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

2   **exogenous *Escherichia coli***

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

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23

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.

Introduction

The spread of β-lactamase-encoding plasmids conferring resistance to broad-spectrum

cephalosporins is of particular concern due to the clinical importance of these antibiotics

in human healthcare (1, 2). One of the β-lactamases most commonly reported in poultry

and other animal reservoirs is CMY-2 (3–5). Various studies suggest that CMY-2-encoding

plasmids of poultry origin may be transferred from animal to human bacteria via

consumption of contaminated poultry meat, as indicated by the detection of almost

identical plasmids in *E. coli* strains from humans, poultry and poultry meat (5–8). *E. coli* is

an integral part of the commensal gut microbiota in both animals and humans but also a

common cause of opportunistic infections. Thus, acquisition of exogenous CMY-2-

encoding plasmids introduced into the gut microbiota by bacteria from food and other

sources can potentially lead to *E. coli* infections that cannot be treated with broad-

spectrum cephalosporins.

The objective of this study was to determine the fate of two nearly identical CMY-2-

encoding plasmids introduced into the human fecal microbiota by exogenous *E. coli* of

human (C20-GM) and poultry meat (1061-1-GM) origin. Strain survival and plasmid

transfer were studied over a period of 24 h using an established *in vitro* gut model called

the CoMiniGut that was inoculated with individual feces from ten human volunteers. In

addition to standard phenotypic counts, donor and transconjugant cells were counted

and sorted by Fluorescence-Activated Cell Sorting (FACS), allowing evaluation of plasmid

host range and transfer dynamics in the non-culturable fraction of the fecal microbiota.

2

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.

3

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.

4

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 Bacteriodetes 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).

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

7

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.

8

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

9

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

10

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

11

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_{CMY-2} 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 *ybe*M 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    $Na_2HPO_4 \cdot 2H_2O$, 1.44; KH_2PO_4, 0.24 in distilled H_2O. PBS 0.1 M pH = 5.6 was prepared from

282   the PBS 1 M stock. NaCl 0.9% solution (g/L) in distilled H_2O. 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

13

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 µl 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   IIIu (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 ~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

15

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 µl 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 5µL 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**ACWCCTACGGGWGGCAGCAG -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 where

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

17

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 μl 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

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

425 **References**

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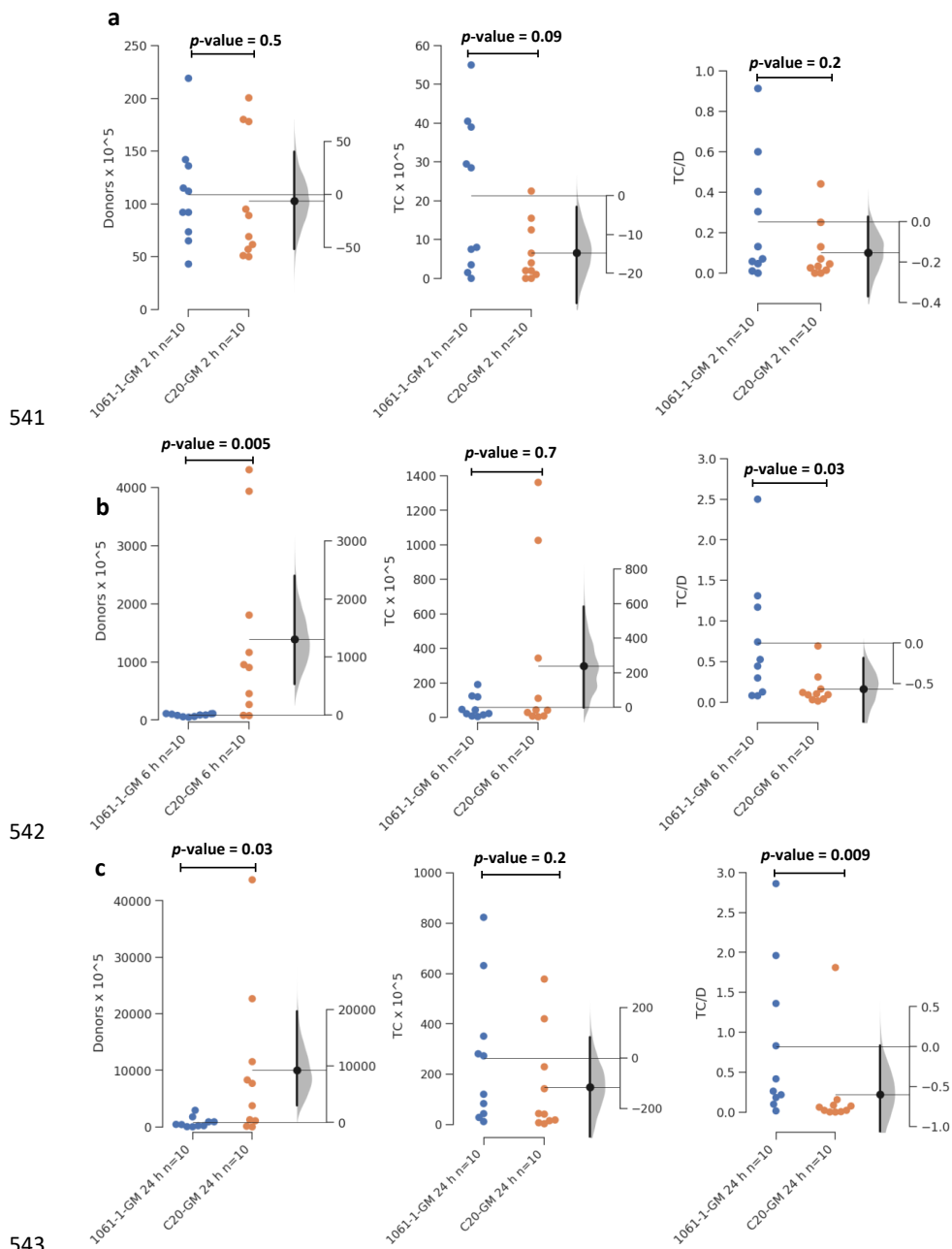
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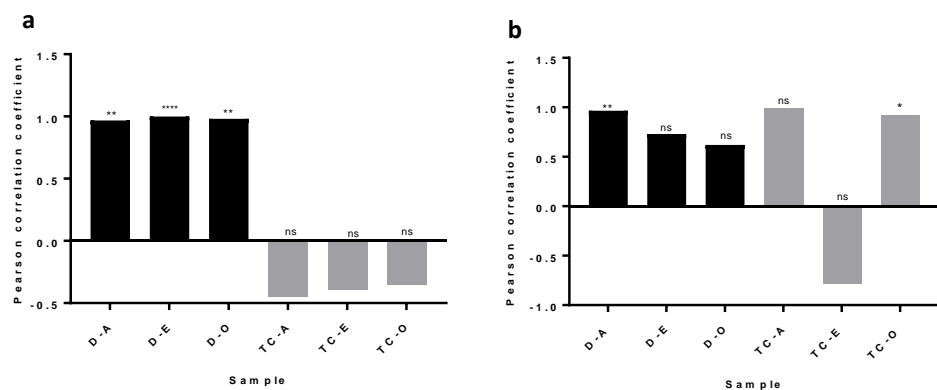
Figure 1: Pearson correlation coefficient (y-axis) between the inoculum concentration and the number of donor cells (D) (black bars), transconjugants (TC) (grey bars) in fecal samples A, E, and O under a) anoxic (An) and b) oxic (O) conditions. ns = non-significant; * = $p < 0.05$; **= $p < 0.005$ and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

540

**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)

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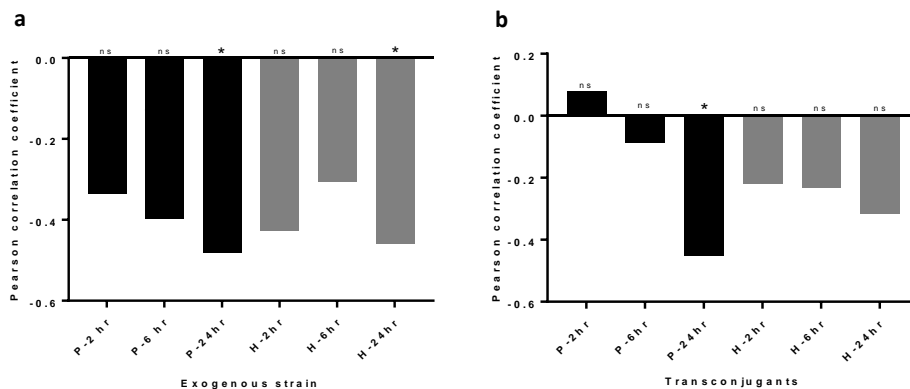
Figure 3: Pearson correlation coefficient (y-axis) indicating the relationship between initial

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; * = $p < 0.05$
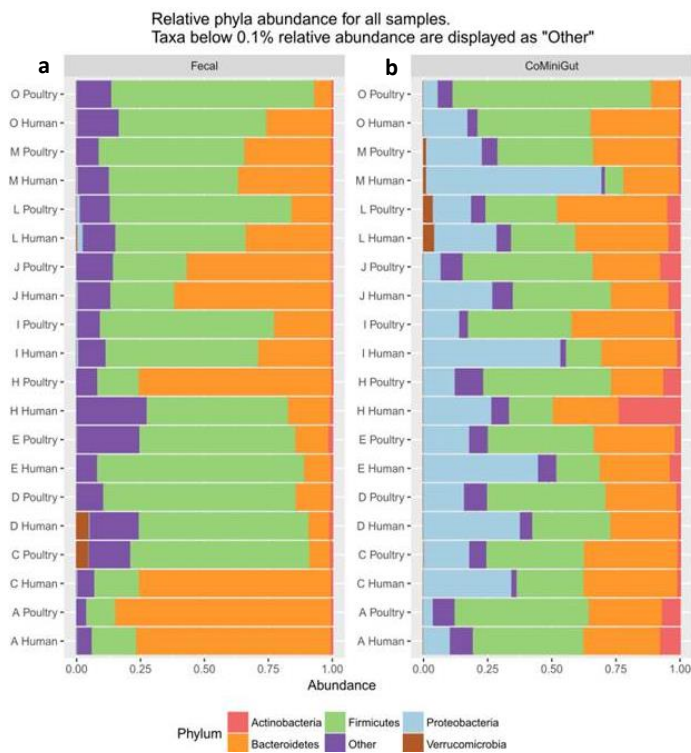
Figure 4: Relative abundance at phylum level in 10 fecal samples (A to O) before (a) and

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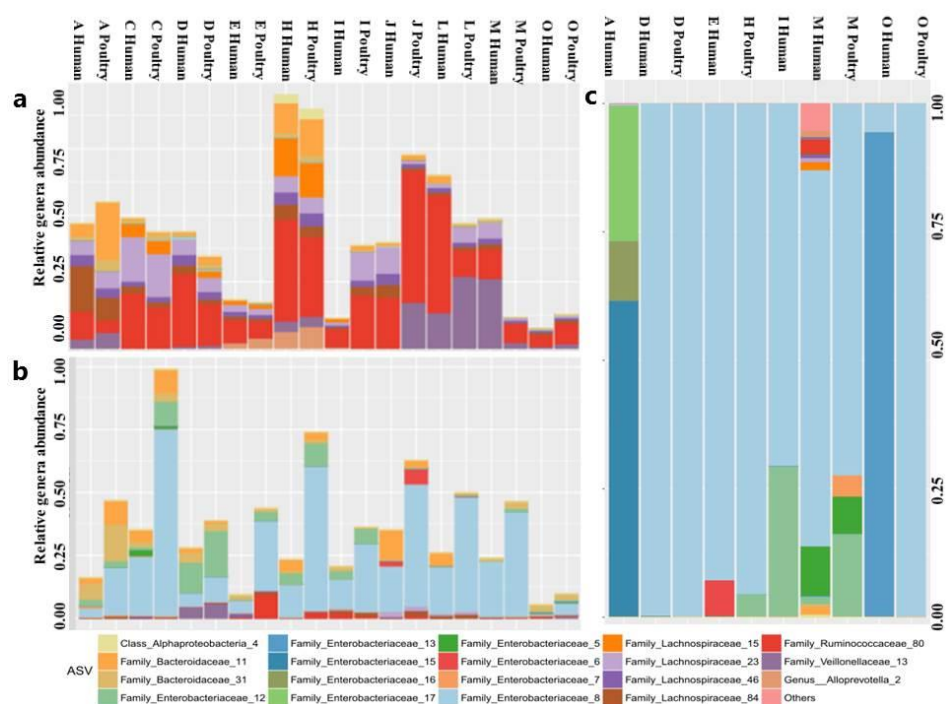
Figure 5: Relative ASV abundance as a function of fecal donor and strain source only ASVs

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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PCoA ordination of unweighted UniFrac distance between samples.

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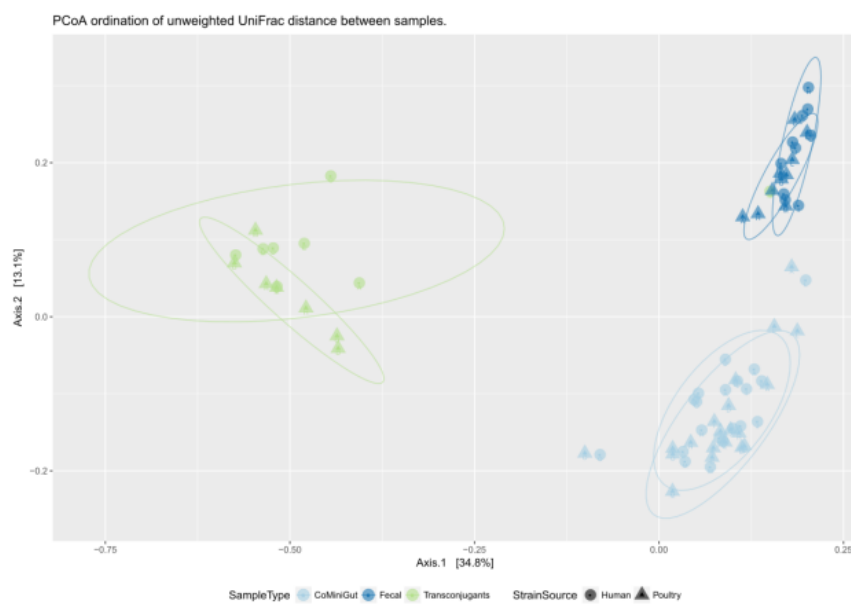
Figure 6: Unweighted UniFrac-based Principal Coordinates Analysis (PCoA) showing the

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.