



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

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

3

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23

24 **Abstract**

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

45 **Introduction**

46 The spread of  $\beta$ -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  $\beta$ -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.

66

67 **Results**

68 **Preliminary experiments on strain inoculum**

69 Five different concentrations of the human UTI strain C20-GM were tested in the *in vitro*  
70 gut model CoMiniGut to determine the strain inoculum to be used in the following  
71 experiments. These experiments were performed under oxic and anoxic conditions using  
72 three randomly selected fecal samples (A, E, O). FACS analysis after 24 hour showed  
73 persistence of the exogenous strain in most all three fecal samples (Fig. S1). Pearson  
74 correlation coefficients revealed a strong positive correlation between the inoculum  
75 concentration and numbers of C20-GM cells under anoxic conditions ( $p$ -value = 0.007,  
76 <0.0001 and 0.003 for sample A, E and O, respectively) (Fig 1a). Such correlation was also  
77 statistically significant for sample A ( $p$ -value = 0.008) but not for samples E and O under  
78 oxic conditions (Fig. 1b).

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

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

89 **Experiments on strain survival and plasmid transfer**

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

95 After 2 h, both strains were detected in all samples (range 33-227 for 1061-1-GM and 45-  
96 231 for C20-GM), and transconjugants were detected in all but one sample for 1061-1-GM  
97 (range 0-68) and in all but two samples for C20-GM (range 0-28) (Fig. 2a).

98 After 6 h, donor numbers were significantly lower for 1061-1-GM (range 23-178) than for  
99 C20-GM (range 52-5572) ( $p$ -value = 0.005). Even though the numbers of transconjugants  
100 did not significantly differ between the two strains (range 1-242 for 1061-1-GM and 2-  
101 1772 for C20-GM), the transconjugant/donor ratio was significantly higher for 1061-1-GM  
102 (range 0.1-3.46) than for C20-GM (range 0.0005-0.45) ( $p$ -value = 0.03) (Fig. 2b). After 24  
103 h, the numbers of 1061-1-GM (range 31-3744) were still significantly lower than for C20-  
104 GM (range 19-46310) ( $p$ -value = 0.03). At this time point, transconjugants were detected  
105 in all samples without significant differences between the two strains (range 8-846 and 1-  
106 661, respectively) but the transconjugant/donor ratio persisted to be significantly higher  
107 for 1061-1-GM (range 0.007-3.6) than for C20-GM (range 0.00002-1.9) ( $p$ -value= 0.009)  
108 (Fig. 2c). Altogether, the different survival dynamics displayed by the two strains in  
109 human feces did not affect their ability to transfer IncI1 CMY-2-encoding plasmids to the  
110 indigenous microbiota.

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

112 ***coli***

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

123

124 **Bacterial community composition of different fecal samples**

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

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

143

#### 144 **Bacterial recipients of plasmids**

145 The diversity of transconjugants was investigated by 16S rRNA gene amplicon sequencing  
146 of the green cells isolated by FACS (gate P7). This was done on samples in which the  
147 transconjugant population was at least 0.1% of the 100,000 bacteria that were analyzed  
148 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  
149 abundant ASVs belonged to the Enterobacteriaceae with multiple sequence variants  
150 detected in all samples except for sample D after inoculation of both strains and sample O  
151 after inoculation of 1061-1-GM. The transconjugants detected in sample M after  
152 inoculation of C20-GM were more diverse compared to other samples and included  
153 Gram-negative Bacteroidaceae *Alloprevotella* and Gram-positive Lachnospiraceae and  
154 Ruminococcaeae (Fig. 6).

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

166

## 167 Discussion

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

177 possibility that low numbers of exogenous strains are sufficient to transfer *bla*<sub>CMY-2</sub> 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.

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  $10^2$ CFU/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

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

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

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

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

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

264

## 265 **Methods**

### 266 **Strains and media**

267 The two genetically modified human and poultry meat *E. coli* strains used in this study  
268 (C20-GM and 1061-GM, respectively) were constructed and validated previously (12). The  
269 strains are typed as ST155 (C20-GM) and ST10 (1061-GM) by MLST and harbor *bla*<sub>CMY-2</sub> on  
270 IncI1 plasmids of sequence type (pST) 12 sharing 99% nucleotide identity over 97% of the  
271 length of the plasmid (European Nucleotide Archives accession number PRJEB9625) (5).

272 The strains were genetically modified by inserting a mCherry fluorescent marker (red) in  
273 the pseudogene *ybeM* on the chromosome and GFP fluorescent marker (green) in a non-  
274 coding region on the IncI1 plasmid (12).

275 Media used were Luria Bertani broth (LB-B), Luria Bertani agar (LB-A), MacConkey agar,  
276 5% blood agar (Oxoid Ltd., Roskilde, Denmark), and complex colon media were prepared  
277 according to Macfarlane et al. (1998) (22). Antibiotics were used at the following  
278 concentrations throughout the work unless mentioned otherwise: 1 mg/L of cefotaxime  
279 and 50 mg/L of kanamycin.

280 Phosphate buffer saline (PBS) 1 M, pH = 7 was prepared as follows (g/L): NaCl, 8; KCl, 0.2;  
281 Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 1.44; KH<sub>2</sub>PO<sub>4</sub>, 0.24 in distilled H<sub>2</sub>O. PBS 0.1 M pH = 5.6 was prepared from  
282 the PBS 1 M stock. NaCl 0.9% solution (g/L) in distilled H<sub>2</sub>O. All chemicals were obtained

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

285

#### 286 **Fecal samples**

287 Fecal samples were collected from 10 healthy human volunteers not exposed to  
288 antibiotics during the last six months. Their ages ranged from 5 to 68 years. Ethical  
289 permission for collection of these samples was waived by the Danish National committee  
290 on health research ethics. The samples were delivered to the laboratory immediately  
291 after collection and kept at -20 °C until processing. All samples were processed within 24  
292 h after they were received. Feces were weighed and equal amount w/v of 20%  
293 glycerol/0.1 M PBS solution was added prior to homogenization in a stomacher for 2 x 60  
294 sec. The resulting fecal suspensions were labeled and frozen in cryotubes at -80 °C.  
295 Immediately before the start of the experiment, each suspension was thawed and diluted  
296 1:5 with 0.1 M PBS at pH 5.6 (working stock).

297

#### 298 **CoMiniGut experiments**

299 CoMiniGut is an *in vitro* system that simulates the colon passage of the human gut (23).  
300 The CoMiniGut has five vessels running in parallel. Each vessel, which has a total of 5 ml  
301 volume comprising of media, fecal sample and donor strain, was inoculated with 10% v/v  
302 of fecal sample in the complex colon medium. During 24 h the pH increased from 5.7 to  
303 6.0 in the first 8 h to simulate the proximal colon. Then in the following 8 h it increased to  
304 6.5 to represent transverse colon and finally it reached 6.9 in the last 8 h to simulate

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

317

### 318 **Cell collection and multiple-gated FACS of transconjugants**

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

327 gates for analysis with FACS. Six gates were defined in bivariate plots to sort for detection  
328 of donors and sorting for transconjugants. On the side scatter-A vs forward scatter-A plot,  
329 a gate for only particles of bacterial size was selected. On the PE-Texas Red-A vs side  
330 scatter-A plot a gate was set that covered all red fluorescent particles and on the  
331 duplicate plot the same gate was set to detect and sort non-red fluorescent particles. On  
332 the FITC-A vs side scatter-A plot, a gate was set that covered all green fluorescent  
333 particles. As the particles from fecal sample and media auto-fluoresced, thus interfering  
334 with the gates selected for detection of mCherry and GFPmut3, a more stringent gate (P7)  
335 was selected for sorting as follows. On the FITC-A vs side scatter-A plot a gate was set up  
336 based on GFP expressing control lab strain in complex colon media that covered  
337 transconjugants with highest GFP expression. An additional non-red gate on the PE-Texas  
338 Red-A vs FITC-A plot, ensured exclusion of small auto-fluorescent particles from fecal  
339 sample, media or leaking donors to sort out only transconjugants. This may have resulted  
340 in loss of sorting of some transconjugants that did not have a high GFP expression but  
341 ensured that the cells sorted were indeed the correct transconjugants. The threshold for  
342 detection was set at 100,000 counting events thereby the numbers of donors and  
343 transconjugants from FACS analysis are given out of  $10^5$  cells analyzed in FACS.  
344 For each sample sorted, a minimum of 15,000 and a maximum of 30,000 transconjugants  
345 were sorted. The cut off for sorting of transconjugants was set so that it was performed  
346 only for the samples from one time point, in which the numbers of transconjugants  
347 detected were at least 0.1% of the whole population in gate P7. Sorted cells were

348 collected in 5 mL sterile polystyrene round-bottom Falcon tubes with 0.5 mL of 0.9% NaCl  
349 solution.  
350 Sorting was also performed for isolation of  $10^6$  cells that were not red for all samples from  
351 both biological replicates. This fraction was plated on blood agar plates containing  
352 cefotaxime and kanamycin and incubated in anoxic conditions at 37 °C overnight. All  
353 colonies were observed with confocal microscopy to detect green and red fluorescence.  
354 The green colonies were subjected to PCR targeting the region on plasmid where the  
355 GFPmut3 cassette was inserted using primers Fwd pC20/1061-1 confirm and Rev  
356 pC20/1061-1 confirm (12). All PCR-positive colonies were identified to species level by  
357 matrix-assisted laser desorption/ionization time-of-flight mass spectrophotometry  
358 (MALDI-TOF MS) (BioMérieux, France).

359

#### 360 **Sequence-based analysis of fecal microbiota**

361 Microbial community profiling was performed on fecal samples prior to CoMiniGut  
362 experiments, after 24 hour CoMiniGut incubation and on the FACS-sorted transconjugants  
363 (from gate P7) by 16S rRNA marker gene amplicon sequencing.  
364 DNA from the original fecal sample and from the 24-hour CoMiniGut culture was  
365 extracted by DNeasy Power Soil kit (Qiagen, Denmark) according to manufacturer's  
366 instructions. DNA was used for amplicon high throughput sequencing of the 16S rRNA  
367 gene using a MiSeq benchtop sequencer (Illumina, CA, USA). Amplicon libraries were  
368 obtained after a PCR reaction targeting the hypervariable V3 region of the 16S rRNA gene.

369 Amplicon libraries for transconjugants analysis were performed by PCR of the cell pellets  
370 using the GenePurgeDirect (Nimagen) direct PCR kit. Sorted cells were transferred to 1.5  
371 mL Eppendorf tubes and centrifuged at 10 000 g for 30 min to collect cell pellets. The  
372 supernatant was carefully removed, cell pellet suspended in 20  $\mu$ l of GenePurgeDirect  
373 lysis matrix. The cell lysis mixture slurries were then transferred to 0.2 mL amplification  
374 tubes. Cell lysis was performed in the thermal cycler using manufacturer's instructions.  
375 PCR reactions were performed with 5 $\mu$ L of lysis mixture using primers targeting bacterial  
376 and archaeal 16S rRNA gene V3 region with overhanging adapters compatible with the  
377 Nextera Index Kit (Illumina): rNXt\_388\_F:5'-  
378 **TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**ACWCCTACGGGWWGCAGCAG -3' and  
379 NXt\_518\_R: 5'-**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**ATTACCGCGGCTGCTGG -  
380 3' (adapters in bold) the PCR reactions and library preparations were conducted as  
381 described previously (25). All individual sample libraries were then pooled in equimolar  
382 proportion and sequenced using MiSeq v2 sequencing kit producing 2x250 bp paired-end  
383 reads on an Illumina MiSeq benchtop sequencer following manufacturer's guidelines.  
384 Amplicon sequences were analyzed using phyloseq R package (26) and used the following  
385 additional R packages: vegan, ggplot2 (27, 28). Unweighted UniFrac distances were  
386 computed using phyloseq implementation of Fast Unifrac (29). Raw amplicon reads were  
387 denoised and clustered in ASV using DADA2 (30) implementation in QIIME2. Each unique  
388 sequence is classified against SILVA NR99 rel. 132 SSU database (31) using q2-feature-  
389 classifier naïve Bayes classifier (32) at the lowest taxonomical rank up to the Genus level  
390 with a confidence threshold of 0.7. Each ASV sequence present above a cumulated

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

393

#### 394 **Culture-based analysis of fecal samples**

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

399

#### 400 **Statistical methods**

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

405 The Gardner-Altman two-group mean-difference plots were drawn using web application:

406 <http://www.estimationstats.com/#/>, which is based on data analysis using Bootstrap-

407 coupled ESTimation (DABEST) (33). Statistical significance was set at  $p < 0.05$ . The

408 reference group in all analysis was assigned to the 1061-1-GM strain and the

409 experimental group was C20-GM strain.

410

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417

418 **Conflict of Interest:** The authors declare that they have no conflict of interest.

419

420 **Ethical Approval:** This article does not contain any studies with human participants or  
421 animals performed by any of the authors. Ethical permission for collection of fecal  
422 samples from human volunteers was waived by the Danish National committee on health  
423 research ethics.

424

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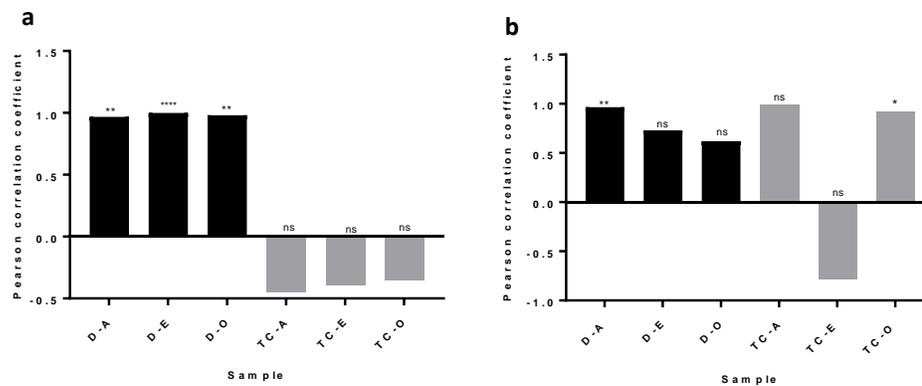
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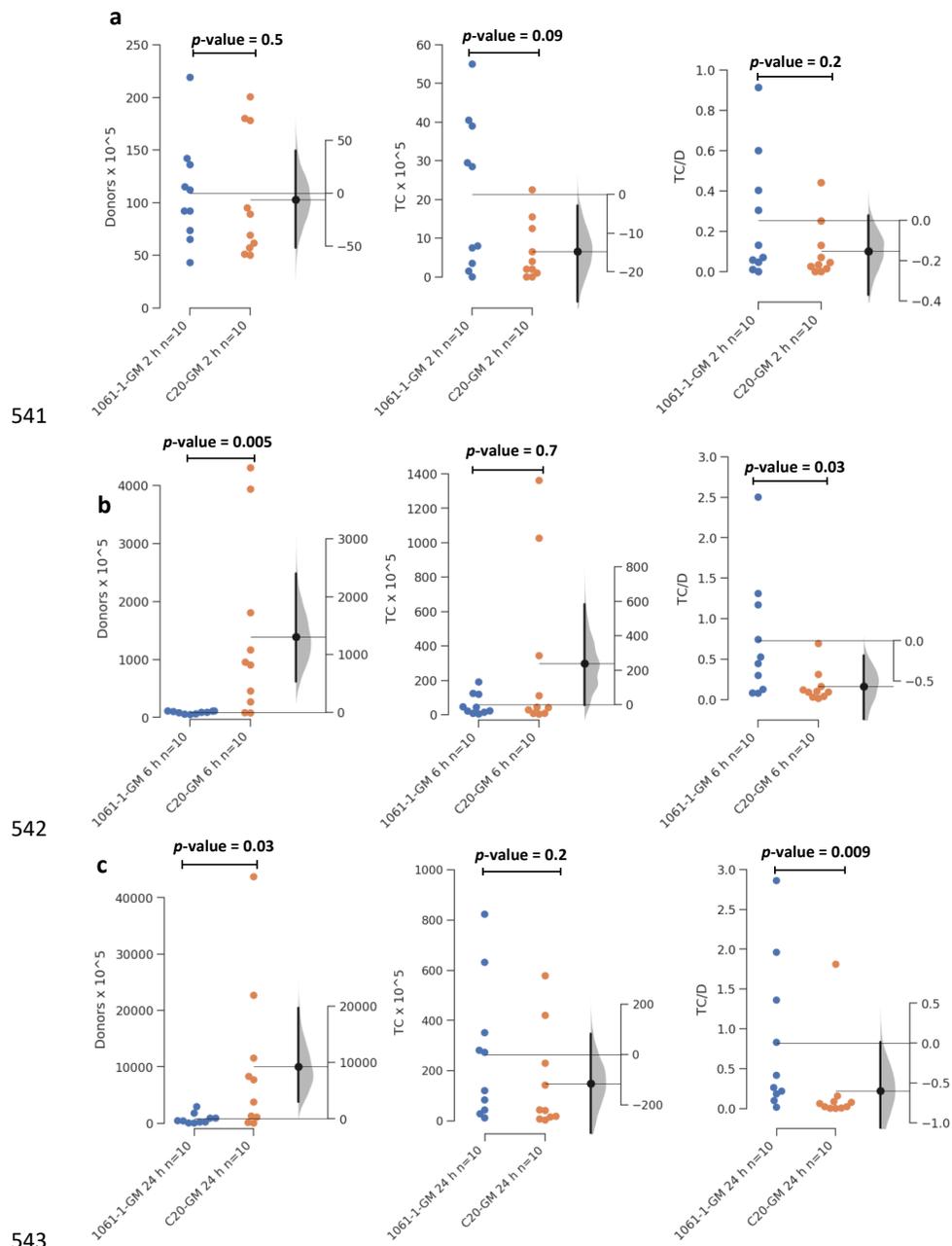
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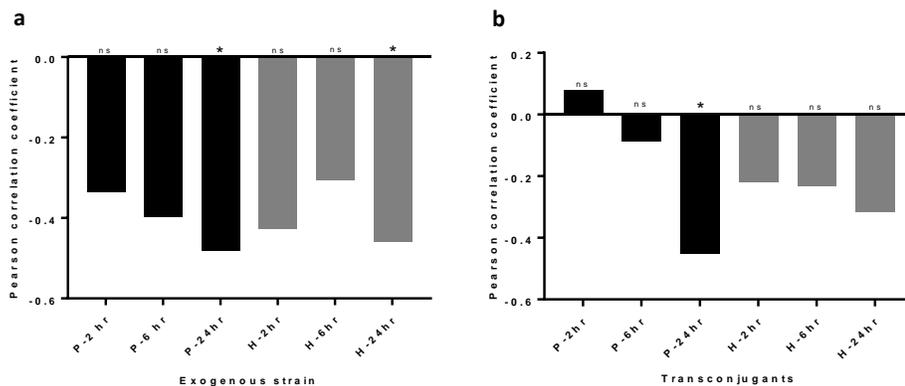
536 **Figure 1:** Pearson correlation coefficient (y-axis) between the inoculum concentration and  
 537 the number of donor cells (D) (black bars), transconjugants (TC) (grey bars) in fecal  
 538 samples A, E, and O under a) anoxic (An) and b) oxic (O) conditions. ns = non-significant; \*  
 539 =  $p < 0.05$ ; \*\* =  $p < 0.005$  and \*\*\*\* =  $p < 0.0001$ .

540



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

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  $\Delta$  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.  
555



556

557 **Figure 3:** Pearson correlation coefficient (y-axis) indicating the relationship between initial

558 Enterobacteriaceae counts in the fecal samples and a) numbers of the exogenous strain

559 poultry strain (black bars) and human strain (grey bars), b) transconjugants that acquired

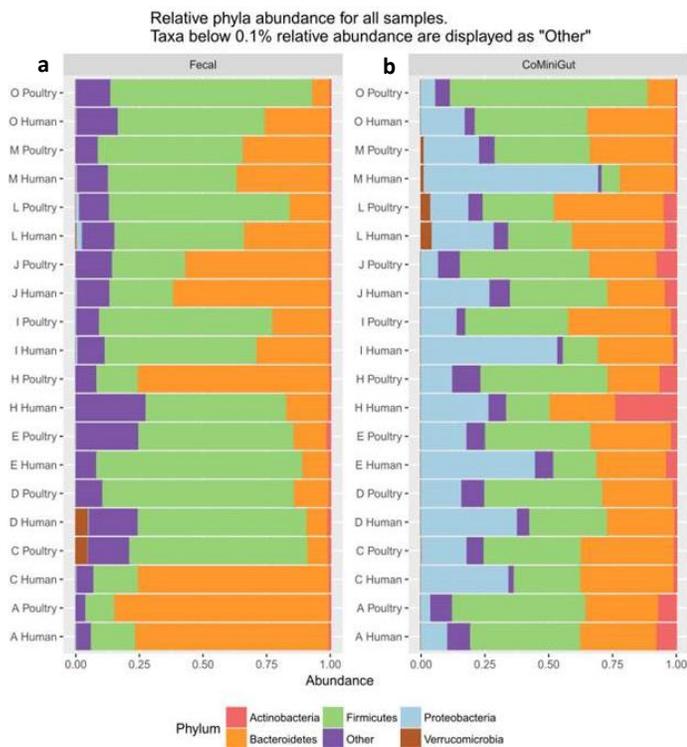
560 their plasmids over time. After 24 h, the numbers of the two exogenous strains negatively

561 correlated with the counts of pre-existing Enterobacteriaceae in the original fecal sample

562 (a). A significant negative correlation was also seen between counts of pre-existing

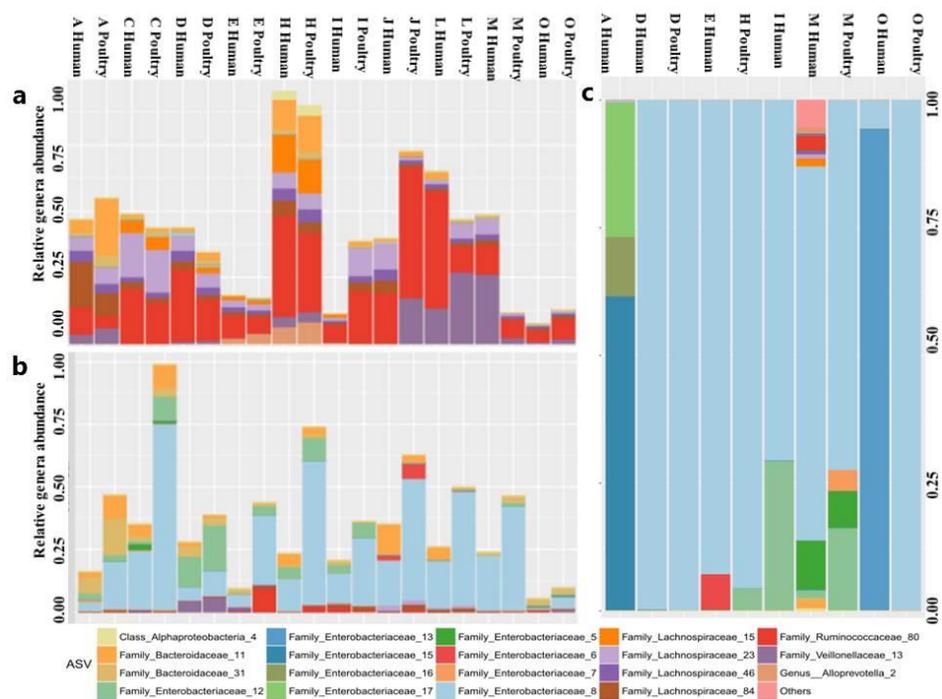
563 Enterobacteriaceae and the numbers of transconjugants that received the plasmid from

564 poultry strain after 24 h. ns = non-significant; \* =  $p < 0.05$



565

566 **Figure 4:** Relative abundance at phylum level in 10 fecal samples (A to O) before (a) and  
 567 24 h after inoculation of the two exogenous strains of human and poultry origin in the  
 568 corresponding CoMiniGut culture (b). The figure shows that the abundance of  
 569 Proteobacteria increased after inoculation of the exogenous strains, even though with  
 570 marked differences between individual fecal samples.



571

572 **Figure 5:** Relative ASV abundance as a function of fecal donor and strain source only ASVs

573 detected in the sorted transconjugants from CoMiniGut culture are shown. ASV &lt; 0.05%

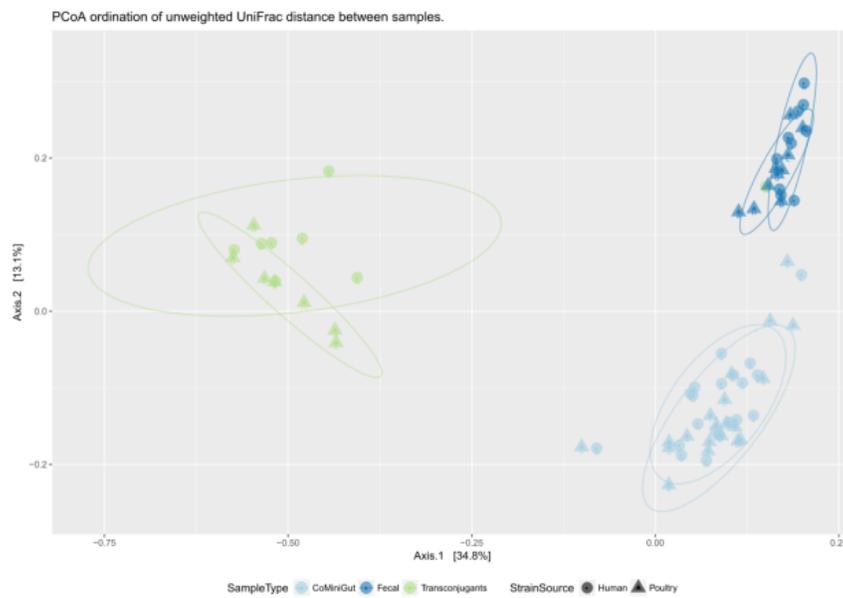
574 relative abundance are grouped in "other". a) Fecal sample b) CoMiniGut samples c)

575 Sorted transconjugants. Annotation in figure legend shows the lowest taxonomic rank

576 (Family/Class/Genus) that could be confidently attributed to each amplicon sequence

577 variants using Bayesian classification.

578



579

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