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# Supplemental Material to:

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## Maternally acquired genotoxic *Escherichia coli* alters offspring's intestinal homeostasis

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# SUPPLEMENTARY INFORMATION

# Maternally Acquired Genotoxic *Escherichia coli* Alters Offspring's Intestinal Homeostasis

**This file includes:**

Supplementary Methods
Supplementary Tables 1 & 2
Supplementary Figures 1 to 8 with Legends

## SUPPLEMENTARY METHODS

### Characterization of Clinical *E. coli* Isolates

One presumptive *E. coli* colony on Drigalski agar was arbitrarily selected per rectal sample for further analysis (if different colonial morphologies were observed, one isolate of each was retained). For all isolates identified as *E. coli* with the automated Vitek II system (bioMerieux), phylogenetic group was determined using the Clermont phylogroup typing method[1], and serogroup was determined by a multiplex PCR targeting the *gnd* gene[2]. The presence of the 54 kb *pks* island was screened by a duplex PCR targeting the *clbA* and *clbQ* genes located at each extremity of the island. Major phylogenetic subgroups of the B2 phylogroup isolates were determined by allele-specific PCRs, as defined by MultiLocus Sequence Typing complexes (MLST)[3].

### Bacterial Strains, Mutagenesis Procedures and Growth Conditions

*Construction of the M1/5 WT streptomycin resistant mutant.* A natural K42R mutation is found in ribosomal protein S12 of *E. coli* MC1400 strain and confers high-level resistance to streptomycin. MC1400 *rps*L gene was amplified using primer IHAP JPN99 and IHAP JPN100 and the PCR products were transformed by electroporation into *E. coli* M1/5 containing the lambda Red recombinase expression plasmid pKD46. After electroporation, streptomycin resistance was used to force mother's

colonization by M1/5 and as a counter-selectable marker for quantification of all M1/5 isogenic strains from fecal contents. *E. coli* M1/5 *rpsl* K42R, *pks*-island positive was then noticed *E. coli* WT.

*Construction of the E. coli ΔclbA mutant.* Mutation in *clbA* coding for phosphopantetheinyl transferase was sufficient to abolish production of colibactin[4]. The *clbA* gene from *E. coli* WT was replaced with a kanamycin resistance cassette, which was amplified from plasmid pKD4 using PCR with primers IHAP JPN44 and IHAP JPN45[4]. Kanamycin resistance cassette was also used as a second selectable marker to detect this mutant by plating dilutions of fecal suspension. This mutant was noted *E. coli* Δ*clbA*.

*Complementation of E. coli ΔclbA mutant.* To answer of molecular Koch's postulate, a fragment consisting of the tetA promoteur and the *clbA* operon, including its putative promoters was subcloned onto the BmgB1 restriction site of pkD3 and the resulting plasmid (pKD3-clbRA; kindly provided by U. Dobrindt, Germany) was transformed into *E. coli* DH5α. This fragment along with the chloramphenicol resistance cassette was amplified using primers clbA_compl and down1960_cat3 (U. Dobrindt, Germany) and transformed by electroporation into *E. coli* Δ*clbA* containing the lambda Red recombinase expression plasmid pKD46 as described above. Complemented mutants, named *E. coli* Δ*clbA::clbA,* were selected on LB agar with chloramphenicol.

**Cell Culture, *in vitro* Infection and Genotoxicity Assay**.

Non transformed rat intestinal epithelial IEC-6 cells (ATCC CRL-1592) were maintained in Dulbecco's modified Eagle's medium (DMEM GlutaMax; Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 80 µg $ml^{-1}$ gentamicin, and bovine insulin (0.1 unit $ml^{-1}$; Sigma) at 37°C in a 5% $CO_2$ atmosphere. IEC-6 (~75% confluent) were washed four times and incubated in infection medium based on DMEM supplemented with 25 mM Hepes and 10% FBS (Invitrogen). Bacteria were pregrown in infection medium to the midlogarithmic phase, and then the infection dose was calculated to a multiplicity of infection of 20 or 100 bacteria per cell. After a 4 h infection at 37°C and 5% $CO_2$, the cells were washed and grown until analysis in cell culture medium supplemented with 200 µg/ml gentamicin.

DNA double strand breaks were demonstrated by γH2AX immunofluorescence analysis 18 h after the end of infection. Mouse anti-γH2AX (Millipore) was diluted 1:500 in blocking solution and incubated overnight at 4°C. FITC-conjugated goat anti mouse secondary antibody (Invitrogen) was diluted 1:100 in blocking solution and incubated for 1 h. DNA was conterstained for 5 min with TO-PRO-3 (Invitrogen). Slides were mounted in Vectashield containing DAPI (Vectorlabs). Images were acquired with an Olympus IX70 laser scanning confocal microscope, in sequential mode, with the Fluoview FV500 software. At least 15 random fields have been evaluated for γH2AX foci counting under 60x apochromic objective.

**Cell Migration**

Three or four 100 days-old rats per group were sacrificed at 12, 24, and 48 h after a single intraperitoneal 5-bromo-2'-deoxyuridine injection (BrdU; Sigma-Aldrich, St Louis, MO, USA; 50 mg/kg body weight in saline solution). Intestinal sections were subjected to BrdU IHC (1/200, abcam ab6326).

To assess migration rates in intestines harvested between 12h and 48h after BrdU injection, locations of the foremost (12 h and 24 h) and least progressed (48 h) BrdU-labeled enterocytes were recorded as the percent of epithelial cell positions between the crypt-villus boundary (0%) and villus tip (100%) in at least 10 well-oriented crypt-villus units per intestinal segment[5]. All analyses were performed using NIS Elements-AR Image J software.

**Supplementary Table 1**: Characterization of the B2 *E. coli* Strains Isolated From 3-Days-old Neonates.

| B2 *E. coli* Isolates | B2 Phylogenetic subgroups* | O-types** | *pks*-island |
|---|---|---|---|
| M093 | I | ND | neg |
| M108 | I | O25b | neg |
| M134 | I | O6a | neg |
| M181 | I | ND | neg |
| M208 | I | O25b | neg |
| M223 | I | O16 | neg |
| M027 | II | O6a | neg |
| M050 | II | O6a | neg |
| M074 | II | O2b | neg |
| M075 | II | O25a | neg |
| M079 | II | O6a | neg |
| M085 | II | O6a | neg |
| M188 | II | O25a | neg |
| M234 | II | O6a | neg |
| M131 | IV | O2b | neg |
| M139 | IV | ND | neg |
| M173 | IV | O2b | neg |
| M177 | IV | ND | neg |
| M055 | IX | O1 | neg |
| M100 | IX | O1 | neg |
| M111 | IX | O1 | neg |
| M115 | IX | O1 | neg |
| M179 | IX | O2a | POS |
| M205 | IX | O1 | POS |
| M210 | IX | O1 | POS |
| M243 | IX | O1 | POS |
| M018 | UG | O75 | POS |
| M021 | UG | O2b | POS |
| M035 | UG | O22 | POS |
| M037 | UG | ND | POS |
| M054 | UG | O75 | POS |
| M104 | UG | O21 | POS |
| M124 | UG | O75 | POS |
| M185 | UG | O2b | POS |
| M190 | UG | O75 | POS |
| M238 | UG | O75 | POS |
| M086 | V | O16 | POS |
| M173 | V | O16 | POS |
| M060 | VI | O4 | POS |
| M125 | VI | O4 | POS |
| M135 | VI | O4 | POS |
| M174 | VI | O4 | POS |
| M217 | VI | O4 | POS |
| M023 | VII | O75 | POS |
| M066 | VII | O75 | POS |
| M106 | VII | O75 | POS |
| M120 | VII | O75 | POS |
| M127 | VII | O75 | POS |
| M133 | VII | O18 | POS |
| M164 | VII | O18 | POS |
| M199 | VII | O75 | POS |
| M215 | VII | O75 | POS |

* B2 phylogenetic subgroups: I=Sequence type complex (STc)131, II=STc73, III=STc127, IV=STc141, V=STc144, VI=STc12, VII=STc14, IX=STc95. UG corresponds to none of the nine B2 subgroups defined in[3]. The correspondance is given with the STc determined using the MLST scheme of Achtman[6].

** O-type detected using multiplex PCR: O1, O2a, O2b, O4, O6a, O7, O12, O16, O18, O21, O22, O25a, O25b, O45a, O75, O83. ND=non detected.

**Supplementary Table 2**. Bacterial Strains and Primers for Mutagenesis Procedures.

| **Bacterial strains & plasmid used** | **Genotype and relevant characteristics** | |
|---|---|---|
| *Escherichia coli* M1/5 (U.Dobrindt, Germany) | O75: K5 | |
| *Escherichia coli* K-12 MC4100 | *rpsL K42R* (Genbank AF312716) streptomycin resistance | |
| ***E. coli* M1/5 strains generated by chromosomal directed-mutagenesis** | | |
| M1/5 WT | *rpsL K42R* | streptomycin resistance |
| M1/5 Δ*clbA* | *rpsL K42R, clbA ::kan* | streptomycin & kanamycin resistance |
| M1/5 Δ*clbA*::*clbA* | *rpsL K42R, clbA ::clbA-cat* | streptomycin & chloramphenicol resistance |
| **Plasmids** | | |
| pKD46 | lambda red disruption system, temperature-sensitive replication of the plasmid | |
| pKD4 | Tn5 neomycin phosphotransferase gene for generation of the kanamycin resistance cassette | |
| pKD3-*clbRA* | *clbA* operon and chloramphenicol acetyltransferase gene for generation of the chloramphenicol resistance cassette | |
| **Targets and primers** | | |
| ***rpsL* mutagenesis (*KR2R*)** | | |
| lHAPJPN99 | ATGGCAACAGTTAACCAGCTGG | |
| lHAPJPN100 | TTAAGCCTTAGGACGCTTCACG | |
| ***clbA* mutagenesis** | | |
| lHAPJPN44 | GGACATACTAGTTTTTTTCATCAAACCAGTAGAGATAACTTCCTTCACTAGTGTAGGCTGGAGCTGCTTC | |
| lHAPJPN45 | TTAGCTGATAGTCGTGGTGATAAAGTTGGGACTGCATAGGAAATAGCTCACATATGAATATCCTCCTTAG | |
| ***clbA* complementation** | | |
| clbA_compl | GGAATACGAATCACGCTATACACATTGCTAACAGGAATGAGATTATCTAAATGAGGATTGATATATTAATTG G | |
| down1960_cat3 | ATATGAAAATCAATATTATCGACGGCTCAGAAGTGTCTAGATTATCCGTGGCGATCATATGAATATCCTCCT TAGTTCC | |

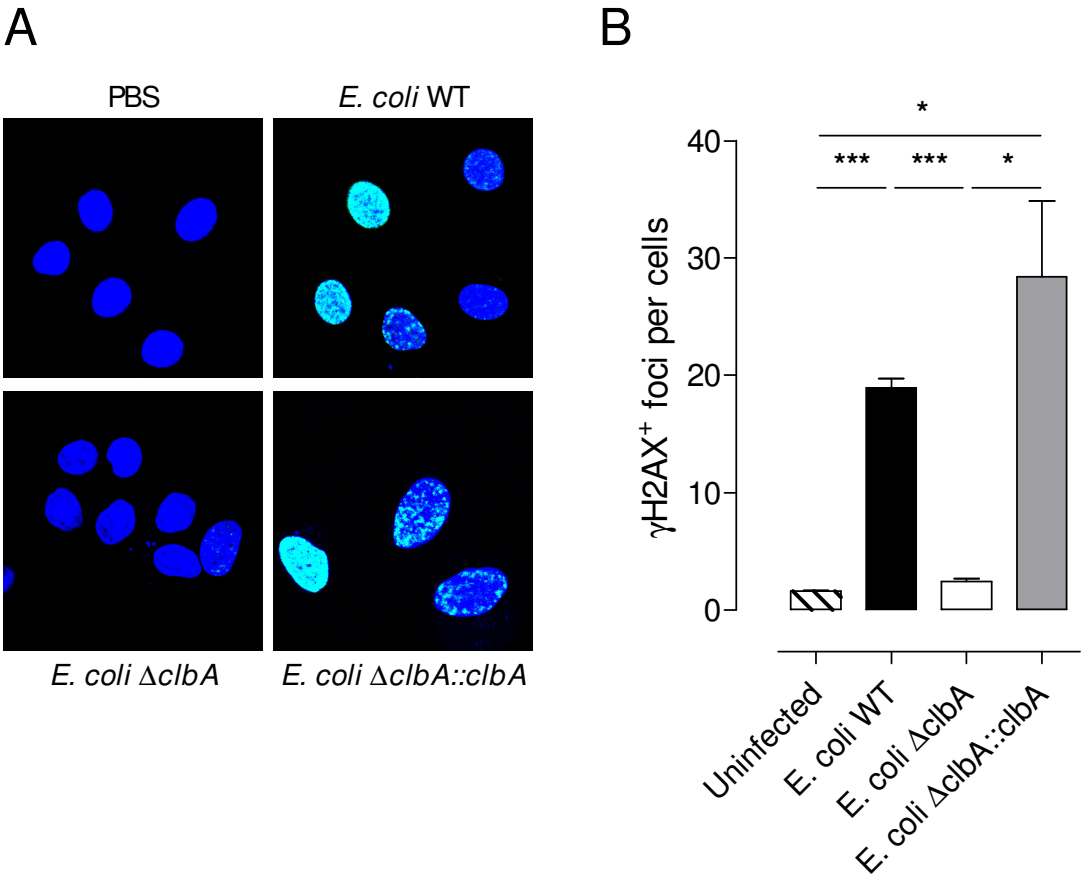



**Figure S1**

**Supplementary Figure 1 – Commensal *E. coli* WT and Δ*clbA*::*clbA* Strains Induce DNA double strand Breaks *in vitro.*** Rat intestinal epithelial cell IEC-6 were transiently infected for 4 h with live *E. coli* WT, Δ*clbA* or Δ*clbA*::*clbA* strains with a multiplicity of infection of 100 bacteria per cell, then washed and cultivated with gentamicin for 18 h.

**(A)** Cells were examined by confocal microscopy for DNA (blue), and phosphorylated H2AX (green). (Scaled bars,10 µm).

**(B)** Quantification of the phosphorylated γH2AX foci was expressed as the mean foci per cell for nuclei with less than 100 foci. 100-200 nuclei were evaluated for each condition. Mean values ± SEM are shown (One-way ANOVA with Bonferroni Multiple Comparison test, * $P \leq 0.05$ and *** $P \leq 0.001$).

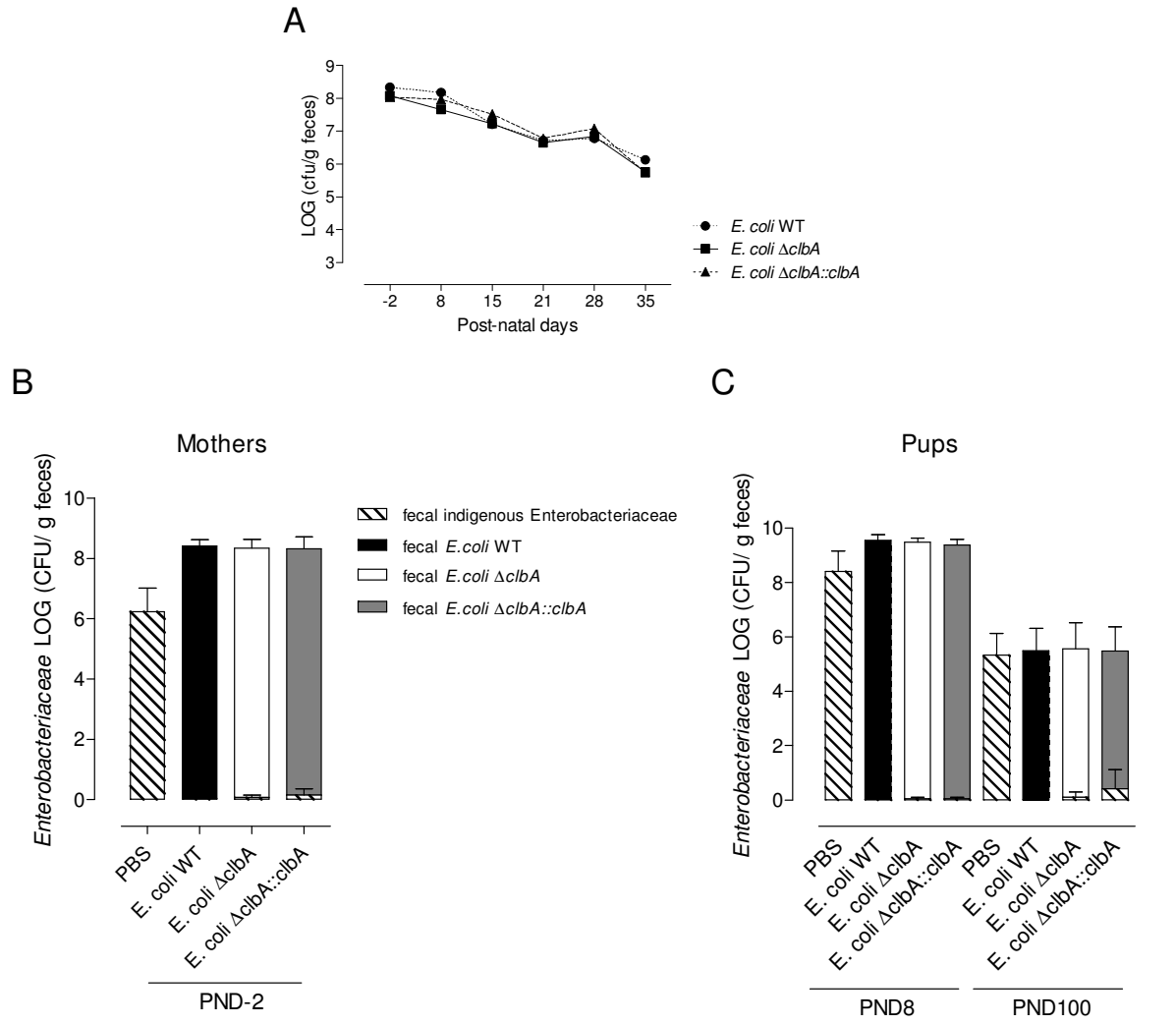



**Figure S2**

**Supplementary Figure 2 – Commensal *E. coli* Is the Main Represent of Mother and Pups Colibiota.** Pregnant rats were treated with streptomycin (5g/L) in drinking water and were inoculated twice with $10^9$ CFU by intragastric gavage before parturition with *E. coli* WT, Δ*clbA* or Δ*clbA*::*clbA* strains or treated with PBS.

**(A)** Evaluation of gut colonization in feces homogenate from pregnancy (PND-2) to post-weaning (PND35).

**(B)** Evaluation of detailed *Enterobacteriacea* colonization in pregnant rats after one oral inoculation.

**(C)** Evaluation of detailed *Enterobacteriaceae* colonization in the progeny at PND8 and PND100. Mean values ± SEM are shown.

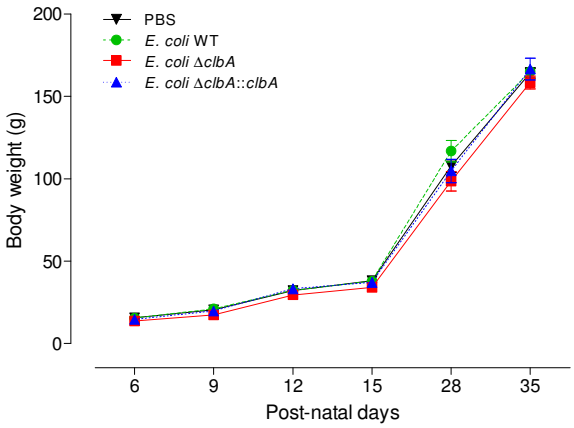



**Figure S3**

**Supplementary Figure 3 – Early Gut Colonization With Commensal *E.* coli Strains Did Not Induce Body Weight Modification.** Pregnant rats were treated with streptomycin (5g/L) in drinking water and were inoculated twice with $10^9$ CFU by intragastric gavage before parturition with *E. coli* WT, Δ*clbA* or Δ*clbA*::*clbA* strains or treated with PBS. Total body weight was evaluated in the progeny from PND6 to PND35. Mean values ± SEM are shown.

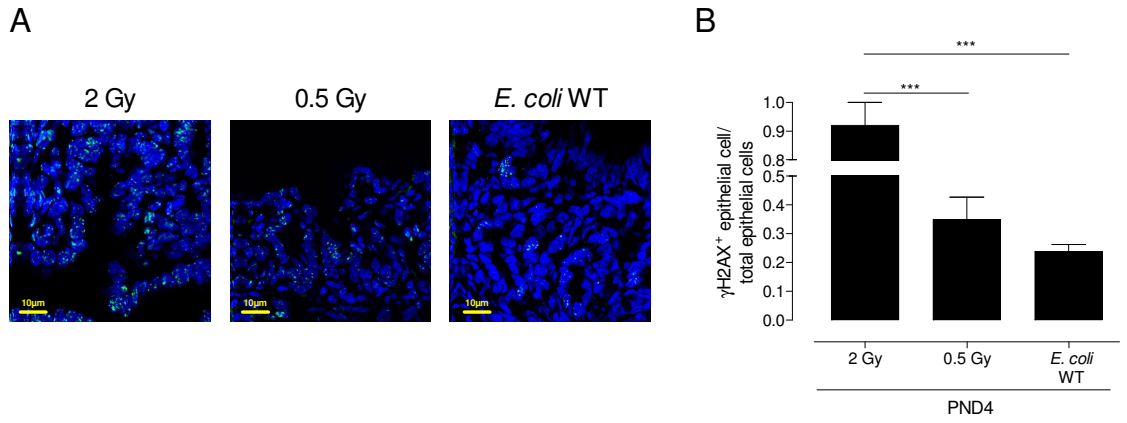



**Figure S4**

**Supplementary Figure 4 – Intestinal Genotoxicity Induced by Early Gut Colonization With Commensal *E. coli* Strains Is Equivalent to 0.5 Gy Total Body Irradiation.** Immunofluorescence analysis of intestinal epithelium of neonates at PND4 colonized since birth with commensal *E. coli* WT or treated with PBS and subsequently irradiated at 0.5 and 2 Gy.

**(A)** Representative colon frozen sections at PND4. DNA was stained in blue and γH2AX foci in green. (Scaled bars, 10 µm).

**(B)** Quantification of γH2AX-positive epithelial cells. Groups of 3-5 rats were analyzed. Mean values ± SEM are shown (One-way ANOVA with Bonferroni Multiple Comparison test, *** $P \leq 0.001$).

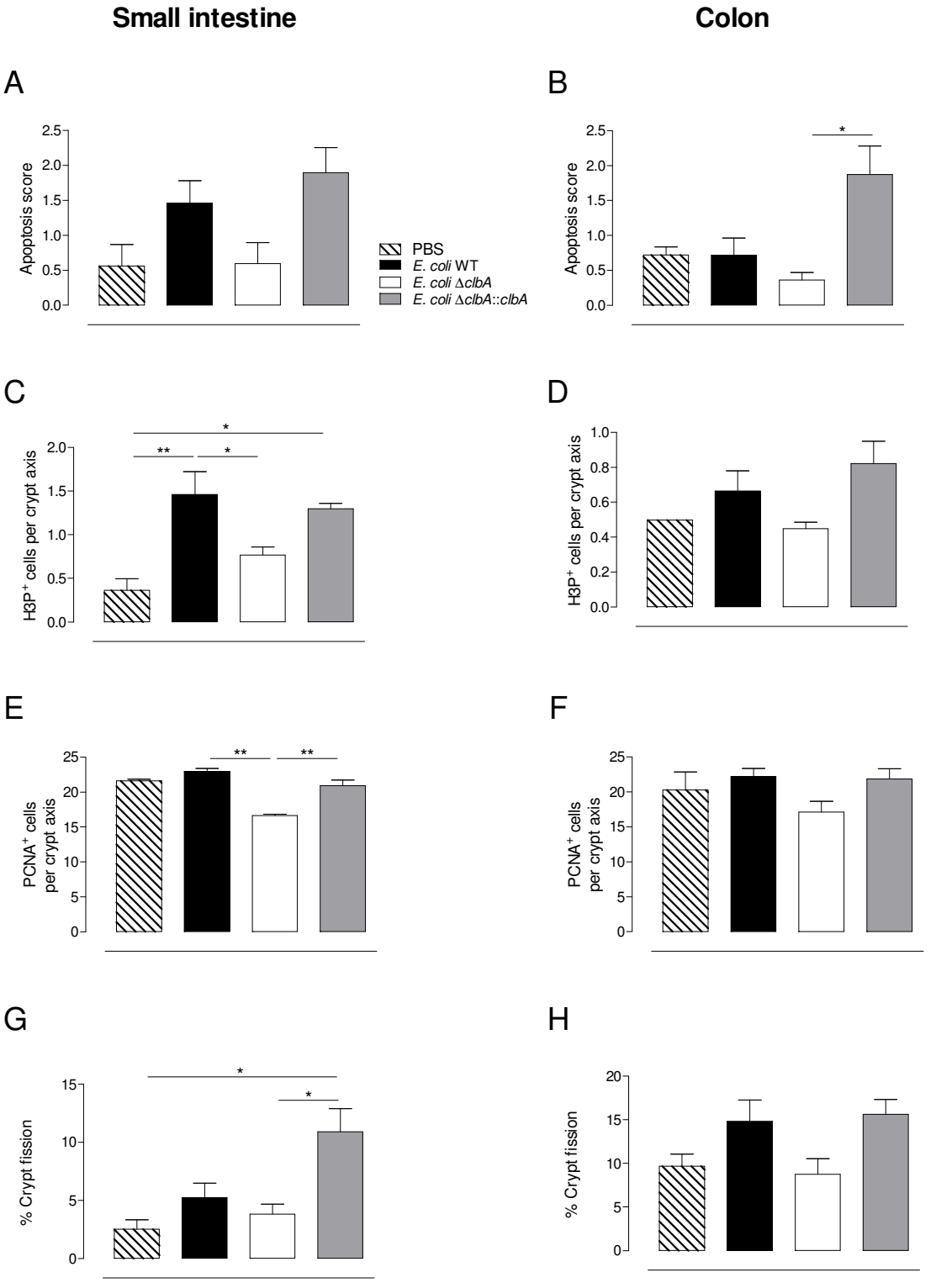

Small intestine
Colon
A
B
C
D
E
F
G
H
Apoptosis score
H3P+ cells per crypt axis
PCNA+ cells per crypt axis
% Crypt fission
PBS
E. coli WT
E. coli ΔclbA
E. coli ΔclbA::clbA
*
**

**Figure S5**

**Supplementary Figure 5 – Early Gut Colonization With Commensal *E.* coli Strains Producing Colibactin Increased the Intestinal Epithelial Cells Apoptosis, Proliferation and Crypt Fission at PND28.** Histological and immunofluorescence analysis of the intestinal and colonic epithelium of young animals at PND28 early colonized by commensal *E. coli* WT, Δ*clbA* or Δ*clbA*::*clbA* strains or treated with PBS.

**(A, B)** Quantification of intestinal apoptotic score (see Methods) in small intestine **(A)** and colon **(B).**

**(C, D)** Quantification of $H3P^+$ cells per crypt in small intestine **(C)** and colon **(D)**.

**(E, F)** Quantification of $PCNA^+$ cells per crypt in small intestine **(E)** and colon **(F)**.

**(G, H)** Quantification of crypt fission in small intestine **(G)** and colon **(h)**.

Groups of 5-10 rats were used. Mean values ± SEM are shown (One-way ANOVA with Bonferroni Multiple Comparison test, * $P \leq 0.05$, ** $P \leq 0.01$).

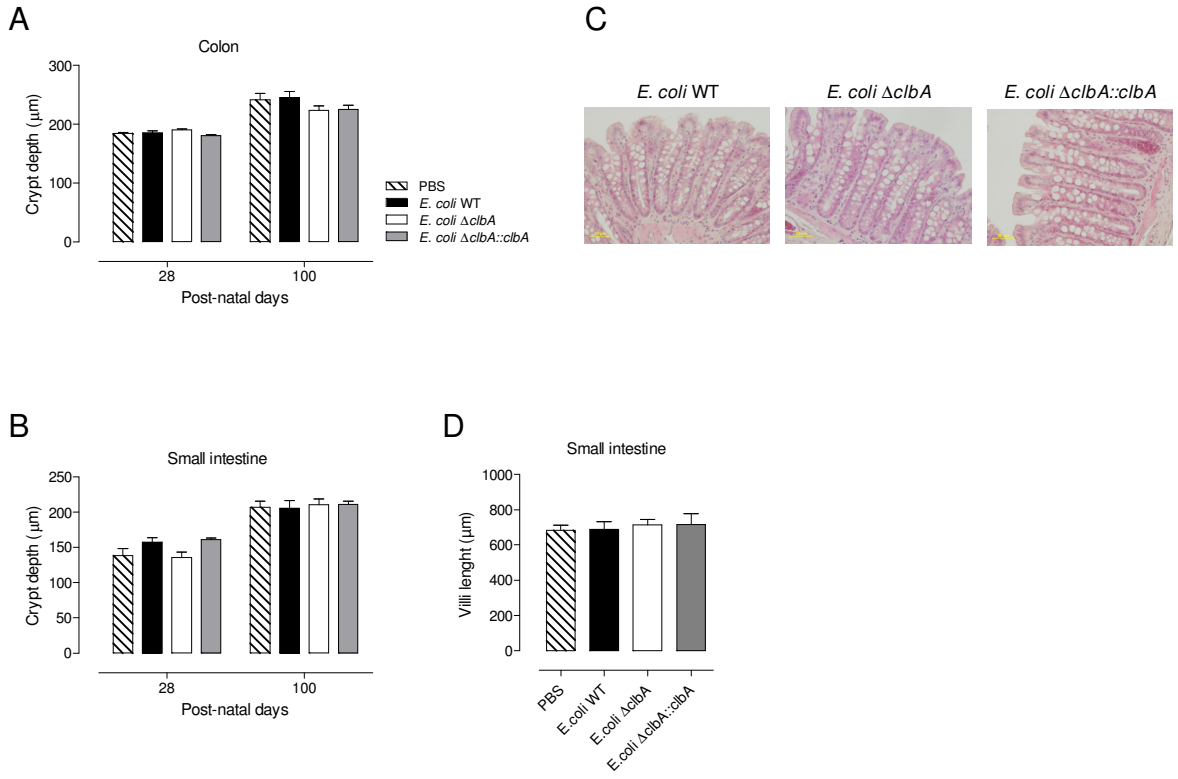



**Figure S6**

**Supplementary Figure 6 – Early Gut Colonization With Commensal *E.* coli Strains Did Not Induce Crypt or Villi Length Modification.** Histological analysis of the intestinal and colonic epithelium of adult animals early colonized by commensal *E. coli* WT*,* Δ*clbA* or Δ*clbA*::*clbA* strains or treated with PBS.

**(A, B)** Evaluation of crypt depth in colon **(A)** and small intestine **(B).**

**(C)** Representative colon sections at PND100 and stained with hematoxylin-eosin. (Magnitude used for photomicrographs: x20).

**(D)** Evaluation of villi length in small intestine.

Groups of 5-10 rats were analyzed. Mean values ± SEM are shown (One-way ANOVA with Bonferroni Multiple Comparison test).

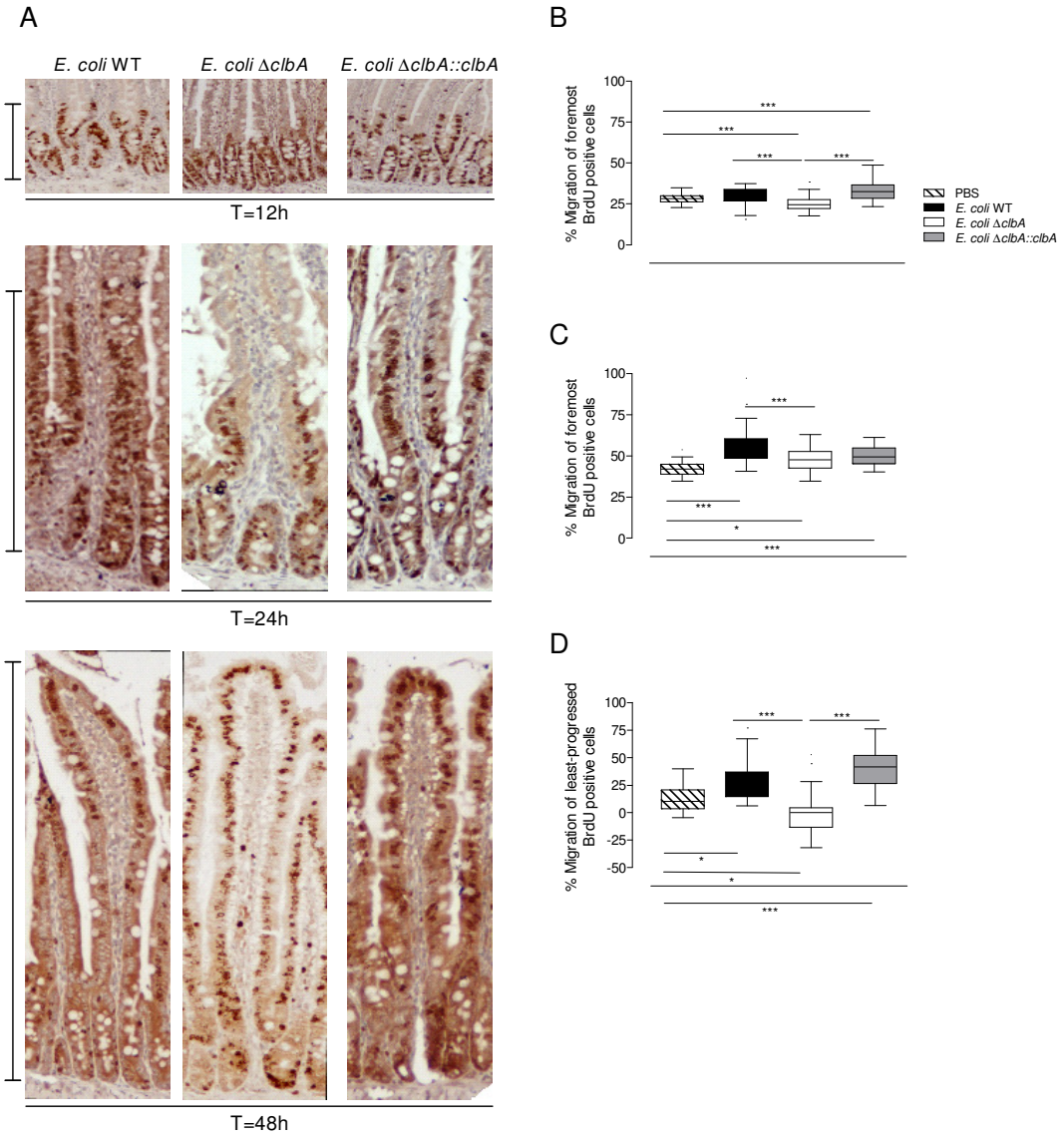

A
E. coli WT
E. coli ΔclbA
E. coli ΔclbA::clbA
T=12h
T=24h
T=48h
B
% Migration of foremost BrdU positive cells
PBS
E. coli WT
E. coli ΔclbA
E. coli ΔclbA::clbA
C
% Migration of foremost BrdU positive cells
D
% Migration of least-progressed BrdU positive cells

**Figure S7**

**Supplementary Figure 7 - Early Gut Colonization With Commensal *E. coli* Strains Producing Colibactin Hastened Enterocytes Migration at Adulthood.** Histological analysis of the intestinal epithelium of adult animals early colonized by commensal *E. coli* WT, Δ*clbA* or Δ*clbA*::*clbA* strains or treated with PBS

**(A)** Representative small intestine sections at PND100, 12 h (upper panel), 24 h (middle panel) and 48 h (lower panel) after oral treatment with BrdU. BrdU was stained in brown. (Magnitude used for photomicrographs: x10).

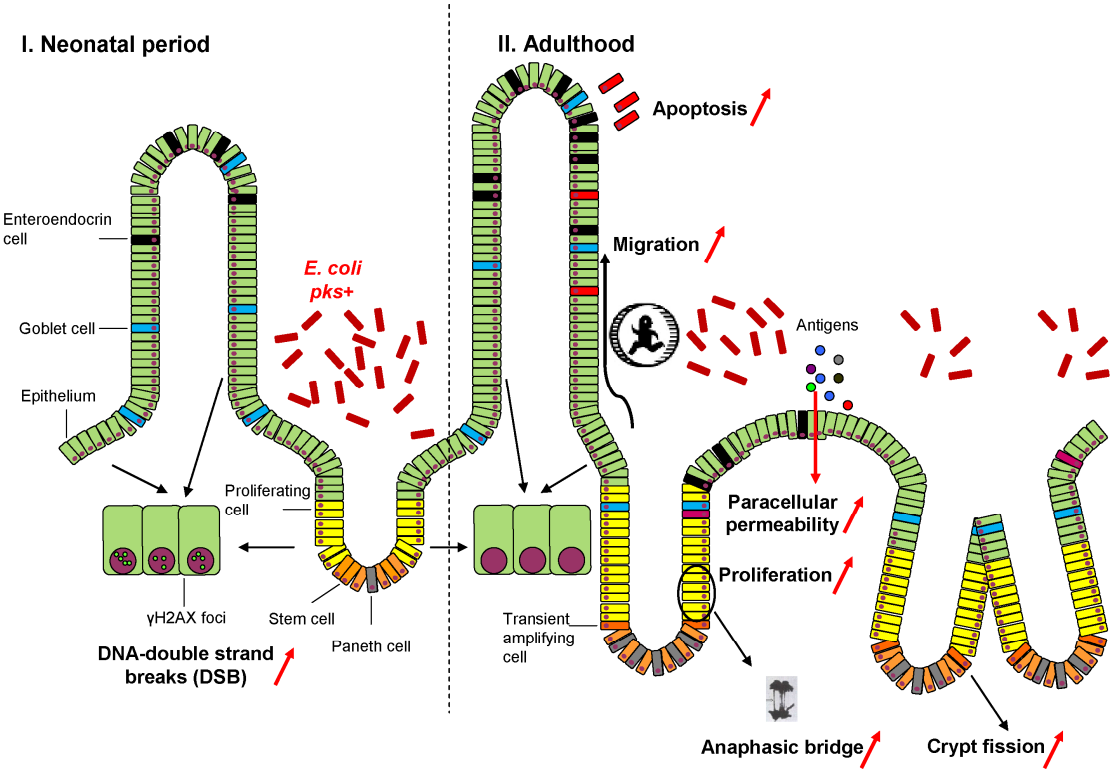
**(B)** Quantification of $BrdU^+$ cells migration along the crypt-villus axis 12 h after oral treatment with BrdU.

**(C)** Quantification of $BrdU^+$ cells migration along the crypt-villus axis 24 h after oral treatment with BrdU.

**(D)** Quantification of $BrdU^+$ cells migration along the crypt-villus axis 48 h after oral treatment with BrdU.

Graphs show mean ± SEM position of foremost and least-progressed $BrdU^+$ enterocytes.

Groups of 3-4 rats were used, 25-100 crypt-villi unit were analyzed. (Non parametric one-way ANOVA with Kruskall-wallis post test. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$)




**Supplementary Figure 8 – Graphical Abstract Summarizing the Main Alterations Observed in the Gut Epithelium of Rats Colonized With a Maternally Transmitted Genotoxic *E.coli*.**


1. Clermont, O., Christenson, J.K., Denamur, E. & Gordon, D.M. The Clermont Escherichia coli phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. *Environ Microbiol Rep* **5**, 58-65 (2013).
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3. Le Gall, T., *et al.* Extraintestinal virulence is a coincidental by-product of commensalism in B2 phylogenetic group Escherichia coli strains. *Mol Biol Evol* **24**, 2373-2384 (2007).
4. Nougayrede, J.P., *et al.* Escherichia coli induces DNA double strand breaks in eukaryotic cells. *Science* **313**, 848-851 (2006).
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6. Wirth, T., *et al.* Sex and virulence in Escherichia coli: an evolutionary perspective. *Molecular Microbiology* **60**, 1136-1151 (2006).