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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; Iftekhar 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  $\gamma$ H2AX (Bossuet-Greif et al., 2018). Immunofluorescence of Ser33-phosphorylated  
103 RPA and  $\gamma$ 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  $\gamma$ 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  $\gamma$ H2AX  
143 in the colon. Nuclear  $\gamma$ 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  $\gamma$ 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  $\gamma$ H2AX foci. Animals  
150 treated with Nissle 1917 exhibited significant levels of nuclear  $\gamma$ 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  $\gamma$ 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 (OD<sub>600</sub>=0.4 to 0.5).

233

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

235  $3 \times 10^6$  bacteria or numbers given in the text were inoculated in 100  $\mu$ 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  $\mu$ 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% CO<sub>2</sub> incubator and maintained by serial  
254 passage in DMEM Glutamax or MEM $\alpha$  (Invitrogen) respectively, both supplemented with 10%  
255 fetal calf serum (FCS), 50  $\mu$ g/ml gentamicin and 1% non-essential amino acids (Invitrogen).  
256  $3 \times 10^5$  cells/well were seeded in 6-wells plates (TPP) or  $3.5 \times 10^4$  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  $\mu$ 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  $\mu$ 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  $\mu$ 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  $3 \times 10^5$  cells using culture medium supplemented with 30  
281  $\mu$ 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  $\mu$ 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  **$\gamma$ 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  $\gamma$ 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  $\mu$ 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  $\gamma$ 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  $\mu$ 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  $\gamma$ H2AX in intestinal tissues, sections (5 or 8  $\mu$ 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  $\gamma$ 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  $\mu$ 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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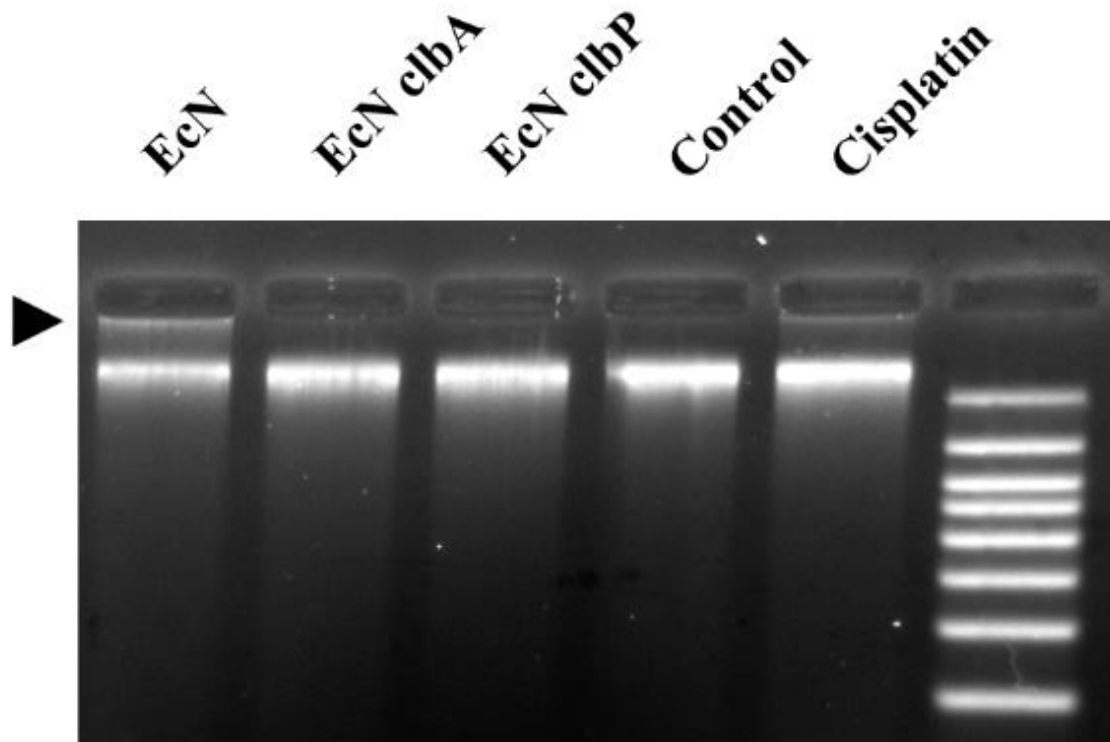
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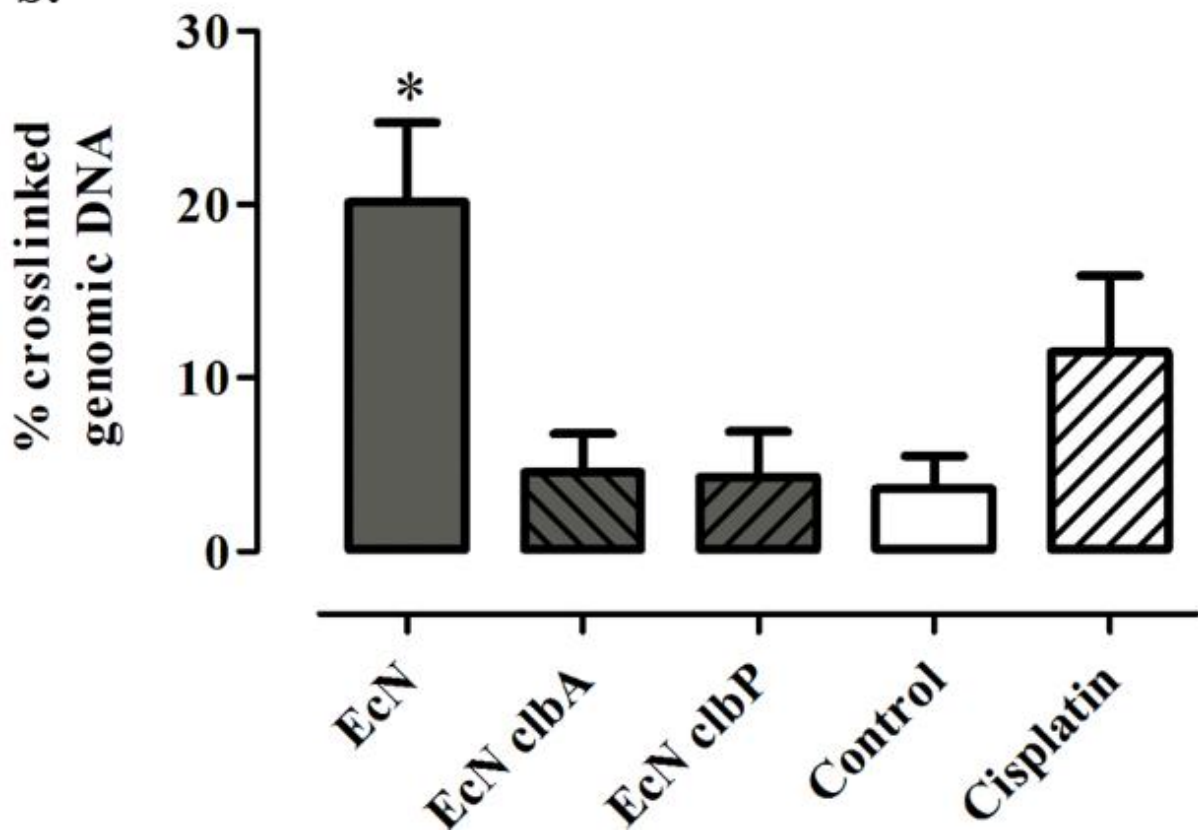
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474 **Figures and table**

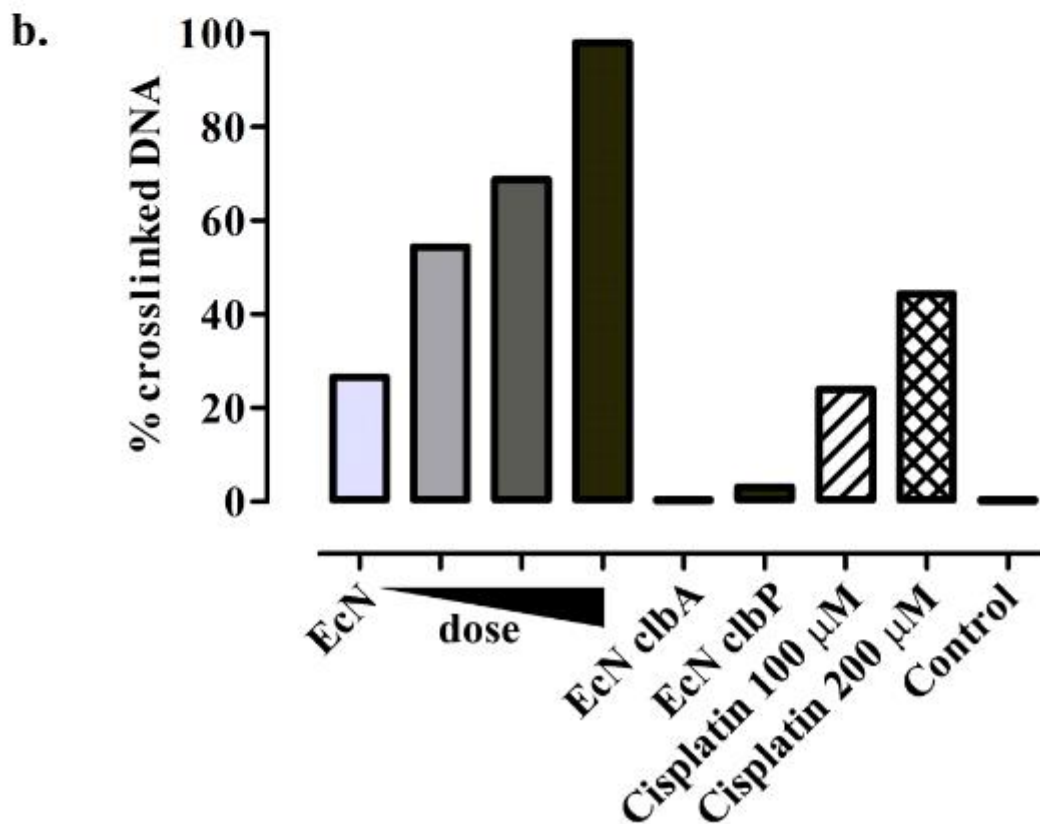
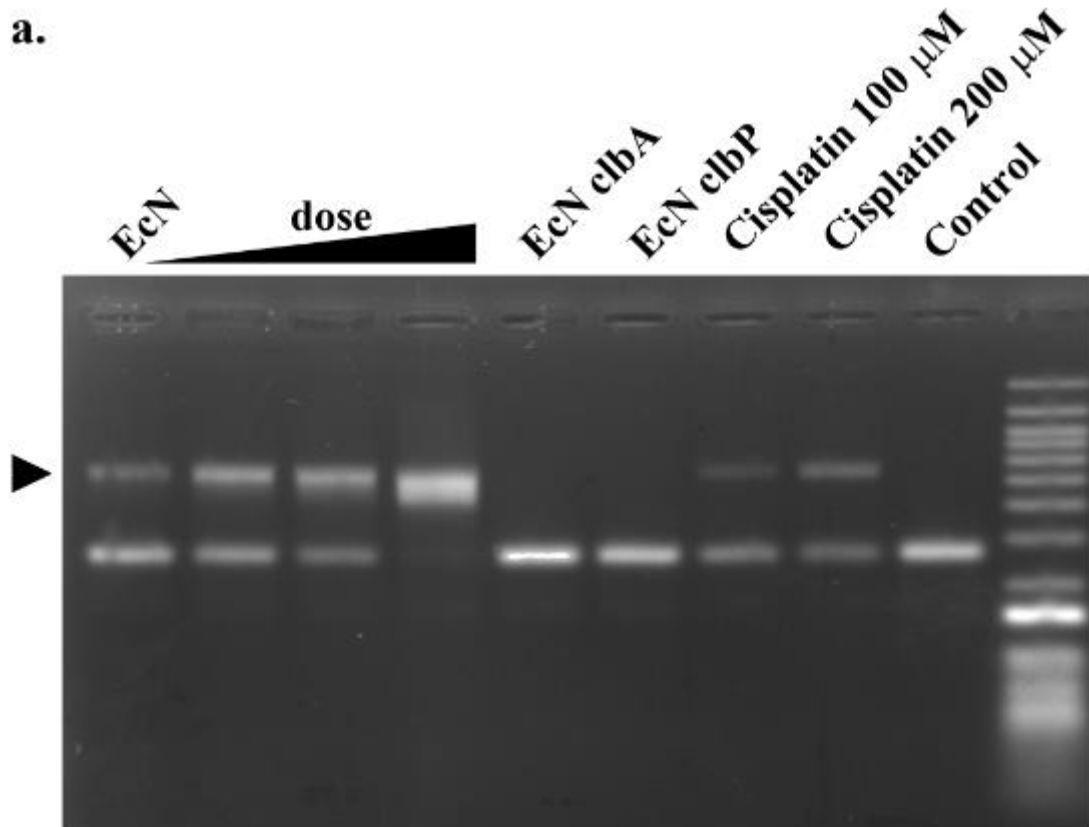
**a.**



**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  $\mu$ 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.



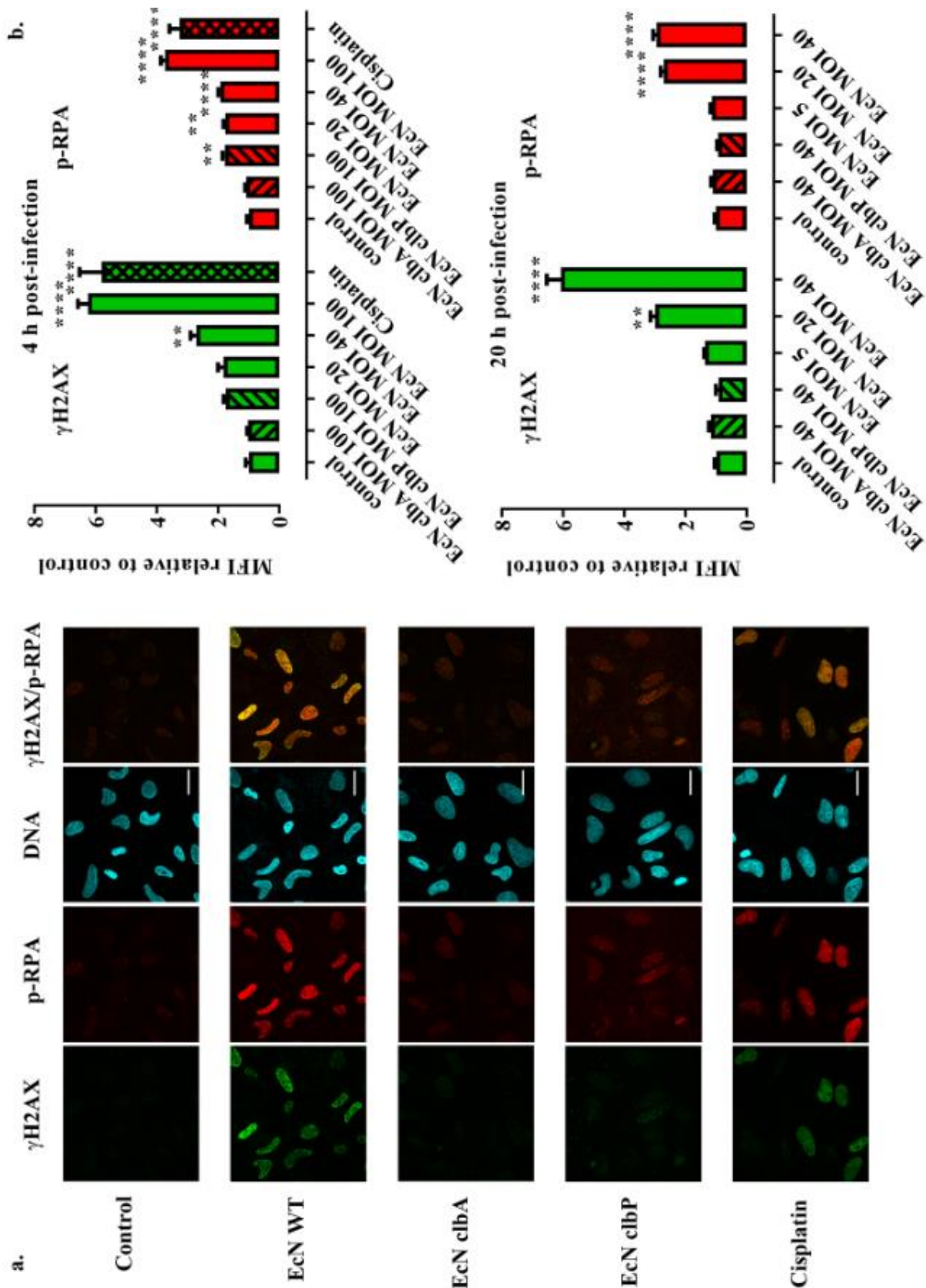
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486 **Supplementary figure 1.** *E. coli* Nissle induces interstrand crosslinks in exogenous DNA.

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 x10<sup>6</sup> bacteria in 100 µl grown 3.5 hours) or with the *clbA* or *clbP*  
489 mutants (6 x10<sup>6</sup> 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.

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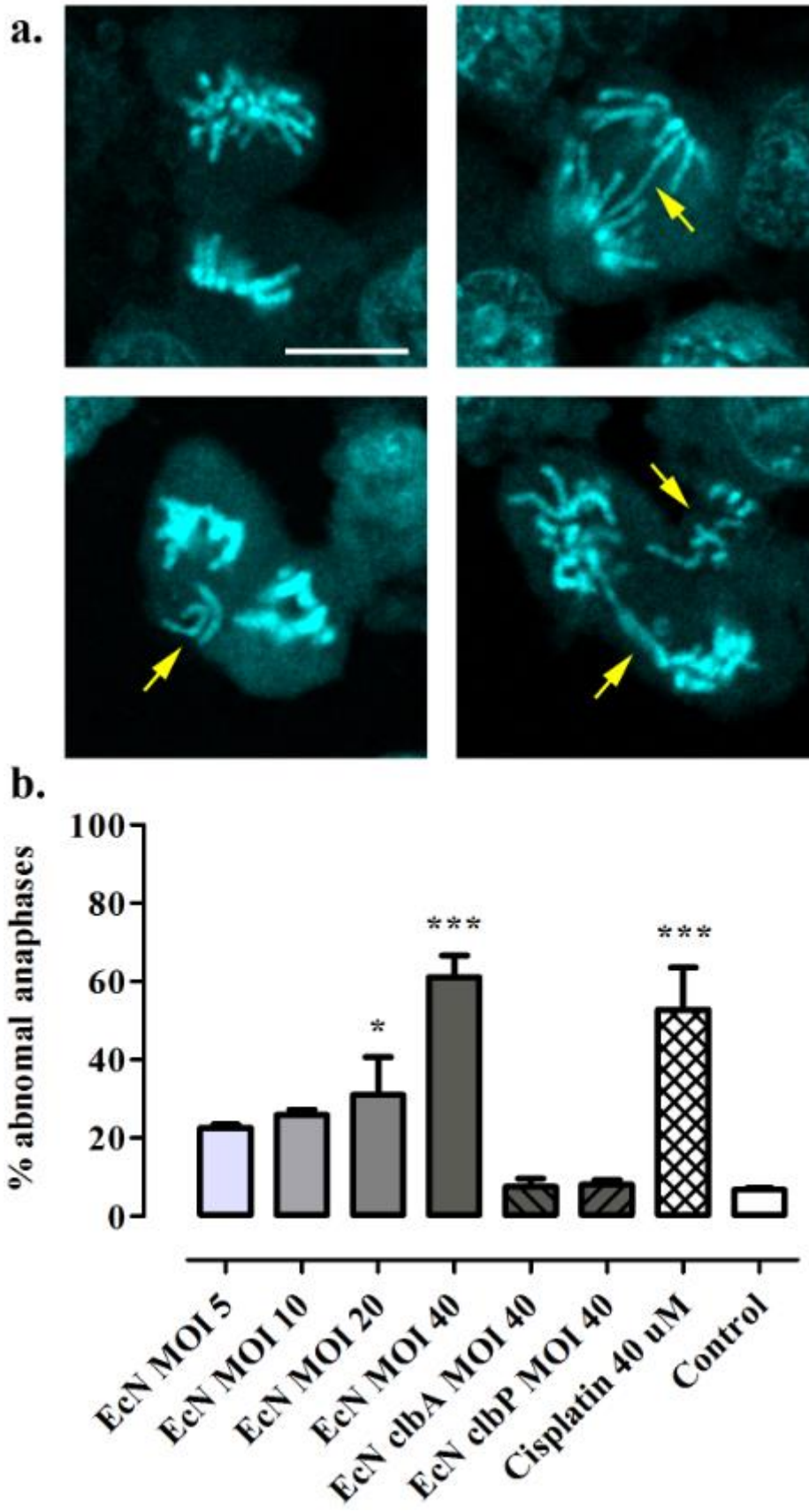
495 **Figure 2.** Formation of phosphorylated RPA and H2AX nuclear repair foci in HeLa cells

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 ( $\gamma$ H2AX) and phosphorylated RPA (p-RPA) 4 hours later. DNA was  
499 counterstained with DAPI. Bar = 20  $\mu$ 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  $\gamma$ 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



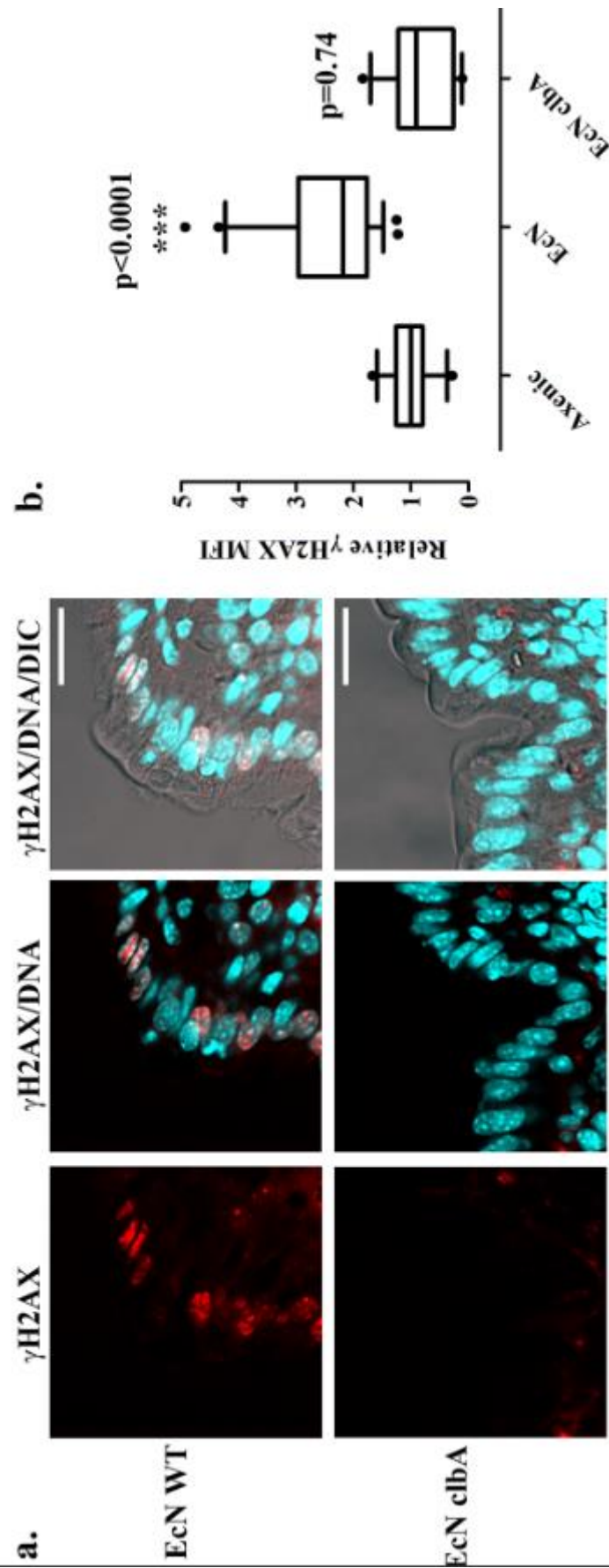
507 **Figure 3.** Infection with *E. coli* Nissle induces aberrant anaphases. (a) Anaphase bridges,  
508 lagging chromosomes and multipolar mitosis (arrows) in CHO cells 20 hours following  
509 infection with *E. coli* Nissle. DNA was stained with DAPI and observed by confocal  
510 microscopy. Bar = 20  $\mu\text{m}$ . (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	<u>Treatment</u>	<u>MF x10<sup>-6</sup></u>	<u>p</u>
521	Control	5.99 $\pm$ 0.98	-
522	Cisplatin 10 $\mu\text{M}$	25.25 $\pm$ 5.83	** 0.006
523	Cisplatin 15 $\mu\text{M}$	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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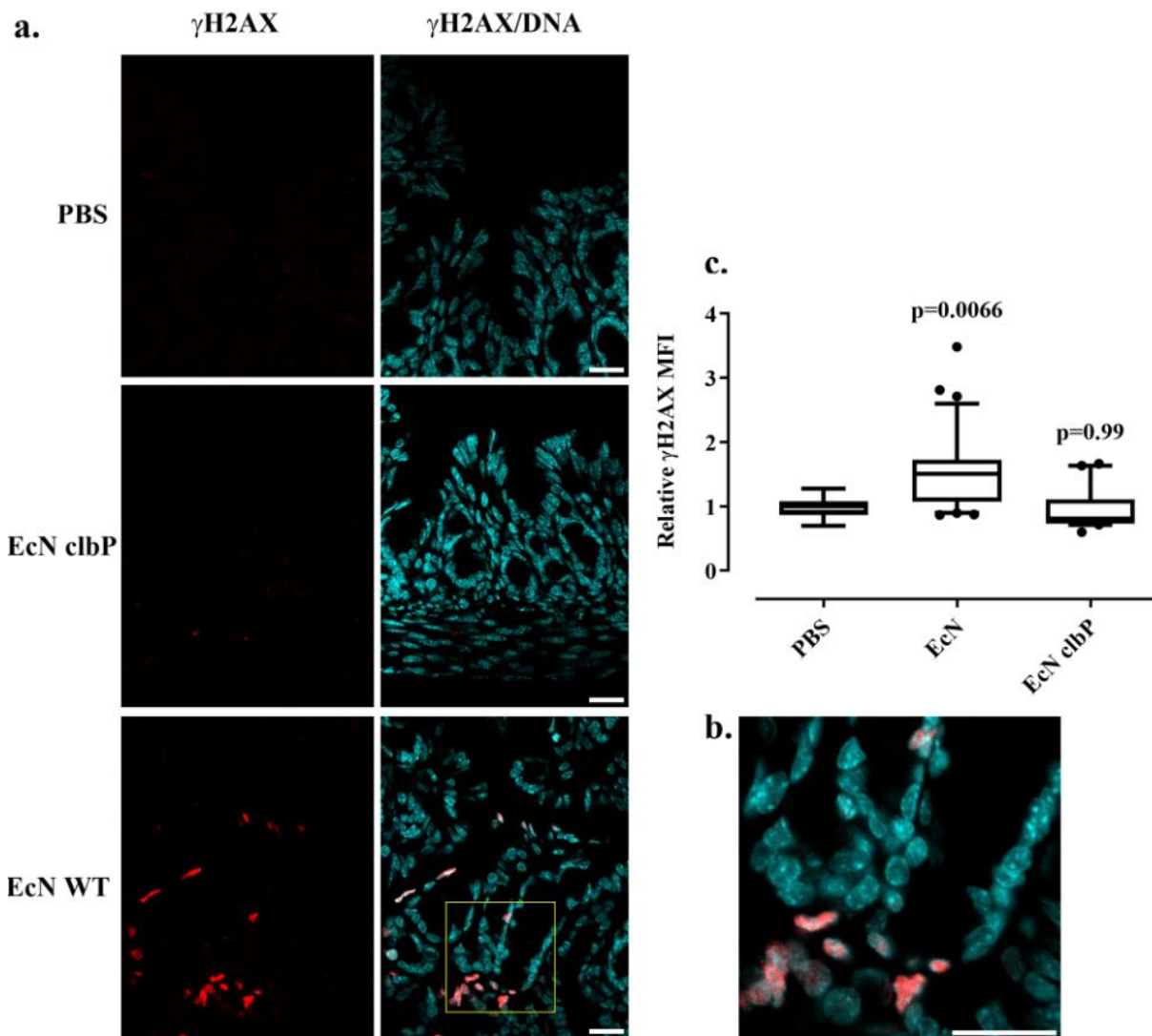
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532 **Figure 4.**  $\gamma$ H2AX foci in gut cells of mice mono-associated with *E. coli* Nissle 1917. Adult  
533 Balb/c mice were mono-colonized 7 days with wild-type *E. coli* Nissle 1917 (EcN WT) or the  
534 *clbA* mutant, or kept axenic. (a)  $\gamma$ 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  $\mu$ m. (b) The  
537 mean fluorescence intensity (MFI) of  $\gamma$ 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.**  $\gamma$ H2AX foci in gut cells of mouse pups treated by *E. coli* Nissle 1917 by gavage. (a)  
545 Mice pups were given orally approximately  $2.5 \times 10^8$  wild-type *E. coli* Nissle (EcN) or the  
546 *clbP* mutant, or the PBS vehicle, then sacrificed 6 hours later. Phosphorylated H2AX ( $\gamma$ 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  $\mu$ m. (b) Close-up of  
549 the region shown in yellow. Bar = 20  $\mu$ m. (c) The mean fluorescence intensity (MFI) of  $\gamma$ 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.