Introduction

Members of the species *Escherichia coli* can be either commensal to humans or pathogenic to the host, depending on the strain in question. The commensal nature of some *E. coli* strains have resulted in their use as probiotics, with the classical example of *E. coli* strain Nissle 1917 (EcN), which has been used as a probiotic for more than 100 years [1]. However, despite this long-term use in the absence of obvious side effects, its safety could be questioned, as the strain contains the polyketide synthesis (pks) locus that is responsible for production and secretion of colibactin [2]. The pks locus is approximately 50-kb long and typically contains 16 genes in the same orientation (clbB–clbQ, sometimes interrupted by the presence of other genes), with the regulatory genes clbA and clbR in the opposite orientation (Figure 1). The pks genes encode 3 polypeptide synthases (ClbC, ClbI, and ClbO), 3 non-ribosomal peptide synthases (ClbH, ClbJ, and ClbN), and 2 proteins with dual functions of both ClbB and ClbK and accessory proteins. In combination, these gene products produce, activate (e.g., peptidase ClbP), and secrete (transporter ClbM) the complex molecule colibactin. Colibactin contains a highly reactive cyclopentanone group. The product of gene clbR regulates the transcription of clbA, whose product ClbA again activates at least 2 promoters to produce polysaccharide messengers [2, 3]. All genes except for clbR are required to actively express colibactin [2, 3]. However, colibactin has not been purified and its structure is only inferred from precursor forms.

Colibactin has been implicated in genotoxicity and may be involved in the onset of colorectal cancer [3–7]. In vitro and in vivo models have demonstrated that pks-containing *E. coli* strains are able to induce DNA damage in exposed eukaryotic cells [3–9]. By means of knock-out mutants, it was demonstrated that the pks locus is responsible for the presumed genotoxicity [3], and heterologous expression of the locus corroborated these findings [10].

The impairment by genotoxic compounds is based on induction of mutations or DNA damage to subjected cells. Induced mutations can revert existing substitutions, a possibility that is assessed by the Ames test [11]. For this test, a potential mutagenic agent is incubated with specific *E. coli* or *Salmonella* reporter mutant strains that are auxotrophic for histidine or tryptophan. If the agent induces mutations, the auxotrophy is repaired by a reverse mutation, and revertant colonies can grow on minimal agar plates lacking this amino acid. The number of revertant colonies is a measurement of the genotoxic activity of the agent, and the Ames test was shown to be a suitable test to screen mutagenicity [12]. However, since the test depends on the growth of *E. coli* or *Salmonella* reporter strains, potential mutagenicity of a living bacterial strain cannot be determined, as it would overgrow the reporter strain, unless the test is modified.

DNA strand breaks induced by genotoxic agents can be visualized by the so-called Comet assay, developed in 1984 [13] and modified 4 years later in order to detect single-strand DNA breaks, too [14]. Recommendations for the Comet assay were expressed in 2003 [15] and in 2012 [16]. The Ames test and the Comet assay are both implemented by the Organization for Economic Co-operation and Development (OECD) to detect genotoxicity of a given compound [17, 18].

No Genotoxicity Is Detectable for *Escherichia coli* Strain Nissle 1917 by Standard In Vitro and In Vivo Tests

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Probiotic *Escherichia coli* strain Nissle 1917 (EcN) has a long history of safe use. However, the recently discovered presence of a pks locus in its genome presumably producing colibactin has questioned its safety, as colibactin has been implicated in genotoxicity. Here, we assess the genotoxic potential of EcN. Metabolic products were tested in vitro by the Ames test, a mutagenicity assay developed to detect point mutation-inducing activity. Live EcN were tested by an adapted Ames test. Neither the standard nor the adapted Ames test resulted in increased numbers of revertant colonies, indicating that EcN metabolites or viable cells lacked mutagenic activity. The in vivo Mammalian Alkaline Comet Assay (the gold standard for detecting DNA-strand breaks) was used to determine potentially induced DNA-strand breaks in cells of the gastro-intestinal tract of rats orally administered with viable EcN. Bacteria were given at 10⁷–10¹¹ colony forming units (CFU) per animal by oral gavage on 2 consecutive days and daily for a period of 28 days to 5 rats per group. No significant differences compared to negative controls were found. These results demonstrate that EcN does not induce DNA-strand breaks and does not have any detectable genotoxic potential in the test animals.

Keywords: genotoxicity, probiotic, *E. coli* Nissle, colibactin, Ames test, comet assay
Although in vitro models that are independent of test animals can be used for primary screening of genotoxicity, in vivo tests should be performed to confirm any measured effect, for which the Comet assay is a cheap, rapid, and sensitive method [19]. In this assay, rats are exposed to a test compound for a given period of time, after which the animals are sacrificed, and the target tissue is collected. Isolated cells from this tissue are embedded in a thin layer of agarose, lysed under high salt concentrations, and the DNA is allowed to unwind and denature using alkaline conditions. The liberated DNA is subjected to agarose gel electrophoresis, allowing any DNA fragments (indicative of damaged DNA) to migrate faster than non-damaged DNA molecules. This produces a comet-shaped pattern, after which the test is named. The relative intensity of the visible ‘tail’ depends on the frequency of DNA breaks, and this can be assessed using computer-based optical analysis. The intensity of the comet tail relative to the total intensity (head plus tail) reflects the amount of DNA breakage [20, 21]. The Comet assay is recommended to be performed in rats, and the genotoxic potential of the test substance should be assayed in the target tissue(s) with respect to the most likely route of exposure [18].

Genotoxic effects of colibactin produced by various E. coli strains have been demonstrated in a number of murine models, which are summarized elsewhere, but all have their limitations [22]. Since rats are more easily colonized by E. coli than mice, and since rats are the standard test animal for genotoxic testing in combination with the Comet Assay, we chose that model to test any genotoxic activity of EcN in vivo.

The aims of this study were therefore to assess any mutagenic or genotoxic properties of EcN by (1) in vitro testing of spent supernatant in the classical Ames test; (2) in vitro testing of viable EcN using an adapted Ames test; and (3) in vivo testing using rats exposed to EcN for 2 time periods followed by the Comet Assay performed on the intestinal tissue in accordance to international guidelines. Before reporting the experimental results, the conservation of 8 pks genes in other species is briefly summarized.

### Materials and Methods

#### Assessment of Conservation of Pks Genes in Bacterial Species.

Eight genes of the pks locus from the E. coli Nissle 1917 genome sequence (National Center for Biotechnology Information [NCBI] accession number CP035486.1) coding for polyketide synthases, non-ribosomal peptide synthases, or both, were used as query in quick-BlastP at NCBI with default settings except for the output, which was increased to 500 hits. The highest percentage of query length (%QL) covered in hits of different bacterial species was recorded for each of the genes, as well as the highest percentage identity (%ID), provided one of these percentages exceeded 50%.

#### In Vitro Ames Test with a Cell-Free Spent Supernatant of EcN.

Escherichia coli strain Nissle 1917 (EcN) and its supernatant were obtained from the producer, Ardeypharma GmbH, Herdecke, Germany. Classical Ames test was performed according to the published guidelines [17] at Eurofins BioPharma Product Testing, Munich. The test item was a cell-free spent supernatant obtained from EcN, batch # 729210 containing 1–2·10^{11} colony forming units (CFU)/mL. It was used in 5 concentrations: undiluted and diluted 1:2, 1:10, 1:20, and 1:100 in sterile salt solution (SS, 40 mM NaCl, 30 mM KCl, 0.8 mM MgSO4, 1.4 mM CaCl2, 0.1 mM MgCl2, and pH 7.0). This corresponded to a volume of cell-free supernatant of 100 μL, 50 μL, 10 μL, 5 μL, and 1 μL tested per agar plate. The highest tested dose would exceed the recommended daily maximum intake of the EcN product administered to young children by 20-fold.

The test was performed according to the published guidelines [17, 23–25] with Ames reporter bacteria of Salmonella enterica serovar Typhimurium strains TA98, TA100, TA1535, TA1537, and TA102. The test was performed without and with metabolic activation. For the latter, S9 mix (Trinova Biochem GmbH, Gießen, Germany) was prepared according to the literature [11, 17]. A solvent control of SS and a negative control (sterile water) were included. As a positive control for Ames reporter strains TA100 and TA1535 without metabolic

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Figure 1. Degree of conservation of pks genes among Gram-negative bacteria. The top drawing shows the pks locus of EcN (genome accession number CP035486.1), with accession numbers of the polyketide synthases and non-ribosomal peptide synthases (grey gene arrows) shown below the analyzed genes. The amino acid sequences of these genes were used as query in BlastP. The blocks below the locus show the reported top scores per species of other Gram-negative bacteria, with percentage identity (%ID, values in black) and percentage of query length coverage (%QL, values in white). Hits to an unspecified Salmonella species (strain HMSC13B08) were ignored. Since the accessory genes (shown in white) may share functional domains with genes not related to the pks locus, their conservation was not assessed.

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activation sodium azide (Sigma) was included at a concentration of 10 μg/plate. The positive control 4-nitro-o-phenylene-diamine (Fluka) dissolved in dimethyl sulfoxide (DMSO) was used at 10 μg/plate for reporter strain TA98 and at 40 μg/plate for reporter strain TA1537. The positive control for reporter strain TA102 without metabolic activation was 1 μL/plate methyl methanesulfonate. The positive control for all Ames reporter strains in combination with S9 metabolic activation was 2-aminoanthracene (Aldrich) dissolved in DMSO.

Following standard procedures, 100 μL of the test item or control was mixed with 500 μL S9 mix for tests with metabolic activation or with 500 μL S9 mix substitution buffer for tests without metabolic activation, 100 μL Ames reporter strain (approx. 10⁸ CFU/mL, early stationary phase), and 2 mL of molten top agar (0.7% agar, 0.6% NaCl, 0.05 mM histidine/biotin) at 44–48 °C. This mixture was poured on Vogel Bonner glucose minimal agar plates and to the top agar, to inhibit the growth of EcN but not inhibiting the background lawn similar to streptomycin-free plates and to the top agar, to inhibit the growth of EcN but not inhibiting the Ames reporter strains (this modification was omitted for strain TA102 that required 10 μg/plate).

In Vitro Modified Ames Test with Viable EcN. Live EcN bacteria from overnight cultures (16–18 h, 37 °C in nutrient broth) were centrifuged (8 min, 5000 rpm), and cell pellets were washed twice with phosphate buffer and resuspended in sterile water to 1–2×10⁹ CFU/mL. Recovered supernatant of the EcN overnight culture was sterile filtrated to obtain a cell-free supernatant containing metabolite products of EcN. These test items were tested in a modification of the classical Ames test. The modification included addition of 20 or 24 μg/mL streptomycin (Roht, Karlsruhe, Germany) to the Vogel Bonner glucose minimal agar plates.

Test Item for the In Vivo Mammalian Alkaline Comet Assay. The in vivo Mammalian Alkaline Comet Assay was performed with EcN viable bacteria containing an average of 1.1×10¹³ CFU/mL (range: 7.5×10¹²–1.6×10¹⁴). Three dose levels of EcN were applied: 10¹⁰ CFU/mL (undiluted EcN), 10⁹ CFU/mL (1:10 dilution in SS), and 10⁸ CFU/mL (1:100 dilution). Sterile SS served as vehicle control. In all rat experiments, single doses of 1 mL, bacterial suspension or vehicle control were given per animal on consecutive days.

Animal Experiments. The animal experiments were conducted at BSL Munich, Germany, in an AAALAC-accredited laboratory. Young healthy SPF Wistar rats (Crl: Wi(Han)) of either sexes were obtained at 6–8 weeks of age from a professional breeder (Charles River, Sulzfeld, Germany) and allowed to acclimatize for at least 5 days in groups of 1 to 3 randomly selected animals per cage, sorted for sex. At the age of 7–9 weeks, EcN was orally administered to the animals via gavage. A negative control group (NC) consisted of 5 untreated animals (only in Experiment A), and a vehicle control group (VC) included 5 animals dosed with SS. A positive control group (PC) consisted of 5 animals treated with ethyl methanesulfonate (Sigma-Alrich/Merck, Darmstadt, Germany) in 0.9% NaCl, at a single dose of 100 mg per kg body weight (bw) in Experiment A and 300 mg/kg bw in Experiments B and C. All animals had free access to water and feed during the course of the experiment.

For the pilot dose-range finding experiment, various doses of EcN suspension were tested to identify the maximum tolerated dose (MTD), defined as the dose that induces the first slight toxic effects with respect to the duration of the main study period. Three animals of each sex were used for this, and since no differences could be determined between male and female animals, all other experiments were performed with male rats only.

Since no toxic effect was detected after application of a maximum feasible dose of 1 mL per day with 1×10¹³ CFU/mL, this dose was chosen as MTD. A higher dose would not be feasible as the bacterial suspension would become too viscous to be applied in the manner required.

The marketed EcN product used in infants and children up to the age of 12 (Mutaflor Suspension) is applied at a daily dose of up to 5 mL containing 10⁶ CFU/mL. This corresponds to a daily dose of 1.1×10⁷ CFU/kg bw for a child of 45 kg, or 10 times that much for an infant of 4.5 kg. The latter refers to 1.67×10⁷ CFU for a rat weighing 0.15 kg on average. We exceeded this dose with a factor of 6000.

For determination of acute oral toxicity [24, 26] short-term exposure experiments were performed with 6 groups (Experiment A) and 3 groups (Experiment B) of 5 rats; dosage and treatment are summarized in Table 1. All animals except for groups 2 and 8 (positive controls) were treated orally by gavage on days 1 and 2 and were sacrificed 4 h after the second dose on day 2. The positive control groups only received a single dose on day 2. For long-term exposure (Experiment C), the daily dose was provided on 28 consecutive days according to the guidelines [27] (Table 1). During the period of administration, the animals were carefully observed for signs of toxicity by qualified staff. Following euthanasia at the end of the experiment, the relevant tissues were sampled.

To verify that EcN had reached the target organs and to prove a continuous exposure, animal feces were sampled group-wise at various time points during all experiments and analyzed for the presence of EcN using cultural methods followed by a strain-specific PCR as previously described [28].

Clinical Scoring. All animals were observed for clinical signs during the experiment. General clinical observations were performed at least once a day at approximately the same
time in consideration of the peak period of anticipated effects after dosing. The health condition of the animals was recorded and twice, daily all animals were observed for morbidity and mortality.

Body weight was recorded from all animals before assignment to the experimental groups, on the first day of administration and weekly during the long-term application. All animals subjected to necropsy were additionally weighed on the day of necropsy.

**Intestinal Cell Preparation.** Following euthanasia, the selected tissues were removed and kept in ice-cold mincing buffer (Hank’s Balanced Salt Solution containing 20 mM EDTA, pH 7.5, with 10% DMSO added before use). The tissues were rinsed with this buffer to remove residual blood and kept on ice until further processing. A portion of the small intestine and the upper and lower parts of the colon were minced with a pair of scissors and the cell suspension was filtered through a mesh (70 μm) to yield a single cell suspension. The cell number of isolated cells was adjusted by determination using the trypan blue dye exclusion test and approximately 2–4 × 10^4 cells per sample were scored. The tail intensity was expressed as a percentage of the total intensity in the nucleus.

The median % tail DNA for each slide was determined, and the mean of the median value was calculated for each animal. Statistical analysis was done by one-way ANOVA with Dunnett’s test and by unpaired t-test with Welch’s correction, and in both cases, normality test by Kolmogorov–Smirnov was used.

**Ethics.** In accordance to German animal protection laws, the study type was reviewed and accepted by local authorities, subjected to the Ethical Review Process and authorized by the Bavarian Animal Welfare Administration.

**Results**

**Conservation of Key Pks Genes in Bacterial Species.** To assess the conservation of *pks* genes among bacterial species, BlastP searches were performed with amino acid sequences extracted from the genome of EcN. Although the *pks* locus is not present in every sequenced member of a bacterial species, when present, it is usually strongly conserved within that species (results not shown). The degree of conservation of key genes between bacterial species is reported by 2 parameters in Figure 1: top hits obtained with BlastP are shown with the percentage of the query length producing overlap (%QL, the query being the EcN gene) and by the percentage of identity within the found overlap (%ID). As can be seen, strong conservation was found for members of *Enterobacter hormaechei* (a colonizer of the human gut [29], *Klebsiella pneumoniae* and *Klebsiella aerogenes*, and *Citrobacter koseri*, an opportunistic pathogen. Less strongly conserved genes were detected in *Klebsiella oxytoca*, *Erwinia oleae*, and *Serratia marcescens*, while the genes of *Frischella serrata* (a gut symbiont of honey bees) are the least conserved (Figure 1).

**In Vitro Testing of EcN Spent Supernatant by the Ames Test.** The results of the classical Ames test, performed with 5 different Ames reporter strains, are summarized in Figure 2. The test item was tested at 5 concentrations, the highest of which (undiluted supernatant) represents a 20-fold excess of the dose under recommended use for infants. The tests were performed in the absence and presence of metabolic activation, while all tests were carried out with and without pre-incubation of the test material with the reporter strain. All tests performed with 5 concentrations of the cell-free spent supernatant were negative, with non-significant differences to the negative and solvent controls in all cases. All positive

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**Table 1. Experiments for in vivo assessment, with 2-day and 28-day colonization**

| Exp. | Group no. n = 5 animals | Treatment | Dose (in 1 mL) per animal, timing |
|------|-------------------------|-----------|----------------------------------|
| Exp. A |                         | Untreated |                                   |
| Exp. B |                         | EcN        | 0.01 MTD: 10^7 CFU EcN daily on days 1–2 |
| Exp. C |                         | EcN        | 0.10 MTD: 10^9 CFU EcN daily on days 1–2 |

Exp: Experiment. EcN: *E. coli* Nissle 1917. MTD: maximum tolerated dose. EMS: ethyl methanesulfonate.
controls resulted in highly significant readings ($P < 0.001$). Thus, no mutagenic or cytotoxic activity could be demonstrated for the spent supernatant of EcN assessed by the classical Ames test, under all tested conditions.

**In Vitro Testing of Viable EcN by the Modified Ames Test.** Since colibactin-induced toxicity depends on direct cell contact [3], we also tested mutagenic activity of viable EcN bacteria by pre-incubation with Ames reporter strains, in a modified Ames tests (see Materials and Methods). By addition of streptomycin to the agar plates, the growth of EcN could be suppressed without affecting the Ames reporter strains. The results are summarized in Figure 3. Only the positive controls resulted in a significant increase of revertants. All other test items produced revertant colonies in numbers not significantly different from those obtained with the solvent control. Thus, even while direct cell contact was enabled, EcN failed to exert any mutagenic effect on the Ames reporter strains.

**In Vivo Genotoxicity of EcN in Rats as Determined by the Comet Assay.** In vivo genotoxic effects of EcN were tested in rats by oral application for 2 days or 28 days, respectively, followed by the Comet assay to analyze the exposed intestinal epithelial cells. Microbiological and PCR analysis of the rats’ feces evidenced that those animals that had received the test item carried living EcN in their feces throughout the experiments (results not shown). This indicates that, as expected, live EcN reached its site of action, the gut, and...
verified that the target tissues had indeed been exposed to the test item.

During application, no clinical signs of toxicity were observed with the test animals, and the post-mortem histopathological examination showed no significant findings (results not shown).

Three sites of the gastrointestinal tract were assessed with the Comet assay: the small intestine, and the upper and lower parts of the colon. The results are presented in Table 2. The positive controls resulted in statistically significant \( (p < 0.05) \) DNA damage of cells from all 3 tested tissues, in all 3 experiments (Table 2). For the highest tested dose of \( 10^{11} \) CFU, a significant \( (p = 0.0032) \) decrease in scores for DNA damage was observed in cells collected from the small intestine, in Experiment B only (Table 2). The comet tail intensity for all other tissues from the EcN application groups was in the same range as the negative (vehicle) control. In none of the tissues from the EcN application group, the comet tail intensity was increased, which would have been an indication of induced DNA strand breaks. The distribution of the findings per animal group is shown as Box-and-Whisker plots in Figure 4. The results clearly show that EcN did not induce DNA damage in the intestinal tissue of the exposed animals.

**Discussion**

A number of in vitro and in vivo tests are available to test the possible mutagenicity or genotoxicity of a given compound. All these tests have advantages and disadvantages, in part depending on the mechanism by which a potential mutagen might cause DNA damage, as discussed elsewhere [30]. The Ames test makes use of certain reporter Ames strains to detect mutagenicity by means of bacterial reverse mutation, which excludes its application to test mutagenic properties of live bacteria. The test is applicable to cell-free supernatant, but in the case of colibactin, testing spent supernatant may be less relevant. It has been described that colibactin, when produced by *E. coli*, is rapidly inactivated upon secretion and that direct cell contact is needed for genotoxic effects [3]. Thus, the finding that EcN spent supernatant did not have any detectable mutagenic properties was not fully informative.

We were able to overcome the practical limitation of the classical Ames test by the addition of a selective antibiotic, which allowed the testing of viable EcN without affecting the spontaneous reversion rates of the Ames reporter strains too much. Again, no mutagenic activity could be demonstrated for EcN, although now EcN was co-incubated with the Ames reporter strains, and direct cell contact was enabled throughout the experiment. These findings demonstrate that neither living EcN bacteria nor its metabolites show genotoxic, cytotoxic, or mutagenic activity in the Ames test.

It has been suggested that colibactin produces DNA strand breaks, which are excellently detectable with the Comet assay. Compared to other in vivo tests, the Comet assay is superior, as it can identify 92% of probable or confirmed human carcinogens, a result that was better than any of the other tests.

### Table 2. Comet Assay, mean tail DNA intensities [%] in cells from the small intestine, upper and lower part of the colon of exposed rats from the three experiments

|                  | Small intestine (group means ± standard deviation) | Upper colon (group means ± standard deviation) | Lower colon (group means ± standard deviation) |
|------------------|---------------------------------------------------|-----------------------------------------------|-----------------------------------------------|
|                  | Exp. A    | Exp. B  | Exp. C       | Exp. A     | Exp. B  | Exp. C       | Exp. A     | Exp. B  | Exp. C       |
| Negative control | 1.87 ± 0.34 | n.d.    | n.d.          | 3.19 ± 0.74 | n.d.    | n.d.          | 2.47 ± 1.15 | n.d.    | n.d.          |
| Positive control | 5.99 ± 2.37 \(^{a}\) | 17.83 ± 7.11 \(^{a}\) | 14.94 ± 7.17 \(^{a}\) | 7.15 ± 0.90 \(^{a}\) | 12.72 ± 5.92 \(^{a}\) | 14.07 ± 5.97 \(^{a}\) | 6.42 ± 1.65 \(^{a}\) | 12.99 ± 3.73 \(^{a}\) | 14.19 ± 8.95 \(^{a}\) |
| Vehicle control  | 2.82 ± 0.72 | 3.77 ± 0.85 | 3.81 ± 0.67 | 3.25 ± 1.14 | 1.87 ± 0.63 | 2.88 ± 1.46 | 2.84 ± 1.47 | 1.10 ± 0.65 | 2.57 ± 1.53 |
| 0.01 MTD        | 1.28 ± 0.20 | n.d.    | 2.54 ± 0.97 | 3.70 ± 0.78 | n.d.    | 2.69 ± 1.70 | 2.17 ± 1.02 | n.d.    | 3.27 ± 1.21 |
| 0.1 MTD         | 1.24 ± 0.66 | n.d.    | 2.95 ± 2.09 | 3.55 ± 1.29 | n.d.    | 3.15 ± 1.84 | 1.13 ± 0.59 | n.d.    | 2.95 ± 0.99 |
| 1 MTD           | 0.98 ± 0.48 | 1.75 ± 0.60 \(^{b}\) | 3.94 ± 2.89 | 3.35 ± 0.82 | 1.59 ± 0.75 | 3.48 ± 2.36 | 2.28 ± 1.59 | 0.82 ± 0.37 | 3.90 ± 2.59 |

\(^{a}\)Significantly increased compared to vehicle control.

\(^{b}\)Significantly decreased compared to vehicle control.
that were compared [31]. Moreover, rats are more easily colonized by *E. coli* than mice, so we consider this animal model to be the most suitable test for the detection of genotoxic activity. Even though the highest tested dose exceeded the daily recommended dose of EcN to infants by a factor of 6000, no cytotoxic or mutagenic effects could be demonstrated, whether the animals were exposed for 2 days or for 28 days.

The findings reported here are in strong contrast with the literature, in which the mutagenicity of colibactin, encoded by the *pks* locus, is hardly doubted. This presumed mutagenicity was demonstrated in a number of in vitro and in vivo models [3–9]. One in vitro model was used to demonstrate that outer membrane vesicles (OMV) from EcN could increase the number of DNA strand breaks in cultured HT-29 cells as demonstrated by the Comet assay, and although those same vesicles did not display cytotoxic activity towards the cells, their proliferation was slightly reduced [32]. The authors speculate that the formed OMVs can pass the intestinal mucus barrier in humans and are taken up by epithelial cells, which would contribute to the beneficial activity of EcN. If, in our experiments, vesicles were formed in the intestine of the rats, they did not generate double-strand DNA breaks, as our data revealed no evidence of DNA damage at all. In line with this observation is the report that a mucus layer attenuates any genotoxic effect of colibactin, as was demonstrated by in vitro experiments [33].

Most in vivo experiments that demonstrated genotoxic effects of colibactin were performed with *pks*-positive *E. coli* strains other than EcN. For instance, a mouse model with germ-free IL-10−/− knockout mice was used to induce colitis by mono-colonization with *E. coli* strain NC101, after which treatment with the carcinogen azoxymethane resulted in tumors. A carcinogenic effect that depended on the presence of the *pks* locus was shown for the used *E. coli* strain [5, 34]. In this model, even *E. coli* strains lacking the *pks* locus caused cancer, but those tumors appeared later and were both fewer and smaller than was observed in animals monoclonized by *pks*-positive *E. coli*.

![Figure 4. In vivo assessment of mutagenic activity of EcN, shown as tail intensity in the Comet Assay. Rats received EcN for 2 days (Experiments A, left column, and B, middle column) or for 28 days (Experiment C, right column), and DNA damage was assessed by the Comet assay with cells from the small intestine (top), upper part of the colon (middle), and lower part of the colon (bottom). Statistical significant findings compared to the vehicle control are indicated with corresponding *p*-values. PC: positive control. VC: vehicle control. NC: untreated animals. MTD: maximum tolerated dose](image)

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Another model compared C57B/6 J-Apc−/− mice (Min mice) with wild-type animals that were treated with streptomycin prior to inoculation. These mice were colonized for 50 days with either a pks-positive or a K12 (pks-negative) E. coli strain, or they were not infected; 50% of the non-infected control animals developed colonic polyps, compared to 57% of K12-colonized and 92% of the animals colonized with the pks-positive strain. Of note, the wild-type animals did not develop neoplasia, despite being colonized with high levels of pks-positive E. coli [7].

A third murine model, that utilized colon loops, was used to demonstrate a rapid DNA-damaging effect of pks-bearing E. coli strain SP15 [4]. It should be noted that all these assays are rather artificial, and none are standard procedures to determine carcinogenic activity of an agent. The gold standard for such testing is the in vivo Comet Assay, with exposed tissues being investigated in a mammalian test system. Even for strains that stably produce colibactin, their genotoxicity is not easily demonstrated. A recent publication was able to demonstrate colibactin activity by means of the Comet Assay after exposing HeLa cells to a pks-positive E. coli strain, but only when the cells were additionally treated by 8 Gy gamma-irradiation. Interestingly, the comets produced in the presence of the colibactin-positive E. coli were significantly shorter than those obtained with cells irradiated in the absence of the bacteria. The authors interpreted this to be due to DNA cross linking [35].

Not all literature agrees with the in vivo effect of colibactin. It was demonstrated that at high multiplicity of infection, E. coli strains expressing pks can actually suppress the proliferation of tumor cells in a murine model using xenografts of E. coli-infected HC116 cells [36]. Since EcN bacteria are attracted to tumor tissue, this ability can even be employed for tumor-targeting bacterial therapy [37]. The authors of that publication demonstrated that genetically modified EcN overproducing colibactin suppressed tumors more strongly in a mouse model [37]. In our experiments, we detected significantly less DNA damage in one group of animals infected for 2 days with the maximum tolerated dose of EcN (Experiment B), but this finding was not replicated in the other experiments, so we are reluctant to conclude that the effect is real. Our data do, however, reproducibly report the lack of mutagenic activity of EcN in rats, as the bacteria did not induce biologically relevant DNA-strand breaks in any of the exposed tissues evaluated. The result indicates no adverse effect of the bacterial strain on the DNA in cells of the small intestine or the colon.

The question that remains to be answered is, how strong and consistently the pks genes of EcN are being expressed. Messenger RNA has been demonstrated for most genes of the EcN pks locus apart from clbR, with slightly higher transcript levels in early-stationary-phase cultures compared to late stationary phase (results not shown). This could suggest that colibactin is produced under nutrient limitation to compete with and eliminate other bacterial cells, an alternative function for colibactin that has been proposed [1]. Presumably, such transcription levels of EcN are insufficient to produce colibactin in amounts high enough to result in genotoxic effects in the assays applied here. The pks genes of EcN are highly conserved in other species (see Figure 1), and in other E. coli strains that were described to have genotoxic activity (results not shown), but a detailed comparison of their transcription activity has not yet been performed. Possibly, genes outside the pks locus affect the expression, production, or secretion of colibactin and these genes may be less conserved between E. coli strains than the pks genes are.

We conclude that E. coli strain Nissle 1917 does not have mutagenic activity in the in vivo mammalian Alkaline Comet assay using rats. These findings corroborate the in vitro findings determined by the Ames test, where neither live EcN bacteria, nor spent culture supernatant including EcN metabolites, were able to induce increased numbers of revertant colonies in different Ames test strains. We conclude that EcN has no mutagenic, genotoxic or DNA-damaging activity.

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**Author's Contributions**

SD, BK, MS, and RvB conceptualized and designed the study. All authors analyzed and interpreted data. TMW performed gene comparison and wrote the first draft. All authors edited and approved the final manuscript.

**Conflict of Interest**

SD, BK, MS, and RvB are employed by Ardeypharm GmbH, the producer of probiotic products containing E. coli Nissle 1917. TMW received financial support from Ardeypharm GmbH.

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