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A toxic friend: Genotoxic and mutagenic activity of the probiotic strain *Escherichia coli* Nissle 1917

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1 **Title:** A toxic friend: Genotoxic and mutagenic activity of the probiotic strain *Escherichia coli*
2 Nissle 1917

3

4 **Running title:** Nissle 1917 is genotoxic

5

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15

16 **Abstract:**

17 The probiotic *Escherichia coli* strain Nissle 1917 (DSM 6601, Mutaflor), generally considered
18 as beneficial and safe, has been used for a century to treat various intestinal diseases. However,
19 Nissle 1917 hosts in its genome the *pks* pathogenicity island that codes for the biosynthesis of
20 the genotoxin colibactin. Colibactin is a potent DNA alkylator, suspected to play a role in
21 colorectal cancer development. We show in this study that Nissle 1917 is functionally capable
22 of producing colibactin and inducing interstrand crosslinks in the genomic DNA of epithelial

23 cells exposed to the probiotic. This toxicity was even exacerbated with lower doses of the
24 probiotic, when the exposed cells started to divide again but exhibited aberrant anaphases and
25 increased gene mutation frequency. DNA damage was confirmed *in vivo* in mouse models of
26 intestinal colonization, demonstrating that Nissle 1917 produces the genotoxin in the gut lumen.
27 Although it is possible that daily treatment of adult humans with their microbiota does not
28 produce the same effects, administration of Nissle 1917 as a probiotic or as a chassis to deliver
29 therapeutics might exert long term adverse effects and thus should be considered in a risk versus
30 benefit evaluation.

31 **Importance:**

32 Nissle 1917 is sold as a probiotic and considered safe even though it is known since 2006 that
33 it encodes the genes for colibactin synthesis. Colibactin is a potent genotoxin that is now linked
34 to causative mutations found in human colorectal cancer. Many papers concerning the use of
35 this strain in clinical applications ignore or elude this fact, or misleadingly suggest that Nissle
36 1917 does not induce DNA damage. Here, we demonstrate that Nissle 1917 produces colibactin
37 *in vitro* and *in vivo* and induces mutagenic DNA damage. This is a serious safety concern that
38 must not be ignored, for the interests of patients, the general public, health care professionals
39 and ethical probiotic manufacturers.

40

41 **Introduction**

42 *Escherichia coli* Nissle 1917 is an intestinal strain originally isolated during the first world war.
43 Nissle 1917 is a potent competitor of different enteropathogens in the gut (Nissle, 1959).
44 Consequently, it has been used for a century as a treatment for diarrhea and more recently for
45 other intestinal disorders such as inflammatory bowel diseases (IBDs). The use of Nissle 1917
46 is recommended for maintaining remission in ulcerative colitis (Floch et al., 2011; Kruis et al.,

47 2004). It is used as a probiotic in human medicine in Germany, Australia, Canada and other
48 countries under the name of “Mutaflor”. Nissle 1917 is also a popular chassis to engineer
49 therapeutic bacteria for vaccine, diagnostics, biosensors and drug development (Ou et al.,
50 2016). The popularity of Nissle 1917 resides not only in its “natural” beneficial properties, but
51 also in the general acceptance that it is harmless and safe. Its safety profile is based in part on
52 the belief that Nissle 1917 does not produce any toxin associated with pathogenic strains of *E.*
53 *coli*. Although this statement is still propagated in the recent biomedical literature, it was shown
54 in 2006 that Nissle 1917 hosts a 54 kb *pks* island coding for non-ribosomal and polyketide
55 synthases (NRPS and PKS) allowing synthesis of a hybrid peptide-polyketide metabolite called
56 colibactin (Homburg et al., 2007; Nougayrède et al., 2006).

57 Colibactin is a genotoxin that binds and crosslinks the opposite strands of DNA, resulting in
58 DNA damages and gene mutagenesis in eukaryotic cells (Bossuet-Greif et al., 2018; Cuevas-
59 Ramos et al., 2010; Dziubańska-Kusibab et al., 2020; Iftexhar et al., 2021; Nougayrède et al.,
60 2006; Pleguezuelos-Manzano et al., 2020; Wilson et al., 2019). Colibactin is a virulence factor
61 during systemic infection (Marcq et al., 2014; Martin et al., 2013; McCarthy et al., 2015), and
62 plays a substantial role in colorectal cancer. Indeed, colibactin-producing *E. coli* promote
63 colorectal cancer in mouse models (Arthur et al., 2012; Cougnoux et al., 2014) and the DNA
64 mutational signature of colibactin has been found in cohorts of patients with colorectal cancer,
65 including in the APC cancer driver gene (Dziubańska-Kusibab et al., 2020; Pleguezuelos-
66 Manzano et al., 2020; Terlouw et al., 2020). A conflicting report claimed that “no genotoxicity
67 is detectable for *E. coli* strain Nissle 1917 by standard *in vitro* and *in vivo* tests” (Dubbert et al.,
68 2020) but the authors used assays that are suboptimal to demonstrate production and
69 mutagenicity of colibactin, such as the use of *Salmonella* reporter bacteria that are killed by the
70 microcins produced by Nissle 1917 (Massip et al., 2020; Sassone-Corsi et al., 2016). Recently,
71 in a study using stem cell-derived human intestinal organoids to evaluate the safety of the

72 probiotic, Nissle 1917 “was found to be safe” (Pradhan and Weiss, 2020), while exposure of
73 such organoids to *pks+* *E. coli* induced the colibactin-specific mutational signature
74 (Pleguezuelos-Manzano et al., 2020). Here, we examined the production and genotoxicity of
75 colibactin by Nissle 1917 *in vitro*, using assays adapted to the described mode of action of the
76 toxin, and *in vivo* in two mouse models.

77

78 **Results**

79

80 **Nissle 1917 produces colibactin and induces DNA crosslinks in infected epithelial cells.**

81 DNA interstrand crosslinks generated by colibactin impair the denaturation of DNA and thus
82 inhibits its electrophoretic mobility in denaturing conditions (Bossuet-Greif et al., 2018). We
83 examined whether infection of epithelial cells with Nissle 1917 could induce crosslinks in the
84 host genomic DNA. Cultured human epithelial HeLa cells were exposed to live *E. coli* Nissle
85 1917 for 4 hours, then the cell genomic DNA was purified and analyzed by denaturing gel
86 electrophoresis. In contrast to the DNA of control cells which migrated as a high molecular
87 weight band, a fraction of the DNA of the cells exposed to Nissle 1917 remained in the loading
88 well (Fig 1). Similar genomic DNA with impaired electrophoretic migration was observed in
89 cells treated with cisplatin, a DNA crosslinking agent (Fig 1a). In contrast, a Nissle 1917 mutant
90 for the phosphopantetheinyl transferase ClbA, required for activation of the NRPS and PKS in
91 the *pks* pathway (Martin et al., 2013), did not induce non-migrating genomic DNA (Fig 1ab).
92 Similarly, no crosslinking activity was detected with the Nissle 1917 strain mutated for the
93 peptidase ClbP that cleaves the inactive precolibactin to generate the mature active colibactin
94 (Brotherton and Balskus, 2013) (Fig 1 ab). We also observed the DNA crosslinking activity in

95 exogenous DNA exposed to the wild-type Nissle 1917 but not to the *clbA* and *clbP* mutants
96 (Sup Fig 1). Thus, Nissle 1917 synthesizes mature DNA crosslinking colibactin.

97

98 **Infection with Nissle 1917 induces the recruitment of the DNA repair machinery.**

99 It was recently shown that upon formation of DNA crosslinks by colibactin, the cells recruit the
100 kinase ataxia telangiectasia and Rad3-related (ATR), which phosphorylate the Ser33 of the
101 replication protein A-32 (RPA) in nuclear DNA repair foci together with phosphorylated
102 histone γ H2AX (Bossuet-Greif et al., 2018). Immunofluorescence of Ser33-phosphorylated
103 RPA and γ H2AX showed nuclear foci of both markers in HeLa cells 4 h after infection with
104 Nissle 1917, or following treatment with the crosslinking drug cisplatin, but not after infection
105 with the *clbA* or *clbP* mutants (Fig 2a). The γ H2AX and p-RPA foci increased with the
106 multiplicity of infection (MOI) with the wild-type Nissle 1917, and remained plainly
107 measurable 20 h after infection, even at the low MOI of 20 bacteria per cell (Fig 2b). Together
108 these results demonstrate that Nissle 1917 induces dose and time dependent DNA crosslinks in
109 exposed cells, resulting in cognate DNA repair machinery recruitment.

110

111 **Exposure to low numbers of Nissle 1917 induces abnormal mitosis and increased gene** 112 **mutation frequency.**

113 Infection with colibactin-producing *E. coli* at low MOI can lead to incomplete DNA repair in a
114 subset of the cell population, allowing cell division to restart and formation of aberrant
115 anaphases, and ultimately increased gene mutation frequency (Cuevas-Ramos et al., 2010). We
116 thus tested whether infection with Nissle 1917 induced these phenotypes, in epithelial CHO
117 cells that have stable chromosomes and are amenable to gene mutation assay. CHO cells

118 exposed to low numbers of wild-type Nissle 1917 showed abnormal mitotic figures 20 h after
119 infection (Fig 3a). We observed lagging chromosomes, multipolar mitosis and anaphase DNA
120 bridges in cells infected with Nissle 1917, or treated with cisplatin (Fig 3ab). The abnormal
121 mitotic index increased with the MOI of the wild-type Nissle 1917 strain, whereas it remained
122 at background level in cells exposed to the highest MOI of the *clbA* or *clbP* mutants (Fig. 3b).
123 Mitotic errors can lead to an accumulation of DNA damage, which in turn favors gene mutations
124 (Chatterjee and Walker, 2017; Levine and Holland, 2018). We thus next assessed gene mutation
125 frequencies at the hypoxanthine-guanine phosphoribosyltransferase (*hprt*) loci after infection
126 of CHO cells (Table). We found a two-fold increase in 6-thioguanine-resistant (*hprt* mutant)
127 colonies after infection with a MOI of 10 of the wild-type Nissle 1917 compared with
128 uninfected cells or cells that were infected with the *clbA* or *clbP* mutant. The mutation
129 frequency was similar to that previously observed with a laboratory *E. coli* strain hosting the
130 *pks* island at the same MOI (Cuevas-Ramos et al., 2010), but did not reach statistical
131 significance. Infection with a MOI of 20 Nissle 1917 resulted in a significant increase of *hprt*
132 mutation frequency. Treatment with cisplatin also resulted in a significant increase of *hprt*
133 mutants, with a mutation frequency similar to that reported in the literature (Silva et al., 2005).
134 We conclude that Nissle 1917 is mutagenic.

135

136 **Nissle 1917 induces DNA damage to intestinal cells *in vivo*.**

137 To test whether Nissle 1917 produces colibactin *in vivo* in the gut lumen and induces DNA
138 damages to intestinal cells, we first used a simplified model of intestinal colonization; adult
139 axenic Balb/c mice were inoculated with Nissle 1917 or the *clbA* mutant, or with sterile PBS.
140 Seven days after inoculation, the mice were sacrificed, fecal and intestinal tissue samples were
141 collected. The mice mono-associated with Nissle 1917 or hosting the *clbA* mutant exhibited

142 similar fecal counts of $\sim 10^9$ CFU/g of feces. We assessed by immune-histology histone γ H2AX
143 in the colon. Nuclear γ H2AX foci were readily observed in the enterocytes exposed to Nissle
144 1917, but not in animals inoculated with the *clbA* mutant, which exhibited background γ H2AX
145 levels similar to that of the axenic controls (Fig. 4ab).

146 Nissle 1917 is used not only in adults but also in infants and toddlers. To further examine
147 production of colibactin *in vivo*, we used a second *in vivo* model in which 8-days old Swiss
148 mouse pups were given *per os* $\sim 10^8$ CFU of Nissle 1917 or the *clbP* mutant or PBS. Six hours
149 after inoculation, the intestinal epithelium was examined for formation of γ H2AX foci. Animals
150 treated with Nissle 1917 exhibited significant levels of nuclear γ H2AX compared to controls
151 treated with PBS (Fig 5). In contrast, the animals treated with the *clbP* mutant that does not
152 produce colibactin showed background levels of γ H2AX (Fig 5). Together these results
153 indicated that Nissle 1917 induces *in vivo* DNA damage to epithelial cells.

154

155 **Discussion**

156 The identification of colibactin mutation signature in human colorectal cancer tissues
157 (Dziubańska-Kusibab et al., 2020; Pleguezuelos-Manzano et al., 2020; Terlouw et al., 2020)
158 and also in colonic crypts from healthy individuals under the age of ten (Lee-Six et al., 2019)
159 proves that colibactin is expressed within the human gut (including in child), and links
160 colibactin exposure to colorectal cancer. Colibactin is now a suspected prooncogenic driver
161 especially in IBD patients (Dubinsky et al., 2020). Nissle 1917 has been used as a probiotic for
162 various clinical applications since its isolation more than 100 years ago. It has shown some
163 efficacy to treat IBDs such as Crohn's disease and ulcerative colitis. In this study, we
164 demonstrate that Nissle 1917 synthesizes colibactin, *in vitro* and *in vivo* in the gut lumen, and
165 inflicts mutagenic DNA damages. Even in low numbers, DNA crosslinks are catastrophic

166 damages that obstruct basic DNA processes, since they prevent the strand separation required
167 for polymerase functions. The crosslinks notably perturb the replication machinery, resulting
168 in replication stress, accumulation of DNA bound by RPA, activation of the kinase ATR that
169 in turn phosphorylates RPA and histone variant H2AX (Bossuet-Greif et al., 2018; Maréchal
170 and Zou, 2015; Vassin et al., 2009). We observed that cells exposed to Nissle 1917 at low MOI
171 (hence numbers of bacteria more relevant to those occurring *in vivo*) entered an error-prone
172 repair pathway, exhibiting mitotic aberrations and increased gene mutation frequency, similar
173 to that observed with other *pks+* *E. coli* strain (Cuevas-Ramos et al., 2010; Iftekhar et al., 2021).
174 Thus, Nissle 1917 is genotoxigenic and mutagenic. This is of concern for patients and
175 participants in clinical trials using Nissle 1917, such as the trial in Finland in which more than
176 250 young children will be inoculated with this strain
177 (<https://clinicaltrials.gov/ct2/show/NCT04608851>)

178 Our results stand in contrast to that reported by Dubbert and colleagues who claimed
179 that Nissle 1917 does not have detectable mutagenic activity using standard tests (Dubbert et
180 al., 2020). However, the assays they used cannot detect colibactin-associated mutagenic
181 damage. Indeed, to examine whether Nissle 1917 could induce mutagenic DNA damages,
182 Dubbert used an Ames test in which *Salmonella typhimurium* reporter bacteria were exposed
183 to Nissle 1917 and then *Salmonella* growth was expected upon mutagenesis. However,
184 *Salmonella* bacteria are readily killed by the siderophores-microcins produced by Nissle 1917
185 (Massip et al., 2020; Sassone-Corsi et al., 2016) and thus the absence of growth of the reporter
186 bacteria was incorrectly interpreted as an absence of effect of colibactin. In addition, Dubbert
187 used a standard comet assay that can detect a variety of DNA lesions through electrophoresis
188 of broken DNA, but which cannot detect DNA crosslinks that inhibit DNA electrophoretic
189 mobility (Bossuet-Greif et al., 2018; Merk and Speit, 1999; Wilson et al., 2019). Thus, the
190 standard assays used by Dubbert were inappropriate, in contrast to the assays used in the present

191 and other works (Vizcaino and Crawford, 2015; Wilson et al., 2019), to highlight the DNA-
192 damaging activity and genotoxicity of colibactin produced by Nissle 1917.

193 We demonstrate using two mouse models that Nissle 1917 synthesizes colibactin in the
194 gut and induces DNA damage in intestinal cells. Obviously, these mouse models do not fully
195 recapitulate the human intestine, in particular its complex microbiota, epithelial and intestinal
196 barrier functions. However, in human patients Nissle 1917 is typically used in the context of
197 IBDs, where the gut is inflamed, the intestinal barrier is dysfunctional and the microbiota is
198 dysbiotic. Importantly, intestinal inflammation was shown to upregulate *pks* genes (Arthur et
199 al., 2014; Dubinsky et al., 2020; Yang et al., 2020). Inflammation and dysbiosis are also known
200 to allow the expansion of the *E. coli* population, including that of Nissle 1917, alongside the
201 epithelium (Cevallos et al., 2019; Dejea et al., 2018; Zhu et al., 2019). Moreover, Nissle 1917
202 is typically administered in very high numbers ($2.5\text{-}25\times 10^9$ bacteria in adults, 10^8 in infants),
203 repeatedly (1-4 times daily), for weeks or even longer in case of ulcerative colitis. Nissle 1917
204 has been reported to persist in the human gut for months after inoculation (Lodinová-Zádníková
205 and Sonnenborn, 1997). Thus, patients treated with this probiotic can be exposed chronically to
206 high numbers of colibactin-producing bacteria, especially in an inflamed context that favors
207 colibactin production, and consequently could be exposed to high levels of mutagenic
208 colibactin. These conditions were shown to promote colon tumorigenesis in colorectal cancer
209 (Arthur et al., 2012).

210 Nissle 1917 is an increasingly popular choice to engineer live biotherapeutics (i.e.
211 bacteria genetically designed to treat or prevent a disease) (Charbonneau et al., 2020). For
212 example, Nissle 1917 has been used successfully as a chassis to deliver an anti-biofilm enzyme
213 against *P. aeruginosa* (Hwang et al., 2017), or a microcin induced upon sensing of *Salmonella*
214 infection (Palmer et al., 2018). Engineered strains of Nissle 1917 have also been constructed to
215 treat obesity through production of N- acylphosphatidylethanolamine (Chen et al., 2014) or to

216 express a phenylalanine-metabolizing enzyme in response to the anoxic conditions in the gut,
217 to treat phenylketonuria (Isabella et al., 2018). Considering the widespread use of Nissle 1917,
218 as a probiotic and as a platform to develop live bacterial therapeutics, ensuring its safety is of
219 paramount importance. Genotoxic carcinogens are classically conceived to represent a risk
220 factor with no threshold dose, because little numbers or even one DNA lesion may result in
221 mutation and increased tumor risk (Hartwig et al., 2020). Production of mutagenic colibactin
222 by Nissle 1917 is thus a serious health concern that must be addressed.

223

224 **Methods**

225

226 ***E. coli* EcN strain, mutants and culture**

227 The *E. coli* strain Nissle 1917 used in this study was obtained from Dr. Ulrich Dobrindt
228 (University of Münster). The *clbA* and *clbP* isogenic mutants were described previously (ref
229 Ollier et Nat Comm). Before infection, the bacteria were grown overnight at 37°C with 240
230 RPM agitation in 5 mL of Lennox L broth (LB, Invitrogen) then diluted 1/20 in pre-warmed
231 DMEM 25 mM Hepes (Invitrogen) and incubated at 37°C with 240 RPM agitation to reach
232 exponential phase (OD₆₀₀=0.4 to 0.5).

233

234 ***In vitro* DNA crosslinking assay**

235 3×10^6 bacteria or numbers given in the text were inoculated in 100 μ l of DMEM 25 mM Hepes,
236 incubated at 37°C for 3.5 hours, then EDTA (1 mM) and 400 ng of linearized (BamHI) pUC19
237 DNA were added and further incubated 40 minutes. As controls, DNA was left uninfected or
238 was treated with 100 or 200 μ M cisplatin (Sigma). Following a centrifugation to pellet the

239 bacteria, the DNA was purified using Qiagen PCR DNA purification kit before analysis by
240 denaturing gel electrophoresis.

241

242 **Denaturing gel DNA electrophoresis**

243 1% agarose gels prepared in a 100 mM NaCl 2 mM EDTA pH 8 solution were soaked 16 hours
244 in 40 mM NaOH 1 mM EDTA electrophoresis running buffer. DNA electrophoresis was
245 performed at room temperature, 45 min at 1 V/cm then 2 h at 2 V/cm. Following neutralization
246 by serial washes in 150 mM NaCl 100 mM Tris pH 7.4, DNA was stained with Gel Red
247 (Biotium) and photographed with flat-field correction and avoiding CCD pixel saturation in a
248 Biorad Chemidoc XRS system. Images were analyzed using NIH ImageJ: the background was
249 subtracted (100 pixels rolling ball) then the lane profiles were plotted and the area of DNA
250 peaks were measured.

251

252 **Cell culture and infection**

253 HeLa and CHO cells were cultivated in a 37°C 5% CO₂ incubator and maintained by serial
254 passage in DMEM Glutamax or MEM α (Invitrogen) respectively, both supplemented with 10%
255 fetal calf serum (FCS), 50 μ g/ml gentamicin and 1% non-essential amino acids (Invitrogen).
256 3×10^5 cells/well were seeded in 6-wells plates (TPP) or 3.5×10^4 cells/well in 8-chambers slides
257 (Falcon) and grown 24 hours. Cells were washed 3 times in HBSS (Invitrogen) before infection
258 in DMEM 25 mM HEPES at given multiplicity of infection (MOI = number of bacteria per cell
259 at the onset of infection). Following the 4 hours co-culture, the cells were washed 3 times with
260 HBSS then incubated in complete cell culture medium supplemented with 200 μ g/ml
261 gentamicin for the indicated times (0, 4 or 20 hours) before analysis.

262

263 ***In cellulo* genomic DNA crosslinking assay**

264 The cells were infected 4 hours or treated 4 hours with 100 μ M cisplatin (Sigma), then collected
265 immediately by trypsination. The cell genomic DNA was purified with Qiagen DNeasy Blood
266 and Tissue kit and analyzed by denaturing gel electrophoresis.

267

268 **Abnormal anaphase scoring**

269 Abnormal anaphase quantification was done as described (Luo et al., 2004). Briefly, 3 hours
270 after the end of infection, the cells were trapped in premetaphase by treatment with 0.6 μ g/ml
271 nocodazole and released 55 min without nocodazole to reach anaphase. The slides were fixed,
272 stained with DAPI and examined by confocal microscopy as described below. The anaphases
273 were scored in three independent experiments.

274

275 **Gene mutation assay**

276 CHO cells were treated 4 days with culture medium supplemented with 10mM deoxycytidine
277 200mM hypoxanthine 0.2mM aminoprotein and 17.5mM thymidine (Sigma) to eliminate
278 preexisting *hprt* mutants. CHO were infected 4 hours with Nissle 1917 or *clbA* or *clbP* mutants,
279 or treated with cisplatin, then washed and cultured one week in normal cell culture medium and
280 passaged in 10 cm dishes seeded with 3×10^5 cells using culture medium supplemented with 30
281 μ M 6-thioguanine (6-TG, Sigma). Cells were also plated without 6-TG to determine plating
282 efficiency. The culture media was changed twice a week for 21 days. Then plates were fixed
283 with 4% formaldehyde and stained with methylene blue.

284

285

286

287 **Animal studies**

288 All procedures were carried out according to European and French guidelines for the care and
289 use of laboratory animals. The experimentations were approved by Regional Council of Ethics
290 for animal experimentation. SPF pregnant Swiss mice obtained from Janvier (Le Genest, St
291 Isle, France) were housed under SPF conditions in the Inserm Purpan animal facility (Toulouse,
292 France). Eight days old mice pups received *per os* a drop (approximately 25 μ l) of bacteria
293 suspended (10^{10} CFU/ml) in PBS, and were sacrificed 6 hours later (protocols 16-U1220-
294 JPN/FT-010 and 17-U1220-EO/PM-461). Germ-free Balb/c mice were housed in the breeding
295 facility of ANAXEM (INRAE, UMR1319 MICALIS, Jouy-en- Josas, France). Axenic animals
296 were inoculated once by intragastric gavage with 10^8 bacteria suspended in PBS, and sacrificed
297 7 days later (protocol APAFIS#3441-2016010614307552 v1). Intestinal tissue samples were
298 fixed 24 hours in neutral buffered formalin, dehydrated in ethanol and embedded in paraffin.

299

300 **γ H2AX and p-RPA immunofluorescence analysis**

301 4 or 20 hours after infection, HeLa cells were pre-extracted 5 min in PBS 0.1% Triton X-100
302 before a 30 min fixation in PBS 4% formaldehyde. Following permeabilization in 0.1% Triton
303 X-100 and blocking in MAXblock medium (Active Motif), the cells were stained 3 hours with
304 antibodies against γ H2AX (1:500, JBW301, Millipore) and S33p-RPA32 (1:500, A300-264A,
305 Bethyl) diluted in MAXblock 0.05% Triton X-100. The cells were washed 3 times in PBS
306 0.05% Triton X-100 and incubated 1 h with anti-mouse AlexaFluor 488 and anti-rabbit
307 AlexaFluor 568 (Invitrogen) diluted 1:500 in MAXblock medium with 1 μ g/ml DAPI (Sigma).
308 The cells were washed again, mounted in Fluoroshield medium (Sigma), and examined with a
309 Leica SP8 laser scanning confocal microscope in sequential mode. The mean fluorescence
310 intensities (MFI) of γ H2AX and p-RPA within the nuclei were analyzed using a NIH ImageJ
311 macro: the nuclei were identified in the DNA image (following a 0.5 μ m Gaussian blur and

312 default auto-threshold) and copied in the ROI manager to measure their corresponding MFI in
313 the green and red channels.

314 For immunohistological staining of γ H2AX in intestinal tissues, sections (5 or 8 μ m) were
315 deparaffinized by serial washes in xylene and ethanol, then rehydrated with water. The antigens
316 were unmasked in HBSS 0.05% trypsin 0.02% EDTA at 37°C for 6 min then in sodium citrate
317 buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) for 30 min at 80-95°C. Following a 1
318 hour cooling to room temperature and blocking 1 hour in 0.3% Triton X-100 MAXblock
319 medium, the tissues were stained 16 hours at 4°C with primary antibodies against γ H2AX
320 (1:200, 20E3, Cell Signaling Technology) diluted in the blocking medium. The slides were
321 washed 3 times in PBS 0.05% Triton X-100 and incubated 1 h with anti-rabbit AlexaFluor 568
322 diluted 1:200 in MAXblock medium with 1 μ g/ml DAPI. The slides were washed again,
323 mounted and examined as above.

324

325 **Statistical analyses.**

326 Statistical analyses were performed using Graph Pad Prism 9. Analysis of mutant frequencies
327 was performed using a two tailed t-test on the log transformed data, to ensure data normality
328 and to correct variance heterogeneity (Silva et al., 2005).

329

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337

338 **References**

- 339 Arthur, J.C., Perez-Chanona, E., Mühlbauer, M., Tomkovich, S., Uronis, J.M., Fan, T.-J.,
340 Campbell, B.J., Abujamel, T., Dogan, B., Rogers, A.B., et al. (2012). Intestinal Inflammation
341 Targets Cancer-Inducing Activity of the Microbiota. *Science*.
- 342 Arthur, J.C., Gharaibeh, R.Z., Mühlbauer, M., Perez-Chanona, E., Uronis, J.M., McCafferty,
343 J., Fodor, A.A., and Jobin, C. (2014). Microbial genomic analysis reveals the essential role of
344 inflammation in bacteria-induced colorectal cancer. *Nat Commun* 5, 4724.
- 345 Bossuet-Greif, N., Vignard, J., Taieb, F., Mirey, G., Dubois, D., Petit, C., Oswald, E., and
346 Nougayrède, J.-P. (2018). The Colibactin Genotoxin Generates DNA Interstrand Cross-Links
347 in Infected Cells. *MBio* 9, e02393-17.
- 348 Brotherton, C.A., and Balskus, E.P. (2013). A prodrug resistance mechanism is involved in
349 colibactin biosynthesis and cytotoxicity. *J. Am. Chem. Soc.* 135, 3359–3362.
- 350 Cevallos, S.A., Lee, J.-Y., Tiffany, C.R., Byndloss, A.J., Johnston, L., Byndloss, M.X., and
351 Bäumlér, A.J. (2019). Increased Epithelial Oxygenation Links Colitis to an Expansion of
352 Tumorigenic Bacteria. *MBio* 10.
- 353 Charbonneau, M.R., Isabella, V.M., Li, N., and Kurtz, C.B. (2020). Developing a new class of
354 engineered live bacterial therapeutics to treat human diseases. *Nature Communications* 11,
355 1738.
- 356 Chatterjee, N., and Walker, G.C. (2017). Mechanisms of DNA damage, repair, and
357 mutagenesis. *Environmental and Molecular Mutagenesis* 58, 235–263.
- 358 Chen, Z., Guo, L., Zhang, Y., Walzem, R.L., Pendergast, J.S., Printz, R.L., Morris, L.C.,
359 Matafonova, E., Stien, X., Kang, L., et al. (2014). Incorporation of therapeutically modified
360 bacteria into gut microbiota inhibits obesity. *J Clin Invest* 124, 3391–3406.
- 361 Cougnoux, A., Dalmaso, G., Martinez, R., Buc, E., Delmas, J., Gibold, L., Sauvanet, P.,
362 Darcha, C., Déchelotte, P., Bonnet, M., et al. (2014). Bacterial genotoxin colibactin promotes
363 colon tumour growth by inducing a senescence-associated secretory phenotype. *Gut* gutjnl-
364 2013-305257.
- 365 Cuevas-Ramos, G., Petit, C.R., Marcq, I., Boury, M., Oswald, E., and Nougayrède, J.-P.
366 (2010). *Escherichia coli* induces DNA damage in vivo and triggers genomic instability in
367 mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* 107, 11537–11542.
- 368 Dejea, C.M., Fathi, P., Craig, J.M., Boleij, A., Taddese, R., Geis, A.L., Wu, X., DeStefano
369 Shields, C.E., Hechenbleikner, E.M., Huso, D.L., et al. (2018). Patients with familial

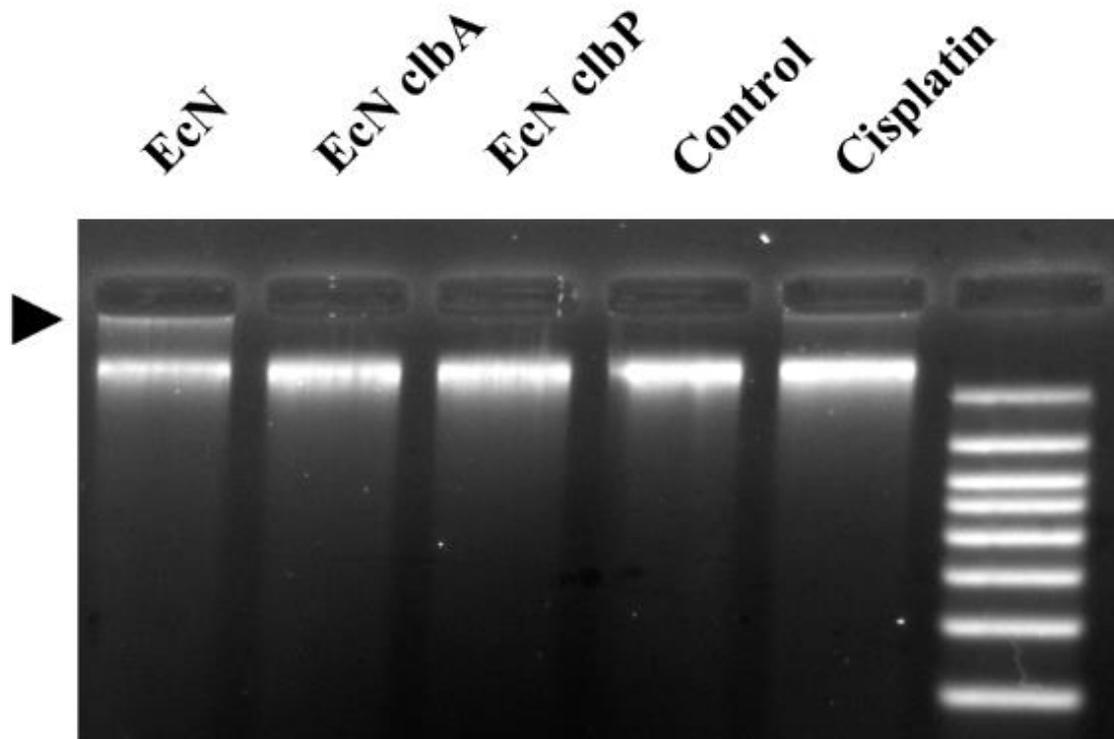
- 370 adenomatous polyposis harbor colonic biofilms containing tumorigenic bacteria. *Science* 359,
371 592–597.
- 372 Dubbert, S., Klinkert, B., Schimiczek, M., Wassenaar, T.M., and von Büнау, R. (2020). No
373 Genotoxicity Is Detectable for *Escherichia coli* Strain Nissle 1917 by Standard In Vitro and In
374 Vivo Tests. *Eur J Microbiol Immunol (Bp)* 10, 11–19.
- 375 Dubinsky, V., Dotan, I., and Gophna, U. (2020). Carriage of Colibactin-producing Bacteria
376 and Colorectal Cancer Risk. *Trends in Microbiology* 28, 874–876.
- 377 Dziubańska-Kusibab, P.J., Berger, H., Battistini, F., Bouwman, B.A.M., Iftekhar, A.,
378 Katainen, R., Cajuso, T., Crosetto, N., Orozco, M., Aaltonen, L.A., et al. (2020). Colibactin
379 DNA-damage signature indicates mutational impact in colorectal cancer. *Nat. Med.*
- 380 Floch, M.H., Walker, W.A., Madsen, K., Sanders, M.E., Macfarlane, G.T., Flint, H.J.,
381 Dieleman, L.A., Ringel, Y., Guandalini, S., Kelly, C.P., et al. (2011). Recommendations for
382 probiotic use-2011 update. *J Clin Gastroenterol* 45 Suppl, S168-171.
- 383 Hartwig, A., Arand, M., Epe, B., Guth, S., Jahnke, G., Lampen, A., Martus, H.-J., Monien, B.,
384 Rietjens, I.M.C.M., Schmitz-Spanke, S., et al. (2020). Mode of action-based risk assessment
385 of genotoxic carcinogens. *Arch Toxicol* 94, 1787–1877.
- 386 Homburg, S., Oswald, E., Hacker, J., and Dobrindt, U. (2007). Expression analysis of the
387 colibactin gene cluster coding for a novel polyketide in *Escherichia coli*. *FEMS Microbiol.*
388 *Lett.* 275, 255–262.
- 389 Hwang, I.Y., Koh, E., Wong, A., March, J.C., Bentley, W.E., Lee, Y.S., and Chang, M.W.
390 (2017). Engineered probiotic *Escherichia coli* can eliminate and prevent *Pseudomonas*
391 *aeruginosa* gut infection in animal models. *Nature Communications* 8, 15028.
- 392 Iftekhar, A., Berger, H., Bouznad, N., Heuberger, J., Boccellato, F., Dobrindt, U., Hermeking,
393 H., Sigal, M., and Meyer, T.F. (2021). Genomic aberrations after short-term exposure to
394 colibactin-producing *E. coli* transform primary colon epithelial cells. *Nat Commun* 12, 1003.
- 395 Isabella, V.M., Ha, B.N., Castillo, M.J., Lubkowicz, D.J., Rowe, S.E., Millet, Y.A.,
396 Anderson, C.L., Li, N., Fisher, A.B., West, K.A., et al. (2018). Development of a synthetic
397 live bacterial therapeutic for the human metabolic disease phenylketonuria. *Nat Biotechnol*
398 36, 857–864.
- 399 Kruis, W., Frič, P., Pokrotnieks, J., Lukáš, M., Fixa, B., Kaščák, M., Kamm, M.A.,
400 Weismueller, J., Beglinger, C., Stolte, M., et al. (2004). Maintaining remission of ulcerative
401 colitis with the probiotic *Escherichia coli* Nissle 1917 is as effective as with standard
402 mesalazine. *Gut* 53, 1617–1623.
- 403 Lee-Six, H., Olafsson, S., Ellis, P., Osborne, R.J., Sanders, M.A., Moore, L., Georgakopoulos,
404 N., Torrente, F., Noorani, A., Goddard, M., et al. (2019). The landscape of somatic mutation
405 in normal colorectal epithelial cells. *Nature* 574, 532–537.
- 406 Levine, M.S., and Holland, A.J. (2018). The impact of mitotic errors on cell proliferation and
407 tumorigenesis. *Genes Dev.* 32, 620–638.

- 408 Lodinová-Zádníková, R., and Sonnenborn, U. (1997). Effect of preventive administration of a
409 nonpathogenic *Escherichia coli* strain on the colonization of the intestine with microbial
410 pathogens in newborn infants. *Biol Neonate* 71, 224–232.
- 411 Luo, L.Z., Werner, K.M., Gollin, S.M., and Saunders, W.S. (2004). Cigarette smoke induces
412 anaphase bridges and genomic imbalances in normal cells. *Mutation Research/Fundamental
413 and Molecular Mechanisms of Mutagenesis* 554, 375–385.
- 414 Marcq, I., Martin, P., Payros, D., Cuevas-Ramos, G., Boury, M., Watrin, C., Nougayrède, J.-
415 P., Olier, M., and Oswald, E. (2014). The genotoxin colibactin exacerbates lymphopenia and
416 decreases survival rate in mice infected with septicemic *Escherichia coli*. *J. Infect. Dis.* 210,
417 285–294.
- 418 Maréchal, A., and Zou, L. (2015). RPA-coated single-stranded DNA as a platform for post-
419 translational modifications in the DNA damage response. *Cell Research* 25, 9–23.
- 420 Martin, P., Marcq, I., Magistro, G., Penary, M., Garcie, C., Payros, D., Boury, M., Olier, M.,
421 Nougayrède, J.-P., Audebert, M., et al. (2013). Interplay between siderophores and colibactin
422 genotoxin biosynthetic pathways in *Escherichia coli*. *PLoS Pathog.* 9, e1003437.
- 423 Massip, C., Chagneau, C.V., Boury, M., and Oswald, E. (2020). The synergistic triad between
424 microcin, colibactin, and salmochelin gene clusters in uropathogenic *Escherichia coli*.
425 *Microbes Infect.* 22, 144–147.
- 426 McCarthy, A.J., Martin, P., Cloup, E., Stabler, R.A., Oswald, E., and Taylor, P.W. (2015).
427 The Genotoxin Colibactin Is a Determinant of Virulence in *Escherichia coli* K1 Experimental
428 Neonatal Systemic Infection. *Infect. Immun.* 83, 3704–3711.
- 429 Merk, O., and Speit, G. (1999). Detection of crosslinks with the comet assay in relationship to
430 genotoxicity and cytotoxicity. *Environmental and Molecular Mutagenesis* 33, 167–172.
- 431 Nissle, A. (1959). [On coli antagonism, dysbacteria and coli therapy]. *Med Monatsschr* 13,
432 489–491.
- 433 Nougayrède, J.-P., Homburg, S., Taieb, F., Boury, M., Brzuszkiewicz, E., Gottschalk, G.,
434 Buchrieser, C., Hacker, J., Dobrindt, U., and Oswald, E. (2006). *Escherichia coli* induces
435 DNA double-strand breaks in eukaryotic cells. *Science* 313, 848–851.
- 436 Ou, B., Yang, Y., Tham, W.L., Chen, L., Guo, J., and Zhu, G. (2016). Genetic engineering of
437 probiotic *Escherichia coli* Nissle 1917 for clinical application. *Appl Microbiol Biotechnol*
438 100, 8693–8699.
- 439 Palmer, J.D., Piattelli, E., McCormick, B.A., Silby, M.W., Brigham, C.J., and Bucci, V.
440 (2018). Engineered Probiotic for the Inhibition of *Salmonella* via Tetrathionate-Induced
441 Production of Microcin H47. *ACS Infect Dis* 4, 39–45.
- 442 Pleguezuelos-Manzano, C., Puschhof, J., Rosendahl Huber, A., van Hoeck, A., Wood, H.M.,
443 Nomburg, J., Gurjao, C., Manders, F., Dalmasso, G., Stege, P.B., et al. (2020). Mutational
444 signature in colorectal cancer caused by genotoxic pks+ *E. coli*. *Nature* 580, 269–273.
- 445 Pradhan, S., and Weiss, A.A. (2020). Probiotic Properties of *Escherichia coli* Nissle in Human
446 Intestinal Organoids. *MBio* 11.

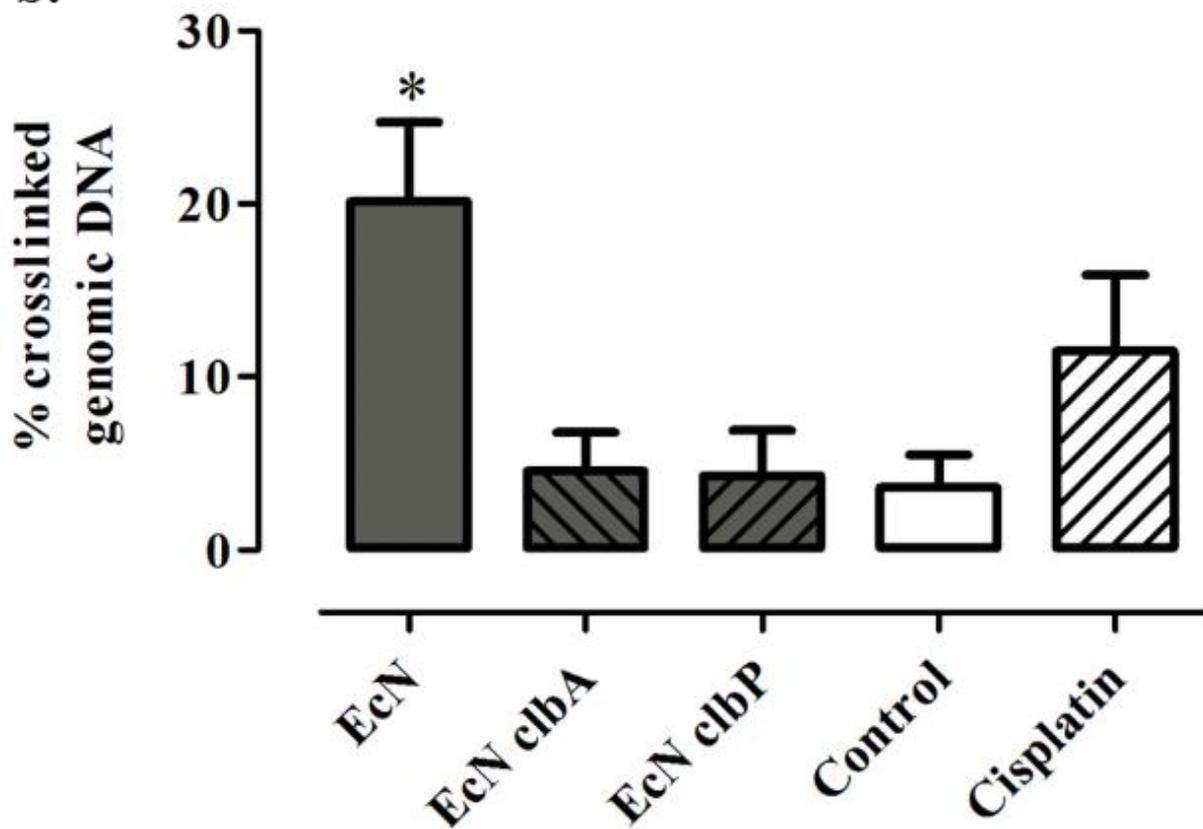
- 447 Sassone-Corsi, M., Nuccio, S.-P., Liu, H., Hernandez, D., Vu, C.T., Takahashi, A.A.,
448 Edwards, R.A., and Raffatellu, M. (2016). Microcins mediate competition among
449 Enterobacteriaceae in the inflamed gut. *Nature* *540*, 280–283.
- 450 Silva, M.J., Costa, P., Dias, A., Valente, M., Louro, H., and Boavida, M.G. (2005).
451 Comparative analysis of the mutagenic activity of oxaliplatin and cisplatin in the Hprt gene of
452 CHO cells. *Environmental and Molecular Mutagenesis* *46*, 104–115.
- 453 Terlouw, D., Suerink, M., Boot, A., Wezel, T. van, Nielsen, M., and Morreau, H. (2020).
454 Recurrent APC Splice Variant c.835-8A>G in Patients With Unexplained Colorectal
455 Polyposis Fulfilling the Colibactin Mutational Signature. *Gastroenterology* *159*, 1612-
456 1614.e5.
- 457 Vassin, V.M., Anantha, R.W., Sokolova, E., Kanner, S., and Borowiec, J.A. (2009). Human
458 RPA phosphorylation by ATR stimulates DNA synthesis and prevents ssDNA accumulation
459 during DNA-replication stress. *J. Cell. Sci.* *122*, 4070–4080.
- 460 Vizcaino, M.I., and Crawford, J.M. (2015). The colibactin warhead crosslinks DNA. *Nat*
461 *Chem* *7*, 411–417.
- 462 Wilson, M.R., Jiang, Y., Villalta, P.W., Stornetta, A., Boudreau, P.D., Carrá, A., Brennan,
463 C.A., Chun, E., Ngo, L., Samson, L.D., et al. (2019). The human gut bacterial genotoxin
464 colibactin alkylates DNA. *Science* *363*.
- 465 Yang, Y., Gharaibeh, R.Z., Newsome, R.C., and Jobin, C. (2020). Amending microbiota by
466 targeting intestinal inflammation with TNF blockade attenuates development of colorectal
467 cancer. *Nature Cancer* *1*, 723–734.
- 468 Zhu, W., Miyata, N., Winter, M.G., Arenales, A., Hughes, E.R., Spiga, L., Kim, J., Sifuentes-
469 Dominguez, L., Starokadomskyy, P., Gopal, P., et al. (2019). Editing of the gut microbiota
470 reduces carcinogenesis in mouse models of colitis-associated colorectal cancer. *Journal of*
471 *Experimental Medicine* *216*, 2378–2393.
- 472
- 473

474 **Figures and table**

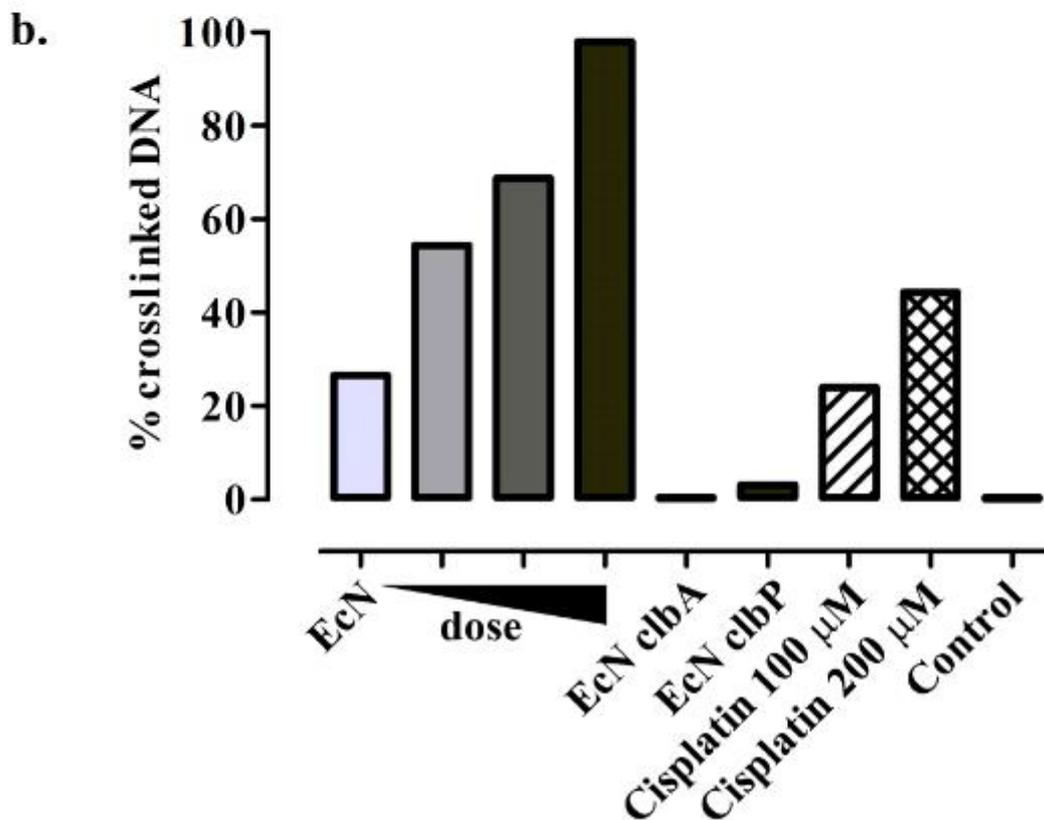
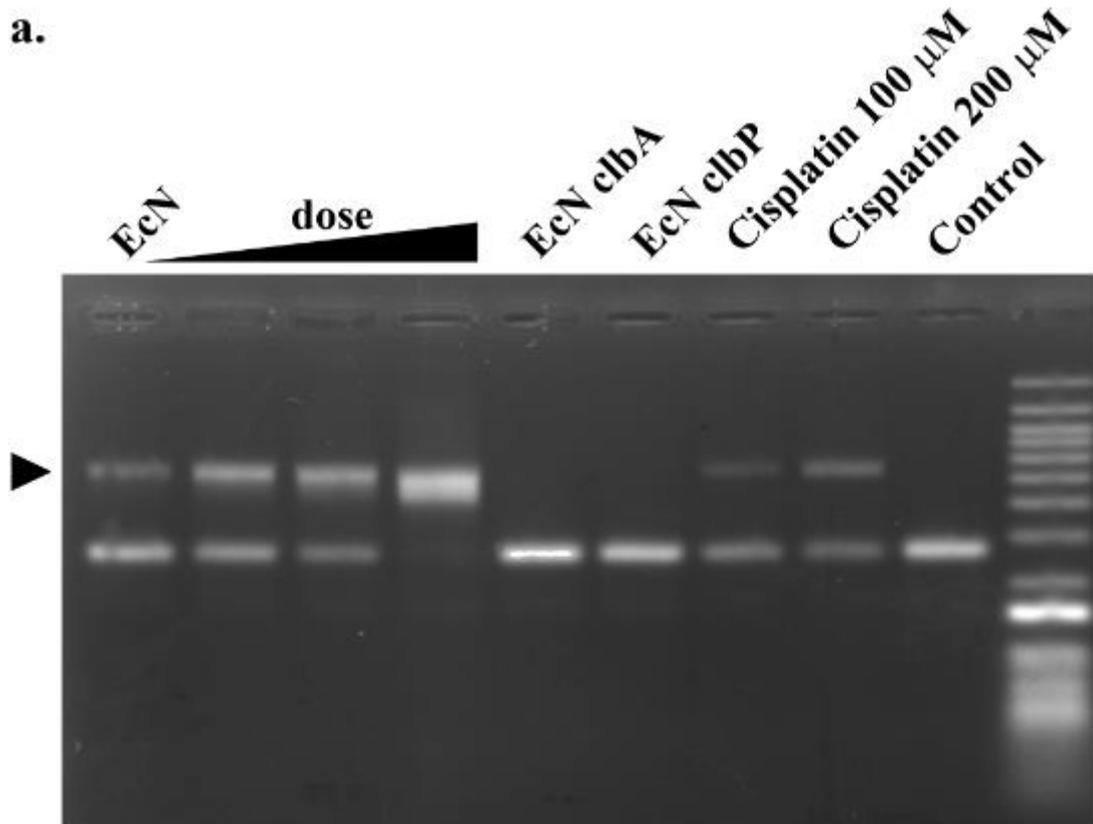
a.



b.



476 **Figure 1.** *E. coli* Nissle 1917 produces colibactin and induces interstrand crosslinks in the host
477 cell genomic DNA. (a) HeLa cells were infected for 4 h, at a multiplicity of infection of 400
478 bacteria per cell, with *E. coli* Nissle (EcN), *clbA* or *clbP* isogenic mutants, left uninfected or
479 treated 4 h with 100 μ M cisplatin. Then, the cell genomic DNA was purified and analyzed by
480 denaturing electrophoresis. The arrow points to the non-migrating DNA that remains in the
481 loading well. (b) The DNA signal in the upper non-migrating band relative to the total DNA
482 signal in the lane was determined by image analysis in ImageJ. The mean % of crosslinked
483 DNA and standard error of the mean (n=3 independent experiments) are shown. * $p < 0.05$
484 compared to control, one-way ANOVA with Dunnett posttest compared to control.

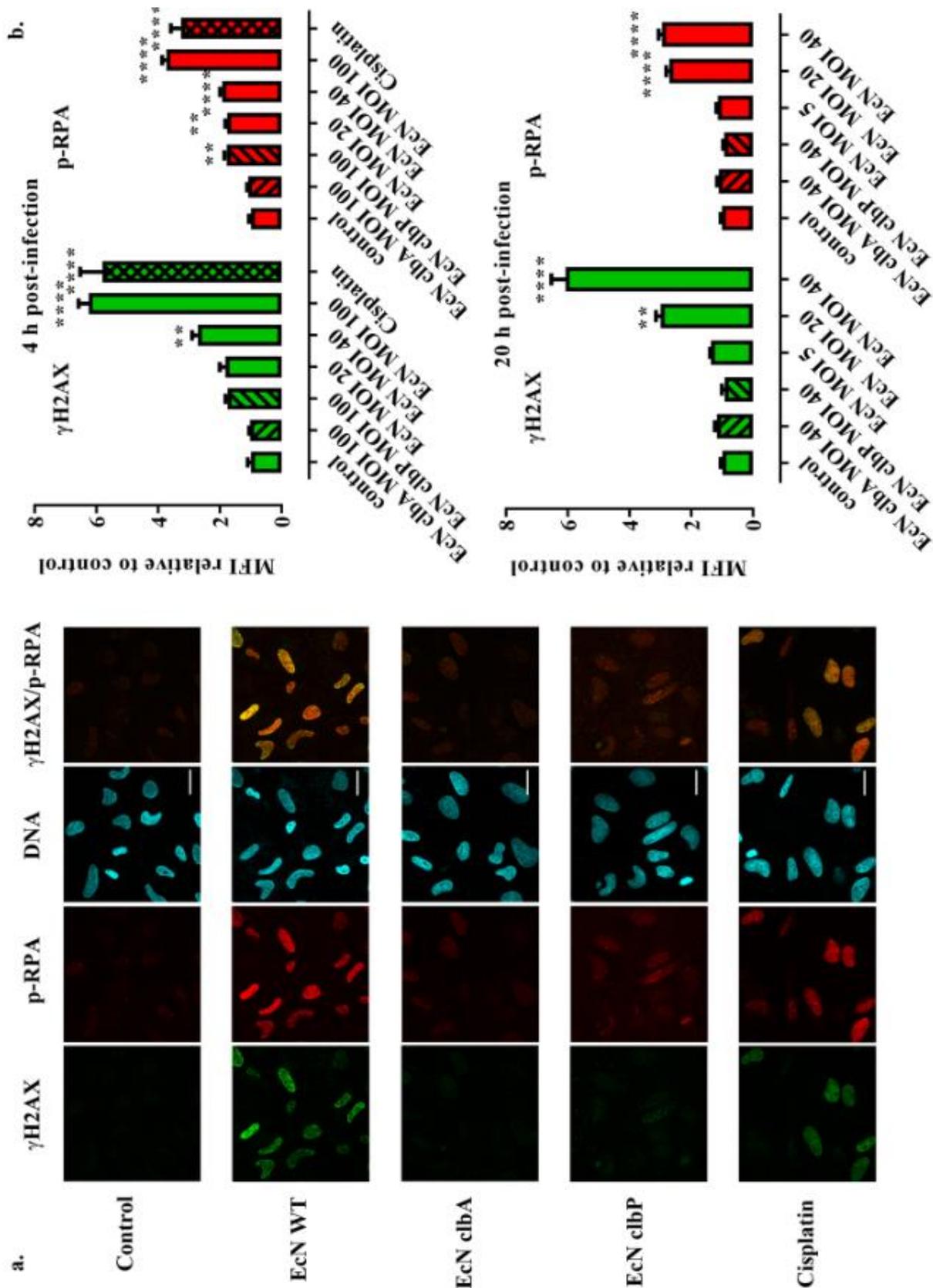


485

486 **Supplementary figure 1.** *E. coli* Nissle induces interstrand crosslinks in exogenous DNA.

487 (a) Linearized plasmid double-strand DNA was incubated 40 minutes with *E. coli* Nissle (EcN)
488 (inoculum of 0.75, 1.5, 3, or 6 x10⁶ bacteria in 100 µl grown 3.5 hours) or with the *clbA* or *clbP*
489 mutants (6 x10⁶ bacteria in 100 µl) or treated 4 h with cisplatin, and then analyzed by denaturing
490 gel electrophoresis. (b) Quantification of panel a; the percentages of the DNA signal in the
491 upper cross-linked band relative to the total DNA signal in the lane was determined by image
492 analysis.

493



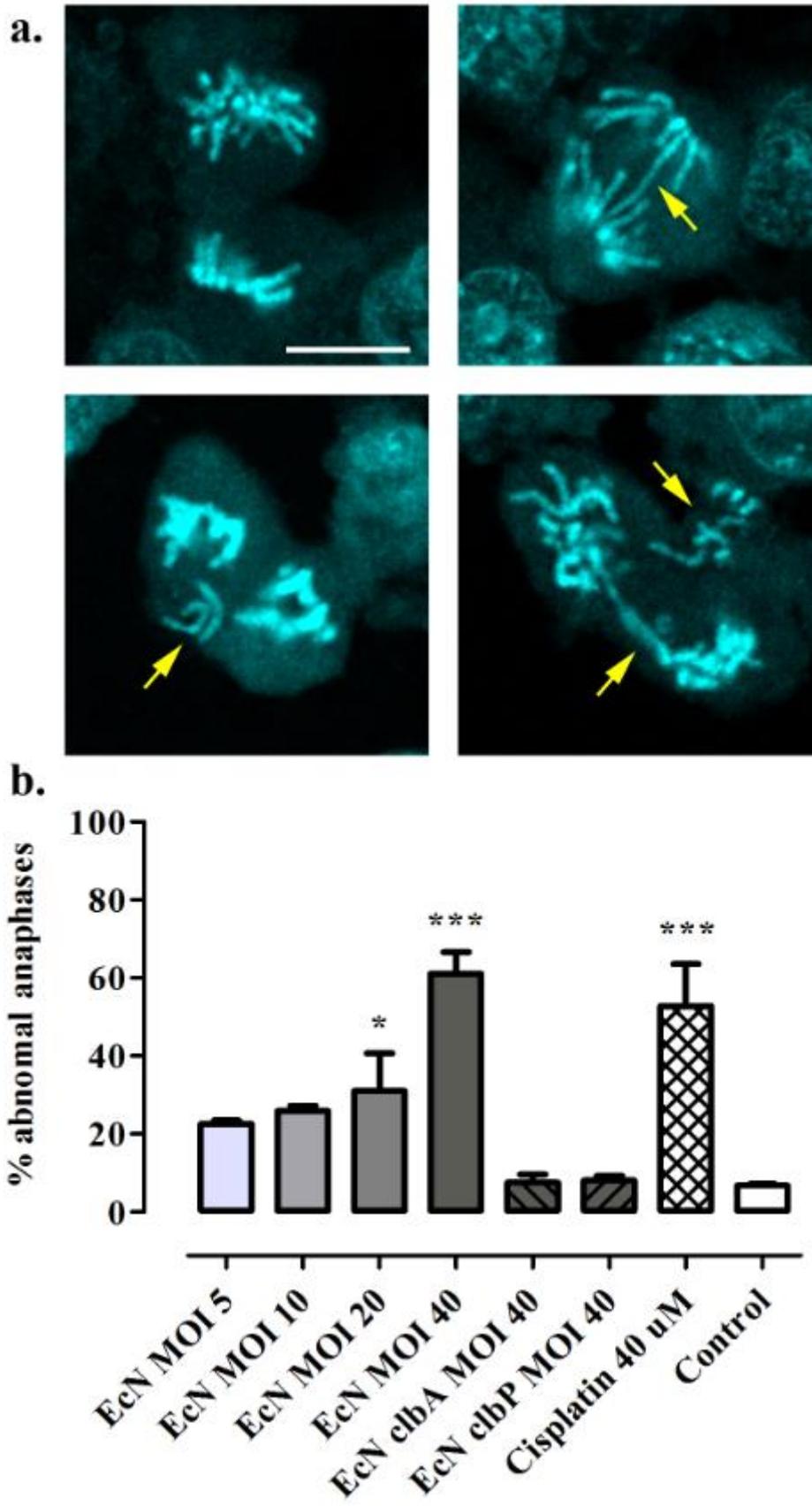
494

495 **Figure 2.** Formation of phosphorylated RPA and H2AX nuclear repair foci in HeLa cells

496 infected with *E. coli* Nissle. (a) HeLa cells were exposed 4 hours to *E. coli* Nissle (EcN) or the

497 *clbA* or *clbP* mutants (MOI = 100) or treated with cisplatin, then immunostained for
498 phosphorylated H2AX (γ H2AX) and phosphorylated RPA (p-RPA) 4 hours later. DNA was
499 counterstained with DAPI. Bar = 20 μ m. (b) Cells were infected with given MOI and
500 immunostained at 4 and 20 hours after infection. The mean fluorescence intensity (MFI) of
501 γ H2AX and p-RPA within the nuclei, relative to that in control uninfected cells, was determined
502 by image analysis using a macro in ImageJ. The means and standard errors, measured in at least
503 70 nuclei for each group, are shown. ** P<0.01, **** P<0.0001 (one-way ANOVA with
504 Dunnett posttest, compared to control).

505



507 **Figure 3.** Infection with *E. coli* Nissle induces aberrant anaphases. (a) Anaphase bridges,
508 lagging chromosomes and multipolar mitosis (arrows) in CHO cells 20 hours following
509 infection with *E. coli* Nissle. DNA was stained with DAPI and observed by confocal
510 microscopy. Bar = 20 μ m. (b) Aberrant anaphase index in CHO cells 20 hours following
511 infection with EcN at given MOI, or with the *clbA* and *clbP* mutants, or treatment with
512 cisplatin. The means and standard errors, measured in three independent experiments, are
513 shown. * $P < 0.05$, *** $P < 0.001$ (one-way ANOVA with Dunnett posttest compared to control).

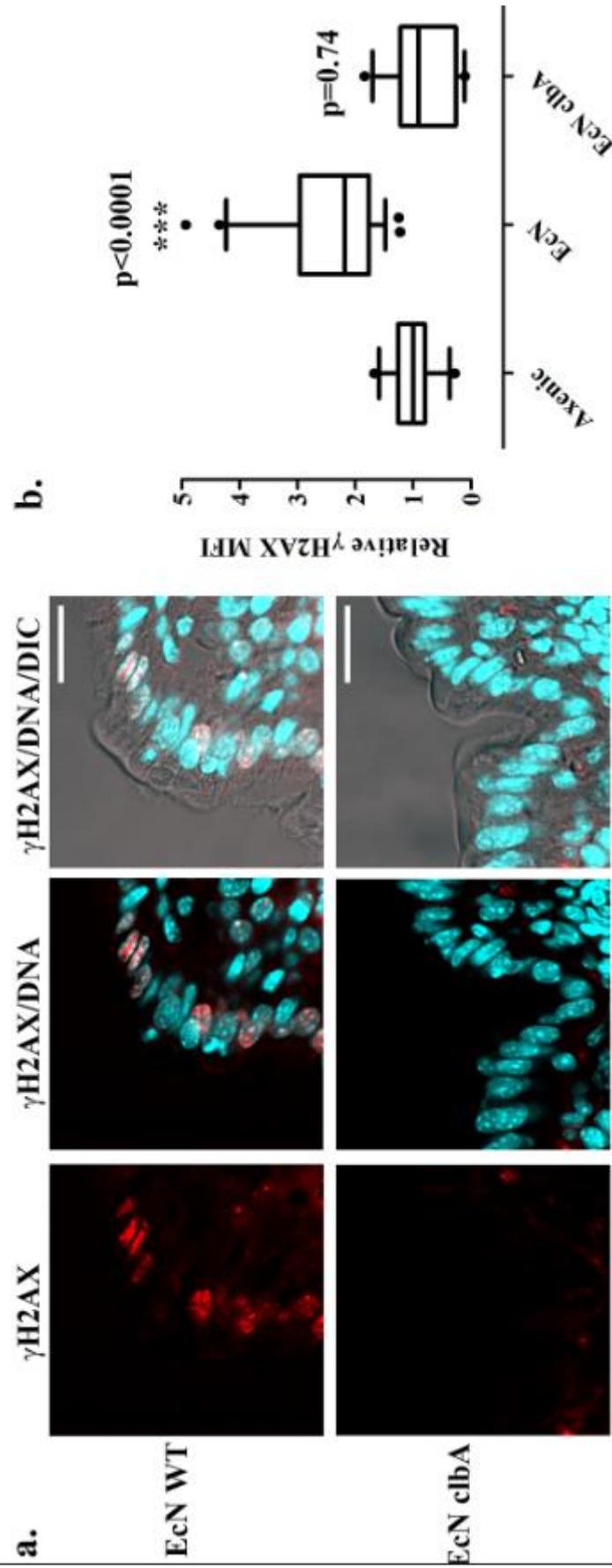
514

515 **Table 1:** *hprt* mutant frequencies (MF) following infection with *E. coli* Nissle 1917 at given
516 multiplicity of infection (MOI), or with the *clbA* or *clbP* mutants, or 1 h treatment with cisplatin.
517 The values are the mean and standard error of three independent infection experiments.
518 Statistical analysis compared to control was performed using a two tailed t-test on the log
519 transformed data.

520	Treatment	MF x10⁻⁶	p
521	Control	5.99 \pm 0.98	-
522	Cisplatin 10 μ M	25.25 \pm 5.83	** 0.006
523	Cisplatin 15 μ M	47.62 \pm 12.60	** 0.002
524	EcN MOI 5	5.66 \pm 0.71	0.685
525	EcN MOI 10	11.98 \pm 5.99	0.425
526	EcN MOI 20	14.49 \pm 8.37	* 0.023
527	EcN <i>clbA</i> MOI 20	5.46 \pm 0.28	0.450
528	EcN <i>clbP</i> MOI 20	4.94 \pm 0.51	0.168

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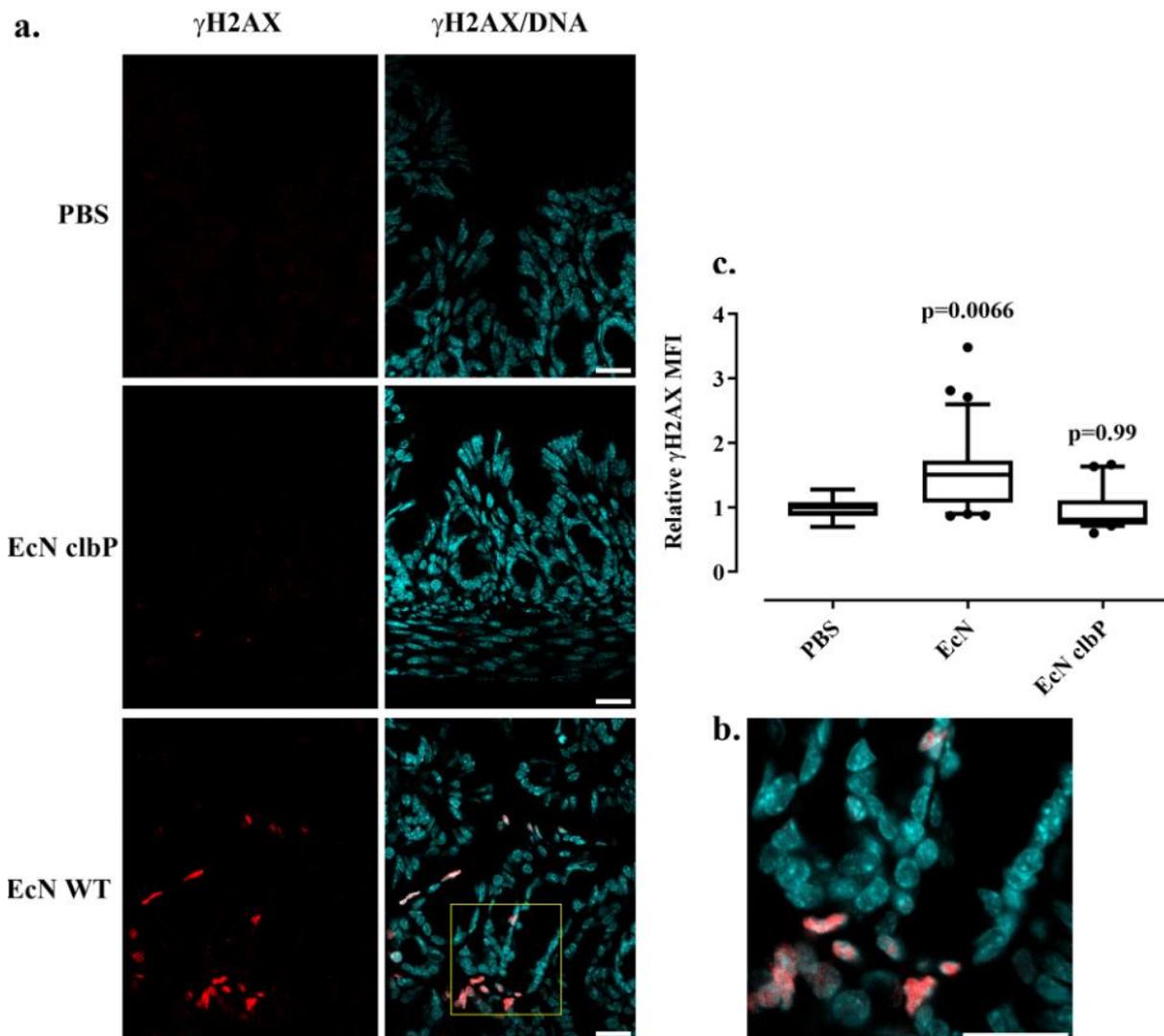
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531

532 **Figure 4.** γ H2AX foci in gut cells of mice mono-associated with *E. coli* Nissle 1917. Adult
533 Balb/c mice were mono-colonized 7 days with wild-type *E. coli* Nissle 1917 (EcN WT) or the
534 *clbA* mutant, or kept axenic. (a) γ H2AX in histological sections of the colon was examined by
535 immunofluorescence and confocal microscopy (red). DNA was counterstained with DAPI, and
536 the tissue was visualized by differential interference contrast (DIC). Bars = 10 μ m. (b) The
537 mean fluorescence intensity (MFI) of γ H2AX within the nuclei, relative to that measured in the
538 axenic animals, was determined by automated image analysis in ImageJ. The whiskers show
539 the median, 10-90 percentile and outliers, measured in at least 20 microscopic fields in 3 axenic
540 and 5 mono-associated animals. The result of a one-way ANOVA with Dunnett posttest
541 compared to axenic is shown.

542



543

544 **Figure 5.** γ H2AX foci in gut cells of mouse pups treated by *E. coli* Nissle 1917 by gavage. (a)
545 Mice pups were given orally approximately 2.5×10^8 wild-type *E. coli* Nissle (EcN) or the
546 *clbP* mutant, or the PBS vehicle, then sacrificed 6 hours later. Phosphorylated H2AX (γ H2AX)
547 in histological sections of the intestinal epithelium was examined by immunofluorescence (red)
548 and confocal microscopy. DNA was counterstained with DAPI. Bars = 20 μ m. (b) Close-up of
549 the region shown in yellow. Bar = 20 μ m. (c) The mean fluorescence intensity (MFI) of γ H2AX
550 within the nuclei, relative to that in the controls, was determined by automated image analysis
551 in ImageJ. The whiskers show the median, 10-90 percentile and outliers, measured in at least
552 10 microscopic fields for each group in 3 controls (PBS) or 5 treated (Nissle 1917 or *clbP*)

553 mutant) animals. The result of a one-way ANOVA with Dunnett posttest compared to PBS is
554 shown.