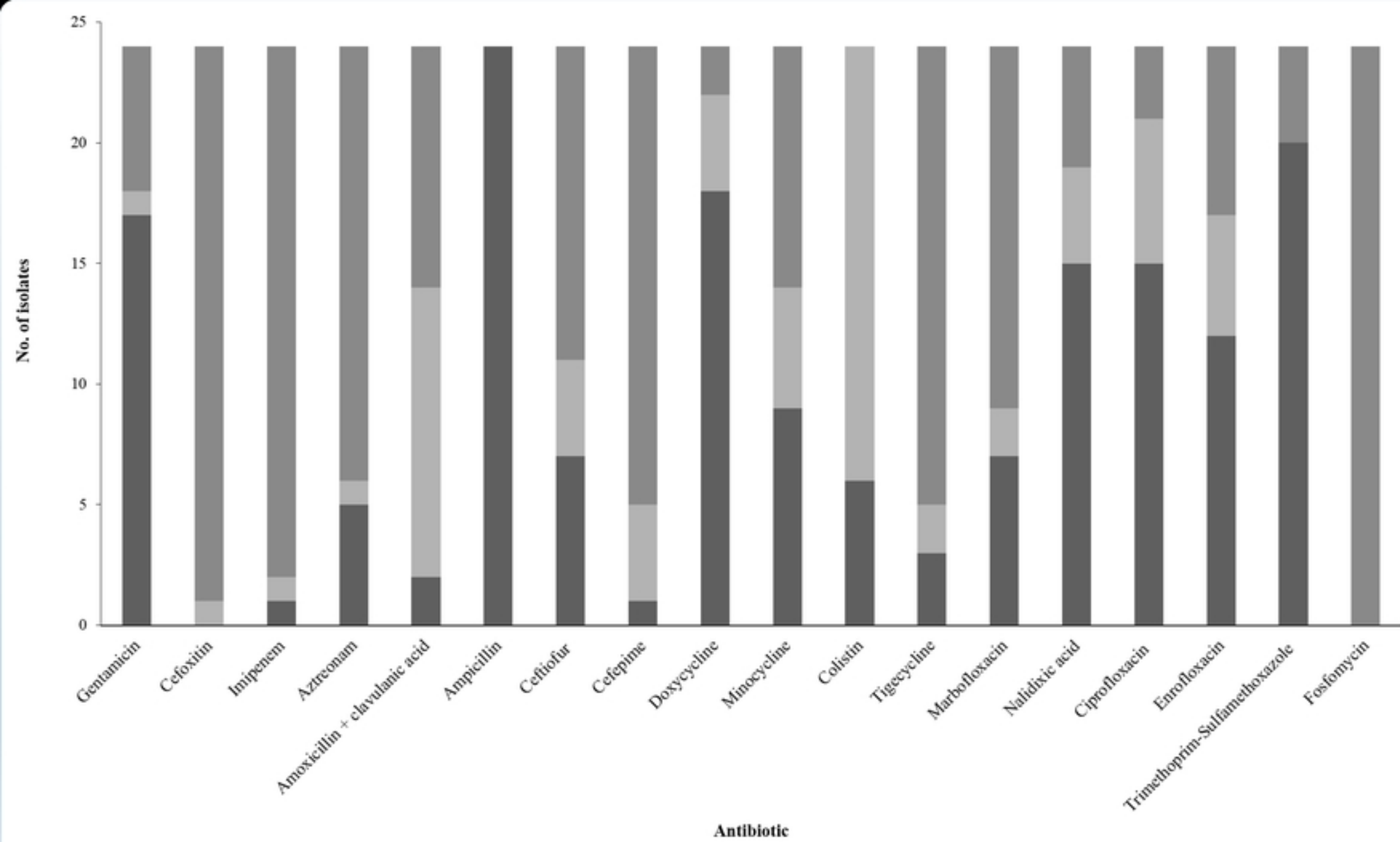
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660 **SUPPORTING INFORMATION**

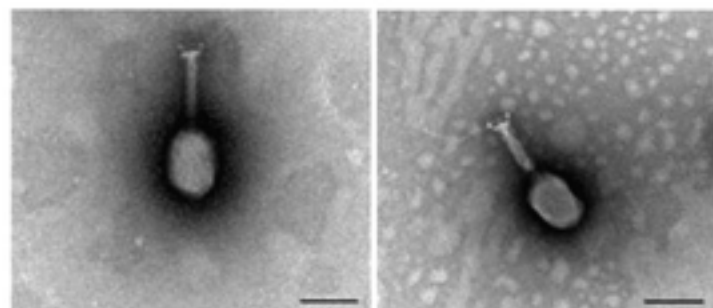
- 661 **S1 Table. Targets and primers associated with ETEC and STEC pathotypes.**
- 662 **S2 Table. Primers used for the detection and/or sequencing of TEM, SHV, CTX-M and**
663 **MCR genes.**
- 664 **S3 Table. Targets and primers to determine phylogroups, clonotypes and sequence**
665 **types by MLST.**
- 666 **S4 Table. Molecular characterization of the 24 *mcr+* Portuguese strains.**
- 667 **S5 Table. Phages (FJ1, FN and SP1) lytic spectra and EOP against 104 ETEC, STEC**
668 **and ETEC/STEC strains (a) and 36 APEC strains (b). The EOP was divided into four**

669 scores: 0 (no lysis), 1 ($\leq 50\%$), 2 ($> 50\% - 100\%$) and 3 ($> 100\%$). LFI stands for lysis from
670 within and LFW for lysis from without. Light gray represents ETEC strains, gray cells are
671 STEC strains and dark gray stands for ETEC/STEC isolates. NA: Not assigned, ONT: O
672 non-typeable, HNM: H non motile.

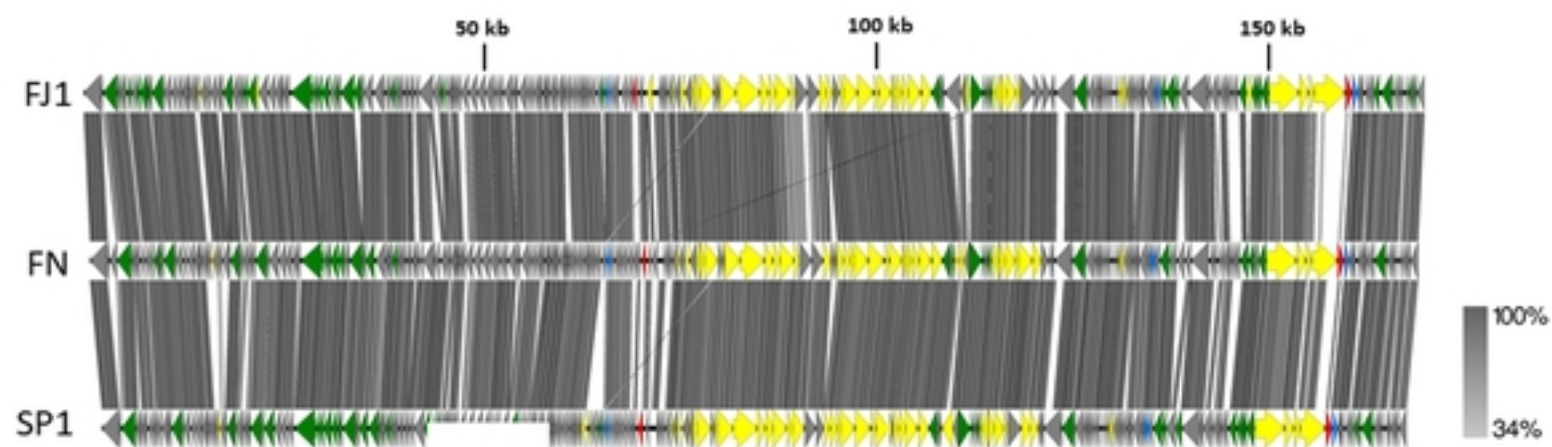
673 **S1 Fig. One-step growth curve of phages FJ1 (a), FN (b) and SP1 (c) on respective hosts**
674 **cells.** Results are shown in PFU per infected cell. Errors bars represent standard deviation for
675 an average of three repeated experiments.



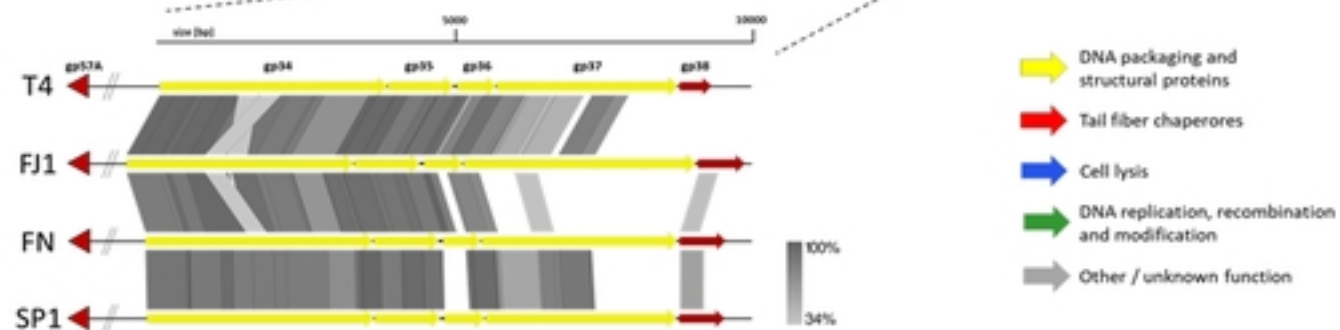
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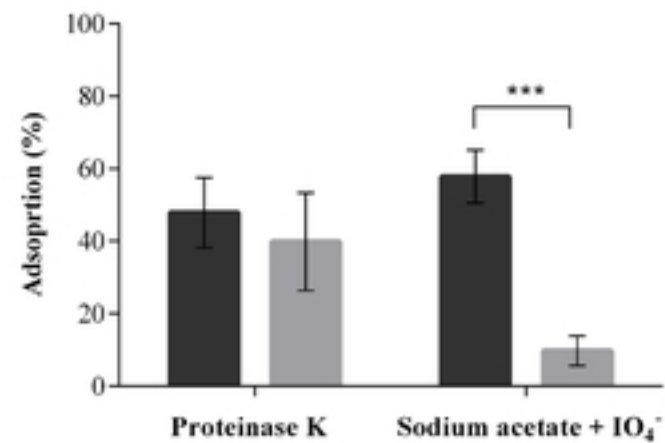
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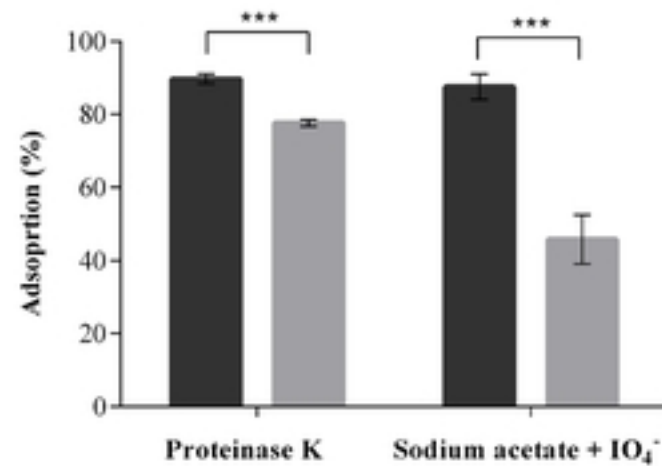
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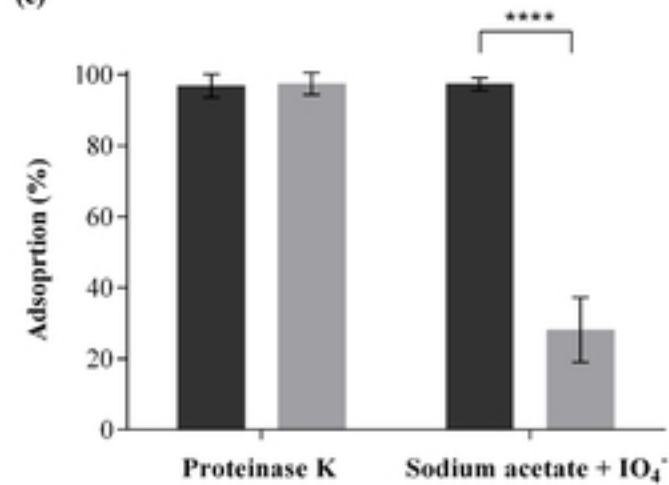
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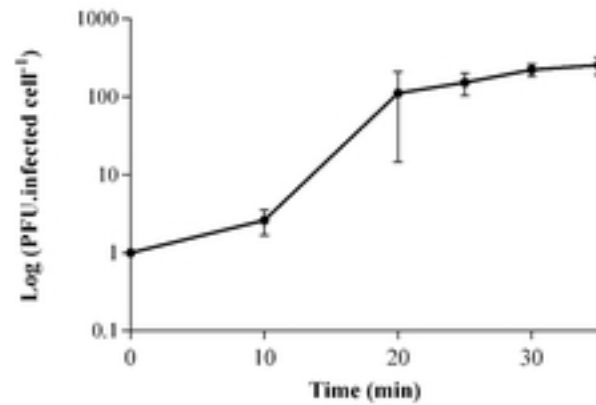
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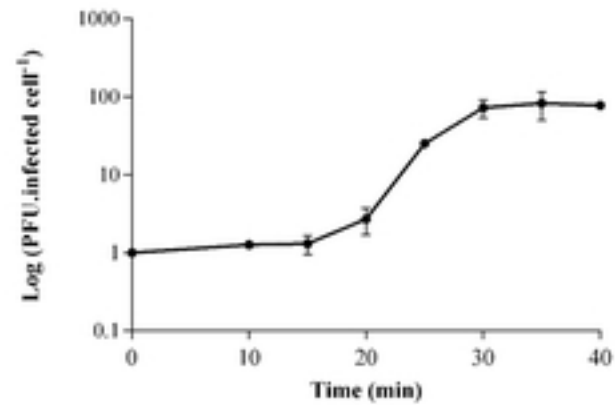
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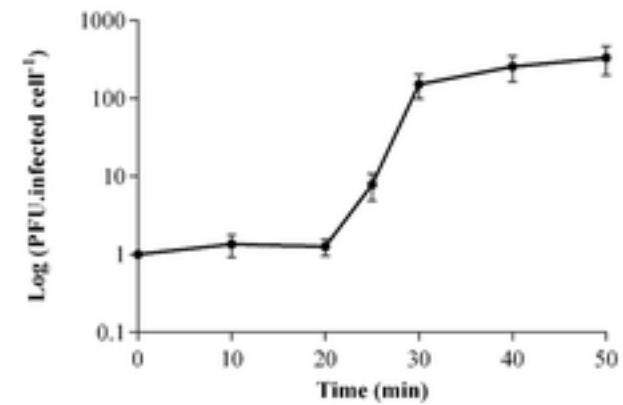
(a)

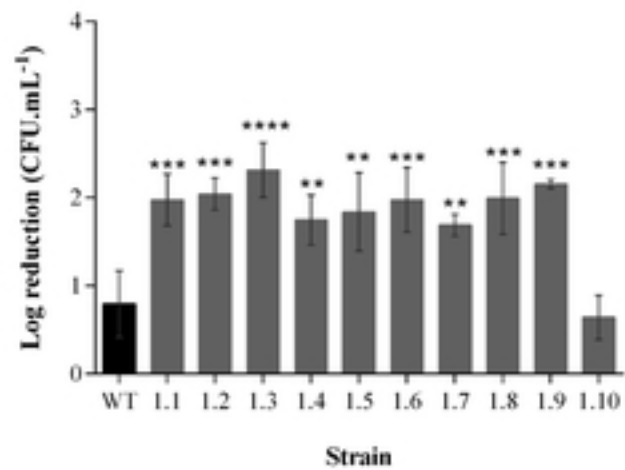
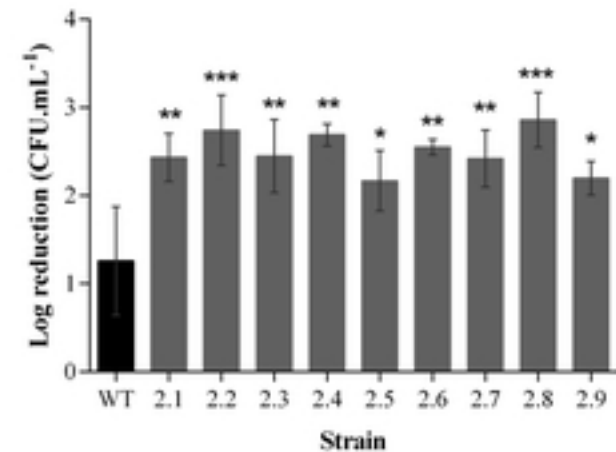


(b)



(c)



(a)**(b)****(c)**