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Fate of CMY-2-encoding plasmids introduced into the human fecal microbiota by exogenous *Escherichia coli*

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Running Title: Fate of CMY-2 plasmids in the human fecal microbiota

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Abstract

The gut is a hot spot for transfer of antibiotic resistance genes from ingested exogenous bacteria to the indigenous microbiota. The objective of this study was to determine the fate of two nearly identical bla_{CMY-2}-harboring plasmids introduced into the human fecal microbiota by two *Escherichia coli* strains isolated from human and poultry meat, respectively. The chromosome and the CMY-2-encoding plasmid of both strains were labeled with distinct fluorescent markers (mCherry and GFP), allowing Fluorescence Activated Cell Sorting (FACS)-based tracking of the strain and the resident bacteria that have acquired its plasmid. Each strain was introduced into an established *in vitro* gut model (CoMiniGut) inoculated with individual feces from ten healthy volunteers. Fecal samples collected 2, 6 and 24 h after strain inoculation were analyzed by FACS and plate counts. Although the human strain survived better than the poultry meat strain, both strains transferred their plasmids to the fecal microbiota at concentrations as low as 10^2 CFU/mL. Strain survival and plasmid transfer varied significantly depending on inoculum concentration and individual fecal microbiota. Identification of transconjugants by 16S rRNA gene sequencing and MALDI-TOF mass spectrometry revealed that the plasmids were predominantly acquired by Enterobacteriaceae such as *E. coli* and *Hafnia alvei.* Our experimental data demonstrate that exogenous *E. coli* of human or animal origin can readily transfer CMY-2-encoding IncI1 plasmids to the human fecal microbiota. Low amounts of exogenous strain are sufficient to ensure plasmid transfer if the strain is able to survive the gastric environment.

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45 **Introduction**

46 The spread of β-lactamase-encoding plasmids conferring resistance to broad-spectrum
47 cephalosporins is of particular concern due to the clinical importance of these antibiotics
48 in human healthcare (1, 2). One of the β-lactamases most commonly reported in poultry
49 and other animal reservoirs is CMY-2 (3–5). Various studies suggest that CMY-2-encoding
50 plasmids of poultry origin may be transferred from animal to human bacteria via
51 consumption of contaminated poultry meat, as indicated by the detection of almost
52 identical plasmids in *E. coli* strains from humans, poultry and poultry meat (5–8). *E. coli* is
53 an integral part of the commensal gut microbiota in both animals and humans but also a
54 common cause of opportunistic infections. Thus, acquisition of exogenous CMY-2-
55 encoding plasmids introduced into the gut microbiota by bacteria from food and other
56 sources can potentially lead to *E. coli* infections that cannot be treated with broad-
57 spectrum cephalosporins.
58 The objective of this study was to determine the fate of two nearly identical CMY-2-
59 encoding plasmids introduced into the human fecal microbiota by exogenous *E. coli* of
60 human (C20-GM) and poultry meat (1061-1-GM) origin. Strain survival and plasmid
61 transfer were studied over a period of 24 h using an established *in vitro* gut model called
62 the CoMiniGut that was inoculated with individual feces from ten human volunteers. In
63 addition to standard phenotypic counts, donor and transconjugant cells were counted
64 and sorted by Fluorescence-Activated Cell Sorting (FACS), allowing evaluation of plasmid
65 host range and transfer dynamics in the non-culturable fraction of the fecal microbiota.
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Results

Preliminary experiments on strain inoculum

Five different concentrations of the human UTI strain C20-GM were tested in the *in vitro* gut model CoMiniGut to determine the strain inoculum to be used in the following experiments. These experiments were performed under oxic and anoxic conditions using three randomly selected fecal samples (A, E, O). FACS analysis after 24 hour showed persistence of the exogenous strain in most all three fecal samples (Fig. S1). Pearson correlation coefficients revealed a strong positive correlation between the inoculum concentration and numbers of C20-GM cells under anoxic conditions (p-value = 0.007, <0.0001 and 0.003 for sample A, E and O, respectively) (Fig 1a). Such correlation was also statistically significant for sample A (p-value = 0.008) but not for samples E and O under oxic conditions (Fig. 1b).

Transconjugants were detected in all tested conditions except in fecal samples A and E using either a very low (10 CFU/mL) or very high (10^8 CFU/mL) inoculum under oxic conditions (Fig. S2). Under anoxic conditions, the Pearson correlation coefficient indicated moderate negative correlation between inoculum concentration and numbers of transconjugants from all samples (Fig. 1a). This pattern was not observed under oxic conditions, where a negative correlation was only observed for sample (E) and was not statistically significant (Fig. 1b).

The lowest inoculum at which both donors and transconjugants were detected in all samples was 10^2 CFU/ml in both oxic and anoxic conditions. Based on these results, we chose to use this inoculum concentration in the following experiments.

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Experiments on strain survival and plasmid transfer

CoMiniGut cultures of 10 fecal samples from healthy volunteers were inoculated separately with the poultry meat strain (1061-1-GM) and the human strain (C20-GM), and incubated under anoxic conditions. Samples were collected at 2, 6 and 24 h after strain inoculation and analyzed in FACS to quantify donor (red fluorescence) and transconjugant (green fluorescence) cells.

After 2 h, both strains were detected in all samples (range 33-227 for 1061-1-GM and 45-231 for C20-GM), and transconjugants were detected in all but one sample for 1061-1-GM (range 0-68) and in all but two samples for C20-GM (range 0-28) (Fig. 2a).

After 6 h, donor numbers were significantly lower for 1061-1-GM (range 23-178) than for C20-GM (range 52-5572) (p-value = 0.005). Even though the numbers of transconjugants did not significantly differ between the two strains (range 1-242 for 1061-1-GM and 2-1772 for C20-GM), the transconjugant/donor ratio was significantly higher for 1061-1-GM (range 0.1-3.46) than for C20-GM (range 0.0005-0.45) (p-value = 0.03) (Fig. 2b). After 24 h, the numbers of 1061-1-GM (range 31-3744) were still significantly lower than for C20-GM (range 19-46310) (p-value = 0.03). At this time point, transconjugants were detected in all samples without significant differences between the two strains (range 8-846 and 1-661, respectively) but the transconjugant/donor ratio persisted to be significantly higher for 1061-1-GM (range 0.007-3.6) than for C20-GM (range 0.00002-1.9) (p-value= 0.009) (Fig. 2c). Altogether, the different survival dynamics displayed by the two strains in human feces did not affect their ability to transfer IncI1 CMY-2-encoding plasmids to the indigenous microbiota.

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Influence of the initial Enterobacteriaceae concentration on survival of exogenous *E. coli*

The correlation between numbers of the two exogenous *E. coli* strains measured by FACS at the three different time points were compared to the initial Enterobacteriaceae counts (Table S1) to determine if strain's survival was influenced by the concentration of indigenous Enterobacteriaceae in the recipient fecal sample. In general, there was a negative correlation between counts of pre-existing Enterobacteriaceae and survival of both exogenous strains, although such a negative correlation was statistically significant only after 24 h (*p*-values= 0.03 for 1061-1-GM and 0.04 for C20-GM) (Fig. 3a). Limited to strain 1061-1, the Pearson correlation coefficient showed a significant negative correlation between counts of pre-existing Enterobacteriaceae and numbers of transconjugants detected by FACS after 24 h (*p*-value= 0.04) (Fig. 3b).

Bacterial community composition of different fecal samples

Bacterial community composition was determined by 16S rRNA gene amplicon sequencing. This analysis was performed in the 10 fecal samples stocks as well as 24 h after the samples were inoculated with the exogenous strains in CoMiniGut, including all biological replicates (n = 30 per strain). The initial bacterial community composition varied between fecal samples with either Firmicutes or Bacteriodetes being the dominant phylum (Fig. 4a). The abundance of Proteobacteria increased in all samples during CoMiniGut culture, most likely due to the experimental conditions favoring fast-growing bacteria, but the magnitude of this increase varied markedly between samples (Fig. 4b).

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Abundance of relative amplicon sequence variants (ASV) found in sorted transconjugants was compared to abundance of those ASVs in the fecal samples and CoMiniGut samples. The most recovered ASVs in sorted transconjugants were not common in the initial fecal community and only moderately enriched after 24 h incubation in the CoMiniGut yet the plasmid was acquired predominantly by specific ASVs from Enterobacteriaceae (Fig. 5). Principal Coordinates Analysis (PCoA) of the unweighted UniFrac distance matrix based on ASV counts for all samples showed that the bacterial communities from the initial fecal sample, after 24 h CoMiniGut incubation and from sorted transconjugants formed tight clusters and that this grouping was significant (p-value<0.001) based on permutational multivariate analysis of variance of the UniFrac distance matrix (Fig. 6).

Bacterial recipients of plasmids

The diversity of transconjugants was investigated by 16S rRNA gene amplicon sequencing of the green cells isolated by FACS (gate P7). This was done on samples in which the transconjugant population was at least 0.1% of the 100,000 bacteria that were analyzed by FACS (D, E, H, M and O for 1061-1-GM, and A, D, E, I, M and O for C20-GM). The most abundant ASVs belonged to the Enterobacteriaceae with multiple sequence variants detected in all samples except for sample D after inoculation of both strains and sample O after inoculation of 1061-1-GM. The transconjugants detected in sample M after inoculation of C20-GM were more diverse compared to other samples and included Gram-negative Bacteroidaceae *Alloprevotella* and Gram-positive Lachnospiraceae and Ruminococcaeae (Fig. 6).

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Additionally, non-red cells (n=10^6) were sorted from all CoMiniGut cultures after 24 h and plated on blood agar plates supplemented with kanamycin and cefotaxime for isolation of presumptive transconjugants. Isolates where the presence of the donor plasmid was confirmed by PCR targeting the region upstream and downstream of the GFP cassette and by confocal microscopy for green fluorescence were identified to the species level by MALDI-TOF MS. Following inoculation of 1061-1-GM, transconjugants were isolated from samples D (n=24), E (n=15), H (n=8) and M (n=46), and all transconjugant isolates were identified as *E. coli*. After inoculation of C20-GM, transconjugants were detected in samples D (n=22), E (n=10), H (n=16), M (n=30) and O (n=12), and all transconjugant isolates were *E. coli* with the exception of transconjugants from sample O that were identified as *Hafnia alvei*, another member of the Enterobacteriaceae family.

Discussion

We investigated horizontal gene transfer of the GFPmut3-tagged IncI1/pST12 CMY-2-encoding plasmids from exogenous *E. coli* of human and poultry origin to the fecal bacterial communities from 10 human donors. GFPmut3-expressing transconjugant cells were isolated by FACS, allowing transconjugant detection and identification in spite of their relative low abundance in the CoMiniGut model. This model was used to simulate the colon environment and mimic the effect of ingesting exogenous CMY-2-producing *E. coli* from contaminated food or by the fecal-oral route. Our results indicate that CMY-2-encoding IncI1 plasmids can readily transfer to the indigenous fecal microbiota at inoculum concentrations as low as 100 CFU (Fig. S1 and S2). This finding highlights the

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177 possibility that low numbers of exogenous strains are sufficient to transfer *bla*$_{CMY-2}$ to the
178 resident gut microbiota, provided that the strains are able to survive the gastric
179 environment of the stomach and reach the colon.
180 Gut colonization by exogenous strains is not a prerequisite for plasmid transfer as
181 indicated by the early detection of transconjugants shortly (2 h) after strain inoculation in
182 eight of the ten fecal samples tested. Accordingly, even a brief transit of exogenous *E. coli*
183 through the colon may lead to acquisition of CMY-2-encoding IncI1 plasmids by the
184 indigenous microbiota. This is important from a public health point of view because once
185 the plasmid has transferred to a resident recipient; the resulting transconjugant can itself
186 act as donor.
187 The numbers of strain detected in FACS after 6 h and 24 h in the fecal microbiota from
188 human volunteers indicated that the human strain survived better compared to the strain
189 isolated from poultry meat (Fig. 2 and Fig. S3). Based on multilocus sequence typing
190 (MLST), both strains belonged to sequence types (ST155 and ST10 for the human and
191 poultry strain, respectively) frequently detected among *E. coli* from food, animals and
192 humans worldwide (9, 10). Even though the general composition of the fecal microbiota is
193 similar between humans and other vertebrates, the poultry fecal microbiota significantly
194 differs from human fecal microbiota (11). Perhaps the strain from human UTI was more
195 adapted to survive within human fecal microbiota than the poultry meat strain. However,
196 this observation cannot be generalized since only single strains of human and poultry
197 origin were tested.

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198 The numbers of transconjugant detected from poultry meat strain were higher than from
199 human UTI strain. The transconjugant/donor ratio was also higher for the poultry meat
200 strain because of the high numbers of transconjugants and lower numbers of donors than
201 the human strain for all samples except one (D) (Fig 2 and Fig. S3). The plasmid transfer
202 efficiency was likely similar for both strains in human fecal microbiota. Indeed, in the *in*
203 *vitro* experiments with lab strain both strains had the transfer efficiency 10^{-5}
204 transconjugant per donor cell (12). The plasmid transfer thus was not dependent on the
205 concentration of the exogenous strain but on transconjugant survival and secondary
206 transfer. Relatively high conjugation frequencies in the range of 10^{-2}-10^{-6}
207 transconjugants/recipients have been previously reported for IncI1 plasmids (13), which
208 are highly prevalent in Enterobacteriaceae (14).
209 Several studies have documented *in vivo* plasmid transfer from a donor of animal origin to
210 a human recipient strain (15–18) but in all these experiments the *in vivo* models were fed
211 with high numbers of donor and recipient strains (10^7-10^9 CFU). Such high numbers of *E.*
212 *coli* are unlikely to be ingested via food in real life. A previous study conducted in Belgium
213 reported 7% and 3% likelihood of humans being exposed to 10 CFU or 100 CFU ESBL-
214 producing *E. coli* from poultry meat, respectively (19). Evers et al. (2016) showed only a
215 6.9% chance that humans can be exposed to 1 CFU of bacteria through consumption of
216 poultry meat (20). Thus, the inoculum size of 10^2CFU/mL (500 CFU) used in our study is
217 more realistic considering the information on human exposure to this type of bacteria,
218 and the expected reduction of the initial inoculum present on poultry meat due to

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219 washing and/or cooking, as well as to the low pH in the stomach (pH=2), which acts as a
220 natural barrier to ingested microbes.
221 The FACS-sorted transconjugants were predominantly identified as members of
222 Enterobacteriaceae, which is consistent with the narrow host range of IncI1 plasmids (14)
223 (Fig. 6). Various anaerobic phyla seemed to acquire the IncI1 plasmid from the human UTI
224 strain in sample M. However, these presumptive anaerobic transconjugants were not
225 verified by cultivation as the agar plates were only incubated under aerobic conditions.
226 The presence of IncI1 plasmids in species other than Enterobacteriaceae has not been
227 shown before but most of the previous studies relied on culture-based detection of
228 transconjugants and did not investigate the fate of these plasmids in complex bacterial
229 communities such as those residing in human feces.
230 Correlation coefficients between the initial Enterobacteriaceae population and donor
231 survival along with plasmid transfer indicated a moderate negative correlation for both
232 1061-1-GM and C20-GM (Fig. 4). *E. coli* are less efficient at establishing themselves in
233 microbiomes when there are higher numbers of Enterobacteriaceae already present in
234 the population possibly because they compete for the same ecological niche.
235 As the experimental setup was limited to 24 h, it is impossible to determine whether the
236 magnitude of plasmid transfer and the number of bacterial taxa involved would have
237 increased if the experiment was continued for a longer period. It should be noted that our
238 experimental setup cannot differentiate between primary transconjugants that have
239 obtained the plasmid from the exogenous donor strain and those that have acquired the
240 plasmid from primary transconjugants acting as donors. In addition, our approach cannot

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distinguish between horizontal and vertical transfer since the transconjugants detected in our experiment may well represent the offspring of transconjugants transmitting the acquired plasmid vertically. Consequently, the observed variations in numbers of transconjugants do not necessary directly reflect the plasmid transfer frequencies, which are generally estimated within one or two bacterial generation times. Another limitation of the study is the antibiotic concentration used for the culture-based detection of transconjugants, which was selected based on breakpoints specific for Enterobacteriaceae. Thus, transconjugants belonging to other bacterial families could fail to grow at these antibiotic concentrations because resistance genes are usually poorly expressed in distantly related heterologous hosts (21).

ASV sequences identified in transconjugants, belonging predominantly to Enterobacteriaceae were present at very low abundance in the initial fecal sample. Such Enterobacteriaceae populations increased after 24 h incubation in the CoMiniGut, but the transfer of plasmids primarily to Enterobacteriaceae also points towards the narrow host range of IncI1 plasmids (Fig. 5). The enrichment of the Proteobacteria in CoMiniGut cultures was mainly due to the experimental conditions (24 h culture) because they are among the fastest growing bacteria.

We conclude that the foodborne and fecal-oral transmission is a possible route for transfer of antibiotic resistance IncI1 CMY-2-encoding plasmids carried by exogenous *E. coli*, provided that the host strain survives cooking and stomach pH, even if in small numbers. To further assess this risk, *in vivo* quantitative studies are needed to evaluate

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the effect of the stomach environment on concentrations of *E. coli* strains transiting through the gut.

Methods

Strains and media

The two genetically modified human and poultry meat *E. coli* strains used in this study (C20-GM and 1061-GM, respectively) were constructed and validated previously (12). The strains are typed as ST155 (C20-GM) and ST10 (1061-GM) by MLST and harbor bla_{CMY-2} on IncI1 plasmids of sequence type (pST) 12 sharing 99% nucleotide identity over 97% of the length of the plasmid (European Nucleotide Archives accession number PRJEB9625) (5). The strains were genetically modified by inserting a mCherry fluorescent marker (red) in the pseudogene *ybe*M on the chromosome and GFP fluorescent marker (green) in a non-coding region on the IncI1 plasmid (12).

Media used were Luria Bertani broth (LB-B), Luria Bertani agar (LB-A), MacConkey agar, 5% blood agar (Oxoid Ltd.,Roskilde, Denmark), and complex colon media were prepared according to Macfarlane et al. (1998) (22). Antibiotics were used at the following concentrations throughout the work unless mentioned otherwise: 1 mg/L of cefotaxime and 50 mg/L of kanamycin.

Phosphate buffer saline (PBS) 1 M, pH = 7 was prepared as follows (g/L): NaCl, 8; KCl, 0.2; $Na_2HPO_4 \cdot 2H_2O$, 1.44; KH_2PO_4, 0.24 in distilled H_2O. PBS 0.1 M pH = 5.6 was prepared from the PBS 1 M stock. NaCl 0.9% solution (g/L) in distilled H_20. All chemicals were obtained

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from the company Sigma-Aldrich (Søborg, Denmark) unless otherwise stated. All solutions were autoclaved before using.

Fecal samples

Fecal samples were collected from 10 healthy human volunteers not exposed to antibiotics during the last six months. Their ages ranged from 5 to 68 years. Ethical permission for collection of these samples was waived by the Danish National committee on health research ethics. The samples were delivered to the laboratory immediately after collection and kept at -20 °C until processing. All samples were processed within 24 h after they were received. Feces were weighed and equal amount w/v of 20% glycerol/0.1 M PBS solution was added prior to homogenization in a stomacher for 2 x 60 sec. The resulting fecal suspensions were labeled and frozen in cryotubes at -80 °C. Immediately before the start of the experiment, each suspension was thawed and diluted 1:5 with 0.1 M PBS at pH 5.6 (working stock).

CoMiniGut experiments

CoMiniGut is an *in vitro* system that simulates the colon passage of the human gut (23). The CoMiniGut has five vessels running in parallel. Each vessel, which has a total of 5 ml volume comprising of media, fecal sample and donor strain, was inoculated with 10% v/v of fecal sample in the complex colon medium. During 24 h the pH increased from 5.7 to 6.0 in the first 8 h to simulate the proximal colon. Then in the following 8 h it increased to 6.5 to represent transverse colon and finally it reached 6.9 in the last 8 h to simulate

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distal colon environment. Preliminary experiments were performed using the human strain C20-GM to determine the strain inoculum concentration. These experiments were performed in oxic and anoxic conditions. Briefly, three fecal samples (A, E, O) were randomly selected and challenged with C20-GM to reach five different concentrations of C20-GM (10^8, 10^6, 10^4, 10^2 and 10 CFU/mL) in each CoMiniGut vessel. The lowest inoculum concentration for which the donor was detected 24 h after inoculation was selected for the final experiment, where all the 10 fecal samples were independently challenged with C20-GM and 1061-1-GM under anoxic conditions. The experimental design was set up to mimic the colon environment of human gut. A volume of 300 µl was collected from each vessel 2, 6 and 24 h after strain inoculation in the CoMiniGut. All experiments were run in two biological replicates. The average value from biological replicates was used for further analysis.

Cell collection and multiple-gated FACS of transconjugants

All samples from the CoMiniGut experiments were analyzed by flow cytometer FACSAria IIIu (Becton Dickinson Biosciences, San Jose, CA, USA). Samples from anoxic cultures were diluted 100-fold in 1M PBS pH 7 and exposed to oxygen by shaking at 110 rpm at 4 °C for up to 3 h. This allowed the fluorescent proteins to mature properly before FACS analysis (24). The settings used were the same as described by Anjum et al., (2018) (12). All samples were diluted in 0.9% NaCl to ~2000 counting events s^1 before FACS to assure for optimal detection of donors and sorting of transconjugants. Control laboratory strains expressing only mCherry or GFP or without any fluorescent marker were used to design

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gates for analysis with FACS. Six gates were defined in bivariate plots to sort for detection of donors and sorting for transconjugants. On the side scatter-A vs forward scatter-A plot, a gate for only particles of bacterial size was selected. On the PE-Texas Red-A vs side scatter-A plot a gate was set that covered all red fluorescent particles and on the duplicate plot the same gate was set to detect and sort non-red fluorescent particles. On the FITC-A vs side scatter-A plot, a gate was set that covered all green fluorescent particles. As the particles from fecal sample and media auto-fluoresced, thus interfering with the gates selected for detection of mCherry and GFPmut3, a more stringent gate (P7) was selected for sorting as follows. On the FITC-A vs side scatter-A plot a gate was set up based on GFP expressing control lab strain in complex colon media that covered transconjugants with highest GFP expression. An additional non-red gate on the PE-Texas Red-A vs FITC-A plot, ensured exclusion of small auto-fluorescent particles from fecal sample, media or leaking donors to sort out only transconjugants. This may have resulted in loss of sorting of some transconjugants that did not have a high GFP expression but ensured that the cells sorted were indeed the correct transconjugants. The threshold for detection was set at 100,000 counting events thereby the numbers of donors and transconjugants from FACS analysis are given out of 10^5 cells analyzed in FACS.

For each sample sorted, a minimum of 15,000 and a maximum of 30,000 transconjugants were sorted. The cut off for sorting of transconjugants was set so that it was performed only for the samples from one time point, in which the numbers of transconjugants detected were at least 0.1% of the whole population in gate P7. Sorted cells were

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collected in 5 mL sterile polystyrene round-bottom Falcon tubes with 0.5 mL of 0.9% NaCl solution.

Sorting was also performed for isolation of 10^6 cells that were not red for all samples from both biological replicates. This fraction was plated on blood agar plates containing cefotaxime and kanamycin and incubated in anoxic conditions at 37 °C overnight. All colonies were observed with confocal microscopy to detect green and red fluorescence. The green colonies were subjected to PCR targeting the region on plasmid where the GFPmut3 cassette was inserted using primers Fwd pC20/1061-1 confirm and Rev pC20/1061-1 confirm (12). All PCR-positive colonies were identified to species level by matrix-assisted laser desorption/ionization time-of-flight mass spectrophotometry (MALDI-TOF MS) (BioMérieux, France).

Sequence-based analysis of fecal microbiota

Microbial community profiling was performed on fecal samples prior to CoMiniGut experiments, after 24 hour CoMiniGut incubation and on the FACS-sorted transconjugants (from gate P7) by 16S rRNA marker gene amplicon sequencing.

DNA from the original fecal sample and from the 24-hour CoMiniGut culture was extracted by DNeasy Power Soil kit (Qiagen, Denmark) according to manufacturer's instructions. DNA was used for amplicon high throughput sequencing of the 16S rRNA gene using a MiSeq benchtop sequencer (Illumina, CA, USA). Amplicon libraries were obtained after a PCR reaction targeting the hypervariable V3 region of the 16S rRNA gene.

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Amplicon libraries for transconjugants analysis were performed by PCR of the cell pellets using the GenePurgeDirect (Nimagen) direct PCR kit. Sorted cells were transferred to 1.5 mL Eppendorf tubes and centrifuged at 10 000 g for 30 min to collect cell pellets. The supernatant was carefully removed, cell pellet suspended in 20 µl of GenePurgeDirect lysis matrix. The cell lysis mixture slurries were then transferred to 0.2 mL amplification tubes. Cell lysis was performed in the thermal cycler using manufacturer's instructions. PCR reactions were performed with 5µL of lysis mixture using primers targeting bacterial and archaeal 16S rRNA gene V3 region with overhanging adapters compatible with the Nextera Index Kit (Illumina): rNXt_388_F:5'-**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**ACWCCTACGGGWGGCAGCAG -3' and NXt_518_R: 5'-**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**ATTACCGCGGCTGCTGG -3' (adapters in bold) the PCR reactions and library preparations were conducted as described previously (25). All individual sample libraries were then pooled in equimolar proportion and sequenced using MiSeq v2 sequencing kit producing 2x250 bp paired-end reads on an Illumina MiSeq benchtop sequencer following manufacturer's guidelines. Amplicon sequences were analyzed using phyloseq R package (26) and used the following additional R packages: vegan, ggplot2 (27, 28). Unweighted UniFrac distances where computed using phyloseq implementation of Fast Unifrac (29). Raw amplicon reads were denoised and clustered in ASV using DADA2 (30) implementation in QIIME2. Each unique sequence is classified against SILVA NR99 rel. 132 SSU database (31) using q2-feature-classifier naïve Bayes classifier (32) at the lowest taxonomical rank up to the Genus level with a confidence threshold of 0.7. Each ASV sequence present above a cumulated

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abundance of 0.05% of reads in transconjugants samples were further identified manually using manual BLAST searches (Table S2).

Culture-based analysis of fecal samples

For the culture-based analysis of species diversity within Enterobacteriaceae, 100 µl from the working stock solution of each fecal sample was spread MacConkey agar plates with or without cefotaxime. At least one colony per morphology observed on MAC agar plate was analyzed by MALDI-TOF.

Statistical methods

The Pearson correlation coefficient was calculated using Microsoft Excel software to assess the relationship of inoculum concentrations with number of donors and transconjugants and transconjugants/donors ratio. The cut off for negative correlation was set at r = > -0.25 and for positive correlation r = < 0.25.

The Gardner-Altman two-group mean-difference plots were drawn using web application: http://www.estimationstats.com/#/, which is based on data analysis using Bootstrap-coupled ESTimation (DABEST) (33). Statistical significance was set at $p < 0.05$. The reference group in all analysis was assigned to the 1061-1-GM strain and the experimental group was C20-GM strain.

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417

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419

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424

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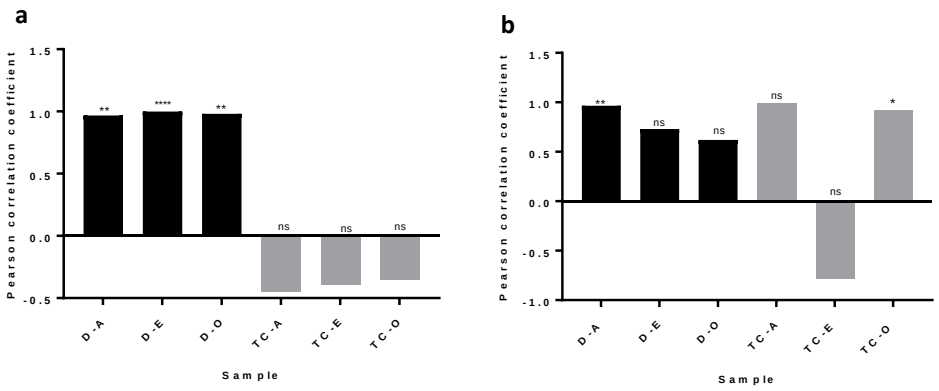

535

536 **Figure 1:** Pearson correlation coefficient (y-axis) between the inoculum concentration and

537 the number of donor cells (D) (black bars), transconjugants (TC) (grey bars) in fecal

538 samples A, E, and O under a) anoxic (An) and b) oxic (O) conditions. ns = non-significant; *

539 = $p < 0.05$; **= $p < 0.005$ and **** = $p < 0.0001$.

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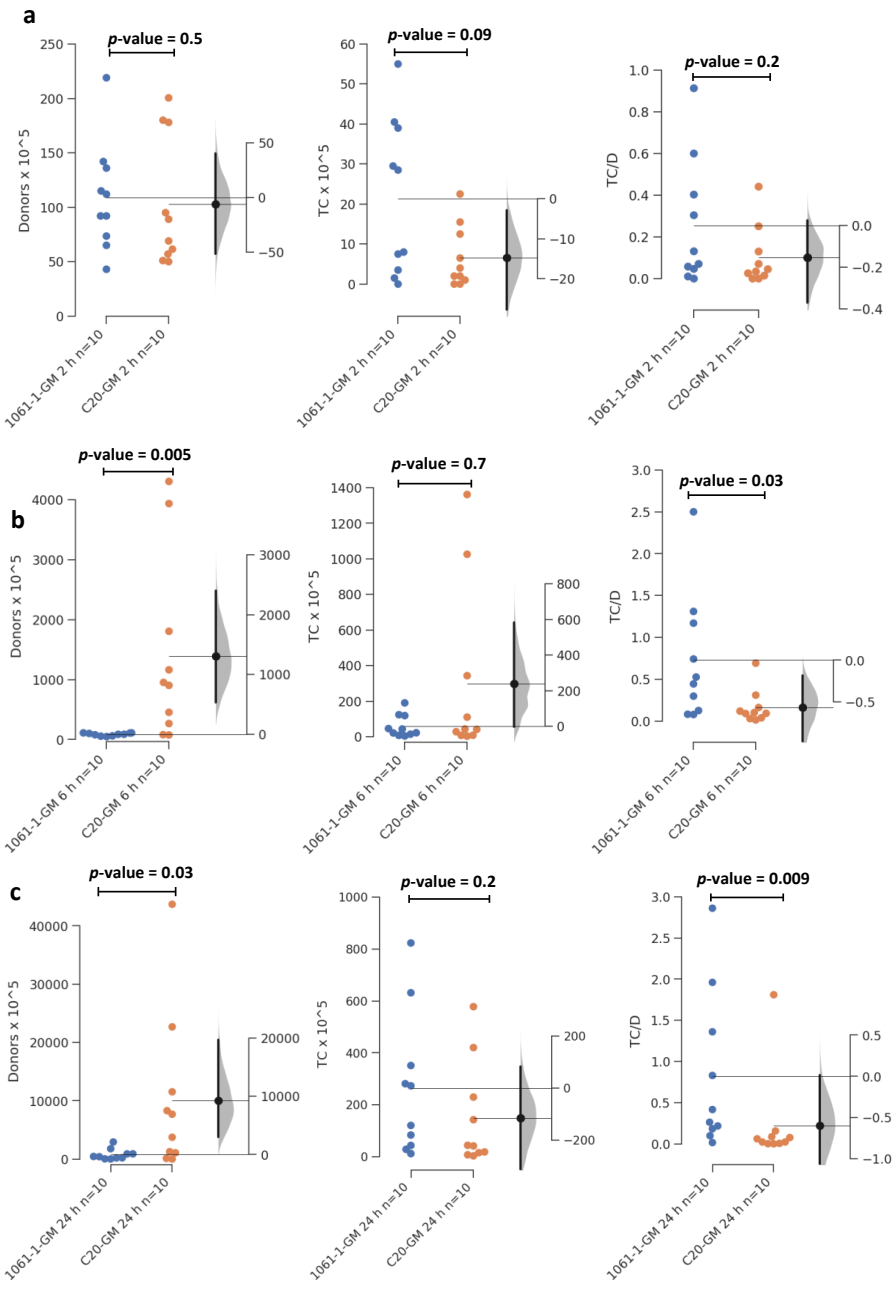

Figure 2: Gardner-Altman two-groups mean-difference plot showing the difference between donors (D) transconjugants (TC) and transconjugants/donors (TC/D) ratios at (a)

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546 2 h, (b) 6 h and (c) 24 h for the poultry meat strain 1061-1-GM (blue) and the human
547 strain C20-GM (orange) in CoMiniGut cultures. The left axis shows the number of donors
548 detected by FACS. On the right axis the filled curve indicates the complete Δ distribution,
549 given the observed data. The human strain C20-GM survives better than poultry strain
550 1061-1-GM however more transconjugants are detected from poultry meat strain than
551 human UTI strain. The low and high bias corrected and accelerated bootstrap interval
552 values are shown as a density plot on the right side. The confidence interval of the mean
553 differences at 95% is illustrated by the thick black line. Significance was determined by
554 Mann-Whitney U test.
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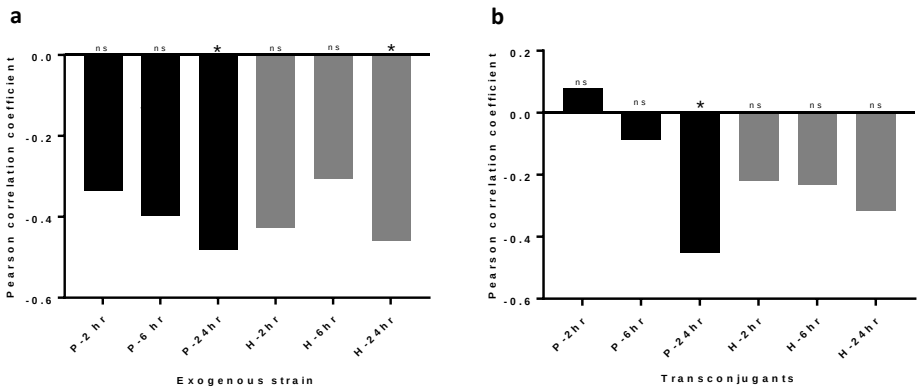

Figure 3: Pearson correlation coefficient (y-axis) indicating the relationship between initial Enterobacteriaceae counts in the fecal samples and a) numbers of the exogenous strain poultry strain (black bars) and human strain (grey bars), b) transconjugants that acquired their plasmids over time. After 24 h, the numbers of the two exogenous strains negatively correlated with the counts of pre-existing Enterobacteriaceae in the original fecal sample (a). A significant negative correlation was also seen between counts of pre-existing Enterobacteriaceae and the numbers of transconjugants that received the plasmid from poultry strain after 24 h. ns = non-significant; * = $p < 0.05$

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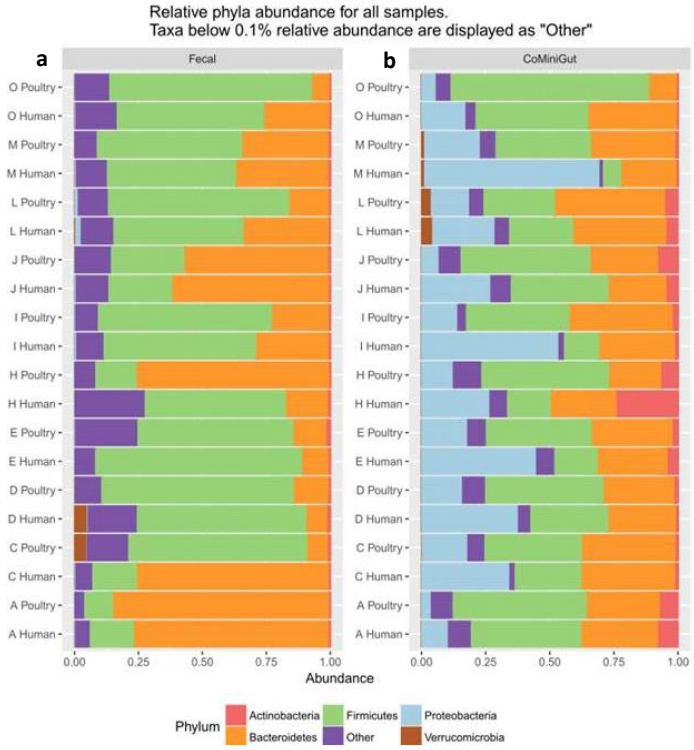

Figure 4: Relative abundance at phylum level in 10 fecal samples (A to O) before (a) and 24 h after inoculation of the two exogenous strains of human and poultry origin in the corresponding CoMiniGut culture (b). The figure shows that the abundance of Proteobacteria increased after inoculation of the exogenous strains, even though with marked differences between individual fecal samples.

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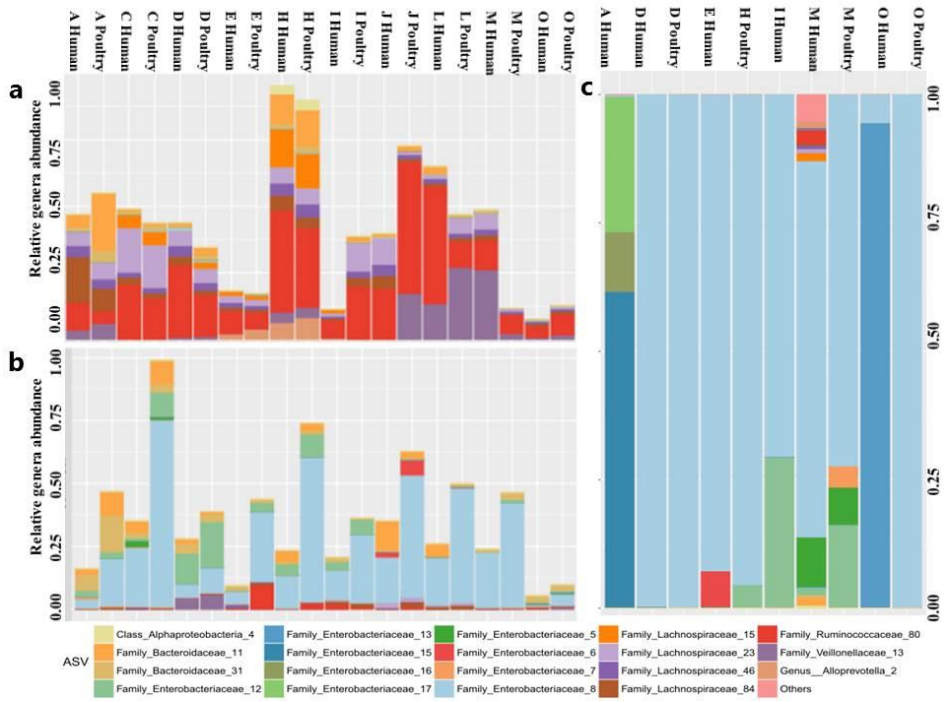

Figure 5: Relative ASV abundance as a function of fecal donor and strain source only ASVs detected in the sorted transconjugants from CoMiniGut culture are shown. ASV < 0.05% relative abundance are grouped in "other". a) Fecal sample b) CoMiniGut samples c) Sorted transconjugants. Annotation in figure legend shows the lowest taxonomic rank (Family/Class/Genus) that could be confidently attributed to each amplicon sequence variants using Bayesian classification.

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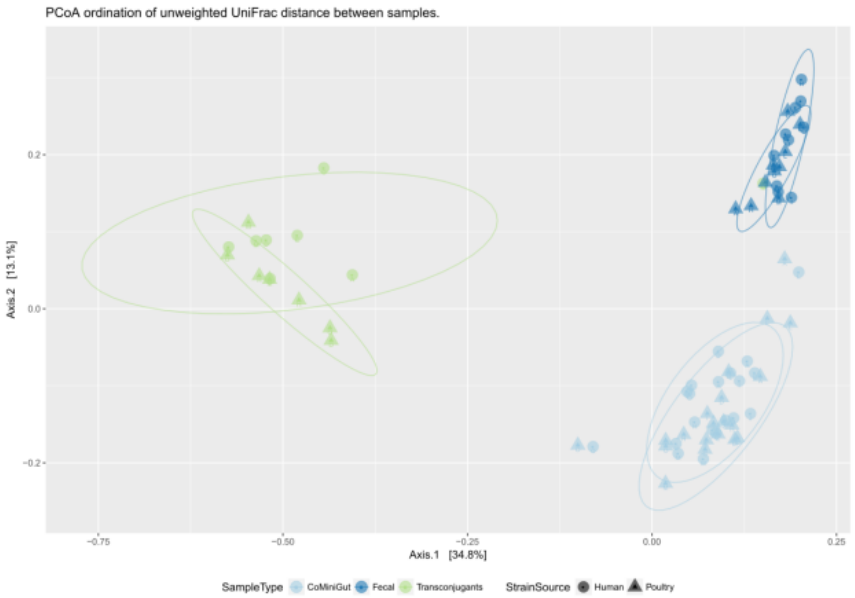

Figure 6: Unweighted UniFrac-based Principal Coordinates Analysis (PCoA) showing the clustering of bacterial communities according to the sample type and strain source. The strains source is human donor assay (circles) or poultry donor assay (triangles). The sample types are CoMiniGut cultures after 24 h (light blue), fecal samples before inoculation (dark blue) and sorted transconjugants from both assays (green). Each dot represents a sample.

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