Mobility of $\beta$-lactam resistance under ampicillin treatment in gut microbiota suffering from pre-disturbance

Alexander Laskey1, John Devenish1, Mingsong Kang1, Mirjana Savic1, John Chmara1, Hanhong Dan1, Min Lin1, James Robertson2, Kyrilo Bessonov2, Simone Gurnik2, Kira Liu2, John H. E. Nash3, Edward Topp3 and Jiewen Guan1,*

Abstract
Ingestion of food- or waterborne antibiotic-resistant bacteria may lead to dissemination of antibiotic resistance genes (ARGs) in the gut microbiota. The gut microbiota often suffers from various disturbances. It is not clear whether and how disturbed microbiota may affect ARG mobility under antibiotic treatments. For proof of concept, in the presence or absence of streptomycin pre-treatment, mice were inoculated orally with a $\beta$-lactam-susceptible Salmonella enterica serovar Heidelberg clinical isolate (recipient) and a $\beta$-lactam resistant Escherichia coli O80:H26 isolate (donor) carrying a bla<sub>CMY-2</sub> gene on an IncI2 plasmid. Immediately following inoculation, mice were treated with or without ampicillin in drinking water for 7 days. Faeces were sampled, donor, recipient and transconjugant were enumerated, bla<sub>CMY-2</sub> abundance was determined by quantitative PCR, faecal microbial community composition was determined by 16S rRNA amplicon sequencing and cecal samples were observed histologically for evidence of inflammation. In faeces of mice that received streptomycin pre-treatment, the donor abundance remained high, and the abundance of S. Heidelberg transconjugant and the relative abundance of Enterobacteriaceae increased significantly during the ampicillin treatment. Co-blooming of the donor, transconjugant and commensal Enterobacteriaceae in the inflamed intestine promoted significantly (P<0.05) higher and possibly wider dissemination of the bla<sub>CMY-2</sub> gene in the gut microbiota of mice that received the combination of streptomycin pre-treatment and ampicillin treatment (Str–Amp) compared to the other mice. Following cessation of the ampicillin treatment, faecal shedding of S. Heidelberg transconjugant persisted much longer from mice in the Str–Amp group compared to the other mice. In addition, only mice in the Str–Amp group shed a commensal E. coli O2:H6 transconjugant, which carries three copies of the bla<sub>CMY-2</sub> gene, one on the IncI2 plasmid and two on the chromosome. The findings highlight the significance of pre-existing gut microbiota for ARG dissemination and persistence during and following antibiotic treatments of infectious diseases.

DATA SUMMARY
All supporting data have been provided within the article or through supplementary data files. Seven supplementary figures and four supplementary tables are available with the online version of this article. The whole-genome sequencing data generated for this study can be found in NCBI BioProject PRJNA674061.

INTRODUCTION
Antimicrobial resistance (AMR; here specifically limited to antibiotics, i.e. antibacterial agents) is a serious public health issue threatening the effective prevention and treatment of an ever-increasing range of bacterial infections [1]. Long-term extensive use of antimicrobials has led to the high prevalence of antibiotic-resistant bacteria (ARB) in clinical settings, agricultural systems and the environment [2–5]. A One Health approach has been taken globally, including reduction of antimicrobial use in human medicine and agriculture, in order to reduce AMR transmission to humans via the environment and food consumption [6–9]. To evaluate the potential health impacts of exposure to food- or waterborne contamination with ARB, it is critical to understand the dynamics of ingested ARB and the antibiotic-resistance genes (ARGs) that they carry in the host...
gut microbiome, and how this varies according to factors such as treatment with antibiotics.

*Firmicutes* and *Bacteroidetes* are the dominant phyla in a healthy gut microbiota [10]. These bacteria produce short-chain fatty acids that maintain a mildly acidic gut environment against the colonization of opportunistic *Enterobacteriaceae* [11, 12]. Members of the gut microbiota also compete with the intruding bacteria for niches and nutrients [13], and educate and maintain the host immune system to generate rapid and efficient response against intruders [14, 15]. Thus, a healthy gut microbiota may resist the colonization of ARB following ingestion and therefore reduce the opportunity for horizontal transfer of plasmid-borne ARGs.

Plasmid conjugation is an efficient means for dissemination of ARGs from ARB to commensal and pathogen bacteria in the gut microbiota [16, 17]. In general, antibiotics cause dysbiosis, thereby enabling ARB colonization and promoting conjugative ARG dissemination in the gut microbiome [18]. However, the results from modelling and *in vitro* experiments [19] show that antibiotics have the potential to promote or suppress conjugation through selection or inhibition of the donor, recipient or transconjugant. In the pre-existing normal mouse gut microbiota, antibiotics also have selective and suppressive effects on donor and recipient that enhance or inhibit conjugation of ARG-bearing plasmids during antibiotic treatments [20]. Since the gut microbiota often suffers from disturbance by various factors, such as diet, bacterial infection and antibiotic treatment, it is important to understand the effects of antibiotics on conjugative transfer of ARGs in microbiota with pre-existing disturbance. As a proof of concept study, we explored the impact of ampicillin on mobility of β-lactam resistance in the gut microbiota that had suffered pre-disturbance by streptomycin.

In a previous study, co-infection of mice with multiple β-lactam-resistant bacterial strains favoured colonization of *Escherichia coli* O80:H26 and enabled conjugative transfer of its blaCMY-2 gene via an IncI2 plasmid under ampicillin treatment [21]. Building on this model, we used the *E. coli* O80:H26 strain as a donor, along with a β-lactam-susceptible *Salmonella* Heidelberg, as a recipient in the present study. To elucidate how antibiotics may influence conjugation in the gut microbiota, we compared the dynamics of the donor, recipient, transconjugant and β-lactam resistance genes in mice that received ampicillin treatment, streptomycin pre-treatment, a combination of streptomycin pre-treatment and lactam pre-treatment, a combination of streptomycin pre-treatment and streptomycin lactam resistance. To facilitate recovery of *S. Heidelberg*, a spontaneous rifampicin-resistant mutant was generated. In brief, *S. Heidelberg* was cultured overnight in Luria–Bertani (LB; Miller formulation, Difco, Fisher Scientific, Ottawa, ON, Canada) broth at 37°C. A 1.0 ml overnight culture was pelleted, resuspended in 100 µL LB broth and spread on LB agar supplemented with 50 µg ml⁻¹ rifampicin (LB-R). After 24 h incubation, resistant colonies were selected and sub-cultured on LB-R agar 20 times to generate and maintain a *S. Heidelberg* rifampicin-resistant mutant culture.

**METHODS**

**Bacteria**

*E. coli* O80:H26 (EC-107) is a multi-antibiotic-resistant strain isolated from a chicken farm in Ontario, Canada. *E. coli* O80:H26 carries five plasmids: IncI2, IncY, IncFII, ColRNAI and a plasmid with no detectable Inc type [21]. IncI2 is a conjugative plasmid encoding a *bla*CMY-2 gene and IncY is a mobilizable plasmid encoding a *bla*TEM-1B gene. *In vitro* and *in vivo* transfer of the IncI2 but not IncY plasmid was detected using *E. coli* O80:H26 as donor and *E. coli* O16:H48 as recipient in the previous study [21].

*S. enterica* serotype Heidelberg (12–6342) is a human clinical isolate that does not carry any *bla* gene and is susceptible to β-lactam antibiotics [22]. *S. Heidelberg* carries two plasmids: IncX1 and ColRNAI. In the present study, *S. Heidelberg* was used as a recipient of β-lactam resistance. To facilitate recovery of *S. Heidelberg*, a spontaneous rifampicin-resistant mutant was generated. In brief, *S. Heidelberg* was cultured overnight in Luria–Bertani (LB; Miller formulation, Difco, Fisher Scientific, Ottawa, ON, Canada) broth at 37°C. A 1.0 ml overnight culture was pelleted, resuspended in 100 µL LB broth and spread on LB agar supplemented with 50 µg ml⁻¹ rifampicin (LB-R). After 24 h incubation, resistant colonies were selected and sub-cultured on LB-R agar 20 times to generate and maintain a *S. Heidelberg* rifampicin-resistant mutant culture.

**Impact Statement**

Plasmid conjugation is an effective means for bacterial dissemination of antibiotic-resistance genes (ARGs) in the gut microbiota. Early mouse studies showed conjugative transfer of ARGs in the gut under positive antibiotic selection pressure. Recent studies demonstrated ARG transfer in the absence of antibiotic selection pressure in mice with pre-diminished gut microbiota. This study was the first to explore the impacts of interaction between antibiotic selection pressure and pre-existing gut microbiota on the dynamics of conjugative transfer of ARGs. Our findings showed that the combination, compared to either one of the two factors, positive antibiotic selection pressure and pre-existing gut dysbiosis, promoted significantly higher and possibly wider dissemination of ARGs and prolonged the persistence of ARGs in the gut microbiota. This study points to a new direction for exploring pre-existing gut microbiota for better elucidation of the mechanisms of conjugative transfer of ARGs during anti-biotic treatments of infectious bacterial diseases.

**In vitro conjugation**

*In vitro* conjugation between *E. coli* O80:H26 (donor) and *S. Heidelberg* (recipient) was assessed in 10⁻¹ × LB broth as described by Laskey *et al.* [21]. Enumeration of the donor, recipient and transconjugant bacteria was performed using Chromocult agar (EMD Millipore, Toronto, ON, Canada) supplemented with 4 µg ml⁻¹ cefotaxime (CHR-F), XLT4 agar (Difco) with 50 µg ml⁻¹ rifampicin (XLT4-FR), XLT4- FR), respectively. Conjugation frequency is expressed as the ratio of transconjugant to donor enumerated at the end of the mating incubation.

**In vivo conjugation**

Experiments and procedures involving mice conformed to guidelines established by the Animal Care Committee at the
Female C57BL/6 mice at the age of 28 days were purchased from Charles River Laboratories (Saint Constant, QC, Canada). Mice were mixed and acclimatized for 2 weeks prior to bacterial inoculation or antibiotic treatment, and then housed three or four per cage (Optimice, Animal Care Systems, CO, USA) with water and feed was provided ad libitum. A total of 68 mice were randomly assigned into 2 sets of 4 groups (a total of 8 groups) to investigate the shedding of the donor and/or recipient bacteria and the transfer of plasmids carrying β-lactam resistance genes under various antibiotic treatments (Table 1). One set of mice were inoculated with only the recipient bacteria and the other set were inoculated with the recipient followed by the donor bacteria 1 h later. Bacterial inocula (100 µl) prepared from log-phase culture containing ~3.0×10^8 colony-forming units (c.f.u.) of the recipient or donor bacteria in buffered peptone water (Difco) were administrated via oral gavage. Four different treatments were tested in this study: (1) ampicillin treatment (Amp), provided immediately following bacterial inoculation via drinking water (0.16 mg ml⁻¹, equivalent to 30 mg ampicillin kg⁻¹ of body weight per day) ad libitum for 7 days; (2) streptomycin pre-treatment (Str), provided once via oral gavage (20 mg per mouse) 24 h before bacterial inoculation; (3) a combination of streptomycin pre-treatment and ampicillin treatment (Str–Amp); and (4) a control without the use of antibiotics (Ctl). Each treatment was applied to a group of mice from each set, one with only the recipient inoculation and the other with both the recipient and the donor inoculation. Fig. 1 shows the schedule of procedures for the group of mice which received inoculation of both S. Heidelberg and E. coli O80:H26, and treatments with both streptomycin and ampicillin. Faecal pellets were collected from all mice on −3 (baseline), 0 (bacterial inoculation), 1, 2, 3, 7, 14, 21 and 42 day post-infection (p.i.). Pellets were processed as described by Laskey et al. [21] for DNA extraction and bacterial enumeration. The three selective agars, CHR-F, XLT4-R and XLT4-FR, were used to enumerate the donor, recipient

| Group       | Donor | Recipient          | Treatment*                     | n† |
|-------------|-------|--------------------|--------------------------------|----|
| SH/Ctl      | No    | S. Heidelberg      | Control (no antibiotic)       | 6  |
| SH/Amp      | No    | S. Heidelberg      | Ampicillin                     | 6  |
| SH/Str      | No    | S. Heidelberg      | Streptomycin                   | 6  |
| SH/Str–Amp  | No    | S. Heidelberg      | Streptomycin followed by ampicillin | 6  |
| SH-EC/Ctl   | E. coli O80:H26 | S. Heidelberg | Control (no antibiotic)       | 10 (4) |
| SH-EC/Amp   | E. coli O80:H26 | S. Heidelberg | Ampicillin                     | 11 (5) |
| SH-EC/Str   | E. coli O80:H26 | S. Heidelberg | Streptomycin                   | 11 (5) |
| SH-EC/Str–Amp | E. coli O80:H26 | S. Heidelberg | Streptomycin followed by ampicillin | 12 (6) |

*Amoxicillin treatment was provided via drinking water (0.16 mg ml⁻¹) immediately following bacterial inoculation for 7 days, streptomycin treatment was provided via oral gavage (20 mg per mouse) once 24 h before bacterial inoculation, streptomycin followed by ampicillin treatment was the sequential combination of streptomycin and ampicillin treatment.

†n is the number of mice used in the experiment, and the number in parentheses represents the number of mice that were euthanized on 7 day post-infection for collection of cecum tissues for histological analysis.

Amp, Amoxicillin; Ctl, control; EC, Escherichia coli O80:H26; SH, Salmonella Heidelberg; Str, streptomycin.

**Fig. 1.** Schedule of procedures for mice that were inoculated with the Salmonella Heidelberg recipient and the Escherichia coli O80:H26 donor, and provided with treatments of streptomycin followed by amoxicillin (SH-EC/Str–Amp). Sample collection was performed on various days post-infection (p.i.), as indicated.
and putative transconjugant bacteria, respectively, with a detection limit of 2.2 log_{10} c.f.u. g^{-1} in faeces. At 7 days p.i., some of the mice inoculated with both recipient and donor were euthanized, consisting of five, five, six and four mice from the groups with the Amp, Str and Str–Amp treatment and the control, respectively. Tissue specimens of the cecum were collected from these mice and immediately stored in 10% neutral buffered formalin for histological examinations.

**Whole genome sequencing**

Putative transconjugant bacteria were whole-genome sequenced and sequence data were analysed using the MOB-suite software tool v2.1.0 [23, 24]. Representative putative transconjugant colonies isolated from selective agar plates (up to five colonies per time point) were subjected to genomic DNA extraction as described by Laskey et al. [21]. Whole-genome sequencing was performed using an Illumina MiSeq system and/or an Oxford Nanopore MinION sequencer (Oxford Nanopore, Cambridge, MA, USA) at the National Microbiology Laboratory (Guelph, ON, Canada). All short- and long-read data were deposited to the National Center for Biotechnology Information (NCBI) under BioProject PRJNA674061. The raw reads along with assemblies of genomes and plasmids were deposited under the BioSample accession numbers (SAMNs) listed in Table S1 (available in the online version of this article). Illumina raw reads were assembled using the shovill v1.1.0 pipeline (https://github.com/tseemann/shovill) with the following parameters: --gsize 5000000 --assembler spades --trim --depth 0 --mincov 0 --minlen 0. Hybrid assemblies utilizing Nanopore and Illumina raw reads were assembled using unicycler v0.4.7 run under default parameters. All assemblies were manually reviewed to confirm the completeness of the chromosome and any plasmids present. As part of the validation process, complete plasmid assemblies were mapped against raw reads using the Snippy [25] pipeline to assess coverage and any potential coverage gaps. The assembled sequences were further analysed using the MOB-suite v2.1.0 and Prokka [26] software tools. An IncI1 plasmid map was rendered using the UGENE software [27] and the plasmid was annotated using Prokka v1.13.3. A gene map in a chromosomal range of an E. coli O2:H6 transconjugant was rendered using the DNA Features Viewer Python library (https://github.com/Edinburgh-Genome-Foundry/DnaFeaturesViewer) and the partial genome was annotated using Prokka v1.13.3.

**16S rRNA gene amplicon sequencing**

DNA extracted from mouse faecal pellets was subjected to 16S rRNA gene amplicon sequencing as described by Laskey et al. [21] at the Ottawa Laboratory-Fallowfield, Canadian Food Inspection Agency (Ottawa, ON, Canada). In brief, the V3–V4 region of the 16S ribosomal RNA gene was amplified through PCR [28]. Libraries were prepared and sequenced using a MiSeq system (Illumina). Raw read data was demultiplexed and then analysed using Qiime2 [29] through a modified version of the Qime2 pipeline created by Forrest Dusseault (https://github.com/forestdussault/AmpliconPipeline). Data analysis and visualization were performed using the R package and GraphPad Prism 8.0 software (San Diego, CA, USA).

**Quantification of β-lactam resistance genes**

The abundance of bla<sub>CMY-2</sub>, bla<sub>TEM-1</sub>, and 16S rRNA genes in mouse faecal pellets was determined by qPCR [30–32, Table S2], Genomic DNA extracted from an overnight culture of E. coli O80:H26 with the DNeasy Blood and Tissue kit (Qiagen, Toronto, ON, Canada) was used for the generation of standard curves. Purified E. coli O80:H26 DNA was quantified and diluted in nuclelease-free water to serial 10-fold concentrations from 4 fg µl^{-1} to 4 ng µl^{-1} to provide a 6 log<sub>10</sub> range of quantitation. DNA extracted from mouse faecal pellets was diluted to 0.4 ng µl^{-1} for quantitation of the bla<sub>CMY-2</sub> and bla<sub>TEM-1</sub> genes and to 0.4 pg µl^{-1} for the 16S rRNA gene. All qPCRs were performed using a QuantStudio 3 Real-Time PCR System (Thermo Fisher, Nepean, ON, Canada). Reaction mixture (25 µl) contained 12.5 µl Power SYBR Green PCR master mix (Thermo Fisher), 1.0 µl forward and 1.0 µl reverse primers (final concentrations of 0.3 µM for bla<sub>CMY-2</sub>, 0.2 µM for bla<sub>TEM-1</sub>, and 0.3 µM for 16S rRNA; primer sequences as shown in Table S2), 0.2 µl (0.2 U) Antarctic Thermolabile UDG (New England Biolabs, Whitby, ON, Canada), 5 µl template DNA and 6.3 µl nuclelease-free water. The PCR programme included an initial incubation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s, annealing and elongation at the given temperatures (Table S2) for 30 s. A final stage with temperature ramping from 65–95 °C was included for analysis of the melting curves of PCR products to confirm the specificity of PCRs. Duplicate wells in duplicate PCR runs were performed for each DNA sample.

**Histology analysis**

Cecum tissue specimens were prepared in Swiss rolls and fixed in 10% (v/v) neutral buffered formalin for at least 24 h. Fixed tissues were embedded in paraffin, sectioned and stained with haematoxylin and eosin [33]. Lesions were evaluated quantitatively and assigned a score based on intensity of infiltrate and inflammation, ulceration and necrosis observed in the most severely affected area of the section. Scores, adapted from methods described by Erben et al. [34], were assigned as follows: 0, normal; 1, minimal; 2, mild; 3, moderate; and 4, marked. The following factors were taken into consideration for scoring: (a) leucocyte density and location: none, 0; minimal mucosal/submucosal, 1; mild mucosal/submucosal, 2; mild to moderate mucosal/submucosal, 3; moderate to marked transmural, 4; (b) goblet cell loss: none, 0; minimal, 1–2; (c) mucosal architecture change related to necrosis (crypt hyperplasia, erosion/ulceration, crypt abscess): none, 0; minimal, 1; minimal/mild, 2; mild, 3; moderate/marked, 4.

**Statistical analysis**

Differences in the conjugation frequency, mean abundance of each target bacterium and relative abundance of each phylum or family in the 16S rRNA gene community profiles between the treatment groups on the same sampling day
RESULTS

In vitro and in vivo conjugation

β-lactam resistance was transferable from the E. coli O80:H26 donor to the S. Heidelberg recipient in vitro and in vivo based on enumeration of the donor, recipient and putative transconjugant according to their phenotypes. The in vitro conjugation frequency was 1.5×10⁻⁵ (data not shown). The in vivo frequency at 1 day p.i. was 3.9×10⁻⁴, 2.5×10⁻³ and 1.3×10⁻⁶ of the Amp, Str and Str–Amp groups, respectively (Fig. 2e). Conjugation was not detected at a limit of 1.0×10⁻⁴ in the control group. The frequency of the Str and Str–Amp groups was significantly [F(3, 32.14)=8.201, P=0.0003; W(3, 18.33)=12.83, P<0.0001] higher than that of the control group. Although the donor abundance in the Str–Amp group was the highest among all groups at 1 day p.i., the transfer frequency of the Str–Amp group was lower than that of the Str group, likely due to limited recipient abundance (Figs 2a-e and S1a). However, from 3 to 42 days p.i., the abundance of donor, S. Heidelberg and S. Heidelberg transconjugant in the Str–Amp group was the highest among all groups (Figs 2a–d and S1). In addition, S. Heidelberg was shed in faeces for a longer period of time following co-infection with the donor and recipient than mono-infection with the recipient under all antibiotic treatments (Fig. 2b–d and f). Furthermore, the abundance of S. Heidelberg was significantly higher in faeces of mice co-infected with the donor and recipient than those mono-infected with the recipient from 2 to 42 days p.i. under Str–Amp treatment (Figs 2d, f and S2d).

Horizontal transfer of conjugative plasmids

To confirm horizontal transfer of β-lactam resistance, putative transconjugants were subjected to whole-genome sequencing analysis and plasmid characterization with the MOB-suite tool v2.1.0. Sequencing information on the representative transconjugants is available in NCBI BioProject PRJNA674061. According to sequence data analysis, three different transconjugant strains were recovered: (1) S. Heidelberg carrying an IncI2 plasmid [SH-(IncI2)], (2) S. Heidelberg carrying both an IncI1 and an IncI2 plasmids [SH-(IncI1, IncI2)] and (3) a mouse commensal E. coli O2:H6 strain carrying both an IncI1 and an IncI2 plasmids [EC-(IncI1, IncI2)], EC-(IncI1, IncI2) appeared a creamy white colour on XLT4- FR agar plates used for selective culturing S. Heidelberg transconjugant. Only SH-(IncI2) was recovered in the Amp group, both SH-(IncI2) and SH-(IncI1, IncI2) recovered in the Str group and all three transconjugant strains recovered in the Str–Amp group (Tables 2 and S1). The IncI1 or IncI2 plasmids in different bacterial strains shared identical size and MOB-suite plasmid cluster code, suggesting plasmid transfer between different bacterial hosts. Specifically, the IncI2 plasmid was transferred from the E. coli O80:H26 donor to the S. Heidelberg recipient, and from either the E. coli O80:H26 donor or the S. Heidelberg transconjugant to E. coli O2:H6. The IncI1 plasmid was possibly transferred from E. coli O2:H6 or other bacteria in the gut microbiota to the S. Heidelberg transconjugant, as neither the E. coli O80:H26 donor nor the S. Heidelberg recipient carries the IncI1 plasmid. The IncI1 conjugative plasmid belongs to cluster 476 (MOB-suite v2.1.0) and contains no ARGs (Fig. S3). Most of the identified genes on the IncI1 plasmid were of E. coli origin, suggesting a stable long-lived plasmid residence in E. coli. These genes are related to stress response, such as SOS response, toxin–antitoxin system and plasmid mobility, and could possibly contribute to conjugative transfer of the IncI1 plasmid. Analysis of the E. coli O2:H6 complete genome (NCBI BioSample SAMN16634233) identified two copies of the blaCMY-2 gene on the chromosome and one copy on the IncI2 plasmid. Both copies of the blaCMY-2 gene on the chromosome are adjacent to transposase IS6 Cp1 (Fig. 3), suggesting a possible movement of the blaCMY-2 gene from the IncI2 plasmid to the chromosome.

Dynamics of the β-lactam resistance genes

The E. coli O80:H26 donor carries one copy of the IncI2 plasmid encoding one copy of the blaCMY-2 gene and one copy of the IncY plasmid encoding one copy of the blaTEM-1B gene. The abundance of both genes was determined by qPCRs for investigating their transmission dynamics. Neither of the two genes was detected at a detection limit of 4.0 log₁₀ copies g⁻¹ of faeces from mice mono-infected with the S. Heidelberg recipient (data not shown). From mice co-infected with the donor and recipient, both genes were detected for only 1 day in the control group, as the donor bacteria passed transiently through the mouse gut (Fig. 4a). In antibiotic treatment groups, the dynamics of blaTEM-1B abundance agreed with E. coli O80:H26 shedding patterns (Figs 4b–d and 2b–d). The abundance of blaTEM-1B and E. coli O80:H26 are positively correlated (Pearson’s correlation coefficient r=0.83, P<0.001, Fig. S4a), suggesting clonal transmission of blaTEM-1B along with the donor. In comparison, the abundance of blaCMY-2 and E. coli O80:H26 are negligibly correlated (Pearson’s correlation coefficient r=0.19, P>0.001, Fig. S4b). The dynamics of blaCMY-2 and blaTEM-1B were similar in the Amp group (Fig. 4b). In comparison, blaCMY-2 abundance remained high while blaTEM-1B abundance decreased in the Str–Amp group after cessation of the ampicillin treatment (Fig. 4d). The ratio of blaCMY-2 to blaTEM-1B was significantly (P<0.05) higher from 2 to 42 days p.i. in the Str–Amp group compared to the Amp group (Fig. 4e, f).
Fig. 2. Enumeration of *Escherichia coli* O80:H26, *Salmonella* Heidelberg and the *S. Heidelberg* transconjugant (mean±se) in faecal samples from mice that received both the recipient and donor inoculation and the treatment of (a) no antibiotic (Ctl), (b) ampicillin (Amp), (c) streptomycin (Str) or (d) streptomycin followed by ampicillin (Str–Amp); *n*=10, 11, 11 and 12 for (a–d), respectively, by 7 days p.i., and *n*=6 per treatment group thereafter. (e) Conjugation frequency (mean±se) at 1 day p.i. is expressed as the ratio of transconjugant to donor, and mean values labelled without common letters are of significant (*P*<0.05) difference based on Brown–Forsythe and Welch’s ANOVA tests. (f) Enumeration of *S. Heidelberg* (mean±se) in faecal samples from mice that only received the recipient inoculation and the treatment of Ctl, Amp, Str or Str–Amp; *n*=6 per treatment group.
Correlations between gut microbiota and transmission of $\beta$-lactam resistance genes

In order to determine correlations between gut microbiota and transmission of the $\beta$-lactam resistance genes, the taxonomic composition of gut microbial communities of mice co-infected with the donor and recipient bacteria was further analysed using 16S rRNA gene amplicon sequencing. In the control group, the composition of gut microbiota was

| Treatment | Transconjugant† (plasmid) | Isolation day | Accession representative‡ |
|-----------|---------------------------|---------------|---------------------------|
| Ctl       | ND                        | 7 days p.i.   | SAMN16634224              |
| Amp       | SH-(IncI2)                | 1, 2, 3 days p.i. | SAMN16634202 |
|           | SH-(IncI1, IncI2)         | 3, 7 days p.i. | SAMN16634220              |
| Str       | SH-(IncI2)                | 1, 2, 3 days p.i. | SAMN16634250 |
| Str–Amp   | SH-(IncI2)                | 7, 14, 21, 42 days p.i. | SAMN16634227 |
|           | EC-(IncI1, IncI2)         | 14, 42 days p.i. | SAMN16634233              |

*Mice received inoculation of the Salmonella Heidelberg recipient and then the Escherichia coli O80:H26 donor bacteria and treatment of no antibiotic (Ctl), ampicillin (Amp), streptomycin (Str) or streptomycin followed by ampicillin (Str–Amp).†nd, not detected; SH=Salmonella Heidelberg; EC=Escherichia coli O2:H6.‡BioSample accession number of the representative isolate, each includes chromosomal and plasmid components of a single isolate.

**Fig. 3.** Gene map of the Escherichia coli O2:H6 (SAMN16634233) genome in the 4650000–4750000 bp range showing the two copies of the $\text{bla}_{\text{CMY2}}$ (red) gene next to IS1380 family transposase ISEcpl (yellow). The following genes were identified in the vicinity of the $\text{bla}_{\text{CMY2}}$ gene: $\text{sugE}$, quaternary ammonium compound resistance protein; $\text{blc}$, outer-membrane lipoprotein; $\text{xerC}$, tyrosine recombinase; $\text{malE}$, maltose-binding periplasmic protein; $\text{malF}$, maltose transport system permease protein; $\text{malG}$, maltose transport system permease protein; $\text{psiE}$, phosphate starvation-inducible membrane protein.
Fig. 4. Quantitation of the *bla*<sub>CMY-2</sub>, *bla*<sub>TEM-1B</sub> and 16s rRNA genes (mean±se) in mouse faecal samples. Mice received inoculation of *Salmonella* Heidelberg and then *Escherichia coli* 080:H26 and treatment of (a) no antibiotic (Ctl), (b) ampicillin (Amp), (c) streptomycin (Str) or (d) streptomycin followed by ampicillin (Str–Amp); *n* = 6 per group. The mean ratio of the *bla*<sub>CMY-2</sub> to *bla*<sub>TEM-1B</sub> gene on each sampling day (e) or individual ratio of all samples (f) from each antibiotic treatment group. In (e), purple stars represent significant difference (**P** < 0.05, ***P** < 0.01, ****P** < 0.001) in the mean ratio between the Str–Amp and any other treatment groups and red stars between the Str–Amp and Amp groups. In (f), the stars represent significant difference between a treatment group and any other treatment groups based on Brown–Forsythe and Welch’s ANOVA tests.
relatively stable. The microbial community was dominated by **Firmicutes**, mainly the families **Ruminococcaceae** and **Lachnospiraceae**, and **Bacteroidetes**, mainly the families **Lactobacillaceae** and **Bacteroidaceae** (Fig. 5a, b). In the Amp group, the relative abundance of **Proteobacteria**, mainly the family **Enterobacteriaceae**, increased during the treatment from 0 to 7 days p.i., decreased after the cessation of ampicillin treatment and at 42 days p.i. returned to normal, a range that was not significantly (P>0.05) different from the control (Tables S3 and S4). In the Str group, the relative abundance of **Proteobacteria**,
mainly the family Enterobacteriaceae, increased on 0, 1 and 2 days p.i. and returned to normal at 7 days p.i. In the Str–Amp group, the relative abundance of Proteobacteria, mainly the family Enterobacteriaceae, increased from 0 to 7 days p.i. and returned to normal at 42 days p.i. (Fig. 5a, b). Expansion of Escherichia–Shigella and Salmonella relative abundance contributed to the increase of Enterobacteriaceae relative abundance (Fig. S5). The gut microbial diversity was reduced by the streptomycin pre-treatment and/or ampicillin treatment (Fig. S6). After treatment cessation, the gut microbiota gradually returned towards the original balance (Figs S6 and S7). The correlogram shows that the abundance of transconjugant and blaCMY-2 gene is positively correlated with the relative abundance of Proteobacteria and Enterobacteriaceae and negatively with Firmicutes, which are unlikely to act as recipients (Fig. 5c). The abundance of E. coli O80:H26 and blaTEM-1B gene are negatively correlated with the relative abundance of Firmicutes, Ruminococcaceae and Lachnospiraceae, and the abundance of S. Heidelberg is also negatively correlated with the relative abundance of Firmicutes.

Inflammation in the mouse gut

Cecum specimens were collected at 7 days p.i. from mice co-infected with the E. coli O80:H26 donor and S. Heidelberg recipient for histopathological analysis. Inflammation was observed in cecum tissue specimens from 100, 20 and 100% of mice in the Amp, Str and Str–Amp groups, respectively (Fig. 6a). No inflammation was found in the control mice. Fig. 6(b, c) shows the inflamed and normal cecum tissues. The inflammatory score of the Str–Amp group was significantly [H (3)=16.90, P<0.001] higher than that of the Str or control groups (Fig. 6a). These scores seemed to be positively associated with the relative abundance of Proteobacteria at 7 days p.i. Specifically, the mean inflammatory scores were 3.7, 2.6, 0.4 and 0, and the corresponding mean relative abundance of Proteobacteria was 0.8391, 0.4852, 0.0012 and 0.0002 for the Str–Amp, Amp, Str and control groups, respectively (Fig. 5a).

DISCUSSION

To explore the impact of antibiotics on ARG mobility in pre-disturbed gut microbiota, mice were subjected to streptomycin pre-treatment. The pre-treatment decreased the relative abundance of Firmicutes, more specifically Ruminococcaceae and Lachnospiraceae, the short-chain fatty acid producers. Thereby, the treatment would likely have lowered short-chain fatty acid concentrations, increased the luminal pH of the intestine and favoured the colonization of opportunistic pathogens [10, 14], such as the E. coli O80:H26 donor and the S. Heidelberg recipient. Colonization of the donor and recipient bacteria provided a base for bacterial cell–cell contact in the gut and facilitated conjugative transfer of the blaCMY-2 gene via the IncI2 plasmid in the Str group. In comparison, in the Str–Amp group, despite benefitting the E. coli O80:H26 donor in reaching high abundance, ampicillin might kill the actively growing β-lactam-susceptible S. Heidelberg at the initial stage of infection in the pre-disturbed gut microbiota. Thus, the limited abundance of recipient might lead to a lower conjugation frequency in the Str–Amp group compared to the Str group at 1 day p.i. In support of our findings, Lopatkin et al. [19] demonstrated with mathematical models and in vitro bacterial culture that antibiotics may reduce conjugation
frequency by reducing the sizes of either or both of the parental populations. Hall et al. [35] also reported that positive selection for plasmid-encoded traits reduced plasmid conjugation frequency in soil bacterial communities. Here, the abundance of S. Heidelberg transconjugants in the Str group increased from 1 to 3 days p.i. and then decreased at 7 days p.i., while that in the Str–Amp group increased from 1 to 7 days p.i. The different dynamics suggest that carrying the IncI2 plasmid to S. Heidelberg is a cost in the absence but a benefit in the presence of ampicillin selection pressure. In addition, the relative abundance of commensal Enterobacteriaceae expanded significantly during ampicillin treatment. The simultaneous blooming of the donor, transconjugant and commensal Enterobacteriaceae in the severely inflamed mouse gut possibly built a strong base for gene transfer among these bacteria. In support of our suggestion, Stecher et al. [17] reported that parallel blooms of S. Typhimurium and mouse commensal E. coli boosted conjugal transfer of a colicin plasmid p2 from S. Typhimurium to E. coli. In our study, the bla\textsubscript{CMY-2} gene was transferred via the IncI2 plasmid to a mouse commensal E. coli O2:H6 strain and incorporated into two locations of its chromosome (Fig. 3), likely through an ISEcp1-mediated transposition [36]. According to the study by Hall et al. [35], such physical movement and duplication of genes between plasmid and chromosome is a common way for bacteria to acquire antibiotic resistance. Furthermore, identical IncI1 plasmids carrying no ARGs were found in both the E. coli O2:H6 and S. Heidelberg transconjugants, suggesting possible transfer of the plasmid from E. coli O2:H6 or other commensal bacteria to S. Heidelberg. Supporting our findings on complex conjugation among bacteria in the Str–Amp group, Conlan et al. [37] reported the dissemination of the carbapenemase gene to multiple bacterial species in a patient during transplant-associated multi-course antibiotic therapies. In the present study, in pre-existing normal microbiota, ampicillin treatment facilitated the co-infection of and conjugation between the donor and recipient. However, the abundance of the donor, recipient and transconjugant were much lower in the Amp group compared to those in the Str–Amp group during the entire study, except that at 1 day p.i. S. Heidelberg abundance was slightly higher in the Amp group than the Str–Amp group. The ampicillin treatment alone without bacterial infection could disturb pre-existing normal gut microbiota and reduce Firmicutes relative abundance, as shown in our previous study [21]. However, compared to the Str–Amp group, the disturbance in the Amp group was smaller, and the less disturbed microbiota might provide greater colonization resistance to the introduced bacteria and limit their growth at lower abundance. Hence, more S. Heidelberg might become dormant at greater colonization resistance in the Amp than Str–Amp group on 1 day p.i., and survive the ampicillin treatment under the protection of β-lactamase producing donor [38, 39]. Yet, low abundance of the donor limited the conjugation frequency in the Amp group. In addition, the donor might depend on co-infection with the recipient in establishing colonization herein, as the donor alone failed to colonize the mouse gut under ampicillin treatment in our previous study [21]. Following cessation of ampicillin treatment or removal of selection pressure, the gut microbiota gradually recovered and diminished the introduced bacteria. The more disturbed gut microbiota likely favoured longer persistence of the introduced bacteria in the Str–Amp group compared to other groups. Moreover, under the Str–Amp treatment S. Heidelberg reached significantly higher abundance and persisted much longer in mouse faeces following co-infection with E. coli O80:H26 compared to S. Heidelberg mono-infection. The findings suggest that antibiotic-susceptible opportunistic pathogens may exploit conjugal transfer of ARGs to propagate and persist in an otherwise hostile environment. Overall, pre-disturbed gut microbiota might promote high-abundance colonization of resistant bacteria under positive antibiotic selection pressure and encourage bacterial conjugation and spread of ARGs.

The E. coli O80:H26 donor carries one copy of the bla\textsubscript{CMY-2} gene and one copy of the bla\textsubscript{TEM-1B} gene. The abundance dynamics of the bla\textsubscript{TEM-1B} gene and the donor was highly correlated, suggesting that the bla\textsubscript{TEM-1B} gene might be transmitted clonally along with the donor. Supporting this suggestion, transconjugant that carries the bla\textsubscript{TEM-1B} gene was not recovered in this and previous studies [21]. Using the ratio of bla\textsubscript{CMY-2} to bla\textsubscript{TEM-1B} as an indicator for dissemination of the bla\textsubscript{CMY-2} gene, the significantly higher ratio of the two genes suggested more efficient dissemination of the bla\textsubscript{CMY-2} gene in the Str–Amp group than in the Amp group during the entire study, except 1 day p.i. The high ratios might be attributed to the transfer of the bla\textsubscript{CMY-2} gene to S. Heidelberg and commensal E. coli, the incorporation of the bla\textsubscript{CMY-2} gene into the commensal E. coli chromosome and possible dissemination of the bla\textsubscript{CMY-2} gene in the gut microbiota. Furthermore, following cessation of the ampicillin treatment, the abundance of the bla\textsubscript{CMY-2} gene remained high, even though the abundance of the donor and transconjugant decreased significantly in the Str–Amp group, suggesting possible persistence of the bla\textsubscript{CMY-2} gene in the gut microbiota at no selective advantage.

CONCLUSION

In this study, pre-disturbed gut microbiota promoted conjugal transfer of the bla\textsubscript{CMY-2} gene from the E. coli O80:H26 donor to S. Heidelberg, commensal E. coli and possibly other commensal Enterobacteriaceae under positive ampicillin selection pressure. Following cessation of ampicillin treatment, shedding of the S. Heidelberg and E. coli transconjugants persisted over 35 days. These findings underline the importance of pre-existing gut microbiota on dissemination of ARGs during antibiotic treatments of bacterial infection.

Funding information

This work was funded by the Government of Canada’s Genomics R&D Initiative Phase VI Shared Priority Project Management Plan on Antimicrobial Resistance (GRDI-AMR).

Acknowledgements

Great appreciation is expressed to Dr R. Reid-Smith at the Public Health Agency of Canada, Guelph, Canada for providing the E. coli O80:H26 isolate; to Dr M. R. Mulvey at the Public Health Agency of Canada.
Author contributions
J.G., E.T. and J.N. designed the experiments. J.G. wrote the manuscript, and all co-authors edited and contributed to revisions. A.L., JD, M.K. and J.G. carried out the animal experiments and bacterial enumeration and analysed the bacterial culture data. H.D. and M.L. performed PCR for quantitation of resistance genes. M.S. performed histology analysis. J.C., M.K. and J.G. conducted the 16S rRNA gene amplicon sequencing analysis. S.G. and K.L. performed the whole-genome sequencing. K.B., J.R. and J.N. analysed the whole-genome sequencing data. All authors have read and agreed to the submitted version of the manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
Experiments and procedures involving mice conformed to guidelines and ethical standards of the Animal Care Committee at the Ottawa Laboratory-Fallowfield, Canadian Food Inspection Agency, Ottawa, Canada.

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