Litter commensal bacteria can limit the horizontal gene transfer of antimicrobial resistance to *Salmonella* in chickens

Running Title: Commensal bacteria reduce HGT

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Abstract

Fostering a 'balanced' gut microbiome through the administration of beneficial microbes that can competitively exclude pathogens has gained a lot of attention and use in human and animal medicine. However, little is known about how microbes affect the horizontal gene transfer of antimicrobial resistance (AMR). To shed more light on this question, we challenged neonatal broiler chicks raised on reused broiler chicken litter – a complex environment made up of decomposing pine shavings, feces, uric acid, feathers, and feed, with *Salmonella* Heidelberg (*S*. Heidelberg), a model pathogen. Neonatal chicks challenged with *S*. Heidelberg and raised on reused litter were more resistant to *S*. Heidelberg cecal colonization than chicks grown on fresh litter. Furthermore, chicks grown on reused litter were at a lower risk of colonization with *S*. Heidelberg strains that encoded AMR on IncI1 plasmids. We used 16S rRNA gene sequencing and shotgun metagenomics to show that the major difference between chicks grown on fresh litter and reused litter was the microbiome harbored in the litter and ceca. The microbiome of reused litter samples was more uniform and enriched in functional pathways related to the biosynthesis of organic and antimicrobial molecules than fresh litter samples. We found that *E. coli* was the main reservoir of plasmids encoding AMR and that the IncI1 plasmid was maintained at a significantly lower copy per cell in reused litter compared to fresh litter. These findings support the notion that commensal bacteria play an integral role in the horizontal transfer of plasmids encoding AMR to pathogens like *Salmonella*.

Importance/Significance

Antimicrobial resistance spread is a worldwide health challenge, stemming in large part, from the ability of microorganisms to share their genetic material through horizontal gene transfer. To address this issue, many countries and international organization have adopted a One health approach to curtail the proliferation of antimicrobial resistant bacteria. This includes the removal and reduction of antibiotics used in food animal production and the development of alternatives to antibiotics. However, there is still a significant knowledge gap in our understanding of how resistance spreads in the absence of antibiotic selection and the role commensal bacteria play in reducing antibiotic resistance transfer. In this study, we show that commensal bacteria play a key role in reducing the horizontal gene transfer of antibiotic resistance to *Salmonella* and provide the identity of the bacterial species that potentially perform this function in broiler chickens and also postulate the mechanism involved.

Introduction

Horizontal gene transfer (HGT) is recognized as the main mechanism by which bacteria acquire antimicrobial resistance (AMR) and exposure to antibiotics has been shown to drive antimicrobial resistance gene (ARG) transfer [1]. Consequently, the rise in AMR in bacteria from hospital settings has been linked to the overuse of antibiotics in humans and their use in food animal production [2, 3]. These public health concerns have led to a reduction in antibiotics used for raising food animals [4, 5], including a ban on antibiotics' use in Europe [6]. We previously showed that neonatal broiler chicks challenged with a nalidixic acid resistant *S*. Heidelberg strain and raised antibiotic-free on fresh litter composed of pine shavings were colonized at a high rate with *S*. Heidelberg strains that harbored IncI1 plasmids encoding AMR [7]. We selected *S*. Heidelberg as the model pathogen because of its promiscuity to plasmids carrying AMR [8, 9].

There is limited research on if and how commensal bacteria reduce AMR in food-borne pathogens [10-16], such as *Salmonella enterica*, and the role it plays in AMR reduction and transfer. Therefore, the goal of this study was to determine the role commensal bacteria play in limiting *Salmonella enterica* from acquiring AMR and to provide information on the bacterial species and mechanism involved. To do this, we compared the dynamics of AMR transfer in neonatal broiler chicks raised on fresh litter to chicks raised on reused litter. We chose reused litter because it is a complex environment made up of decomposing plant-based bedding (e.g., wood shavings, sawdust and rice or peanut hulls) mixed with chicken feces, uric acid, feathers, feed,

insects, and other broiler-sourced materials. Therefore, reused litter carries a unique and complex population of bacteria, fungi, and viruses [17-19], interacting with various forms of eukaryotes, making it a suitable environment to study competitive exclusion. Broiler chickens are commonly raised on litter but how litter is managed differs between countries, producers, and farmers. For instance, the practice of reusing litter over multiple flocks of broiler chickens is a widespread practice in the US and Brazil while Canada [20] and Europe [21] recommend fresh litter bedding for every flock. One argument against litter reuse is that it harbors pathogenic bacteria that can be transferred to the next flock [22]. Contrastingly, proponents of litter reuse argue that it confers competitive exclusion against pathogens when effectively managed and it is cost-effective [23-26]. Beyond broiler chicken production, competitive exclusion by commensal bacteria has received enormous attention in the 20th century, resulting in the bloom of commercially marketed probiotics [27] and the underlying theory behind the application of fecal microbiota transplantation in human medicine [28].

Results

S. Heidelberg abundance and prevalence in ceca and litter.

We confirmed that neonatal chicks were *Salmonella*-free by testing the chick pads used for transportation from the hatchery for *Salmonella*. None of the chick pads were positive for *Salmonella.* The reused litter for this study was confirmed to be *Salmonella*-free in earlier studies [29, 30]. To determine if the microbiome offers protection against *Salmonella* colonization, we challenged neonatal broiler chicks with a nalidixic resistant (nal^R) strain of *S*. Heidelberg and raised the chicks on either fresh pine shavings (here after referred to as fresh litter) (n = 75) or reused litter (litter previously used to raise

116 three flocks of broiler chickens) (n =75). Chicks were challenged either through oral
117 gavage (n = 25) , cloacal inoculation (n =25), or by the seeder method (i.e., a few chicks
118 [n = 5] were challenged orally and comingled with unchallenged chicks [n = 20]) [7].
119 Unchallenged chicks on fresh (n =25) and reused litter (n =25) were used as controls.
120 Broiler chicks on fresh and reused litter were housed separately for 14 days (four
121 separate pens for each treatment in a house) and chicks were not administered any
122 medication or antibiotics for the duration of the study.

123 Afterwards, we determined the concentration of nal^R *S*. Heidelberg in the ceca and
124 litter of chicks 14 days after they were challenged. Average nal^R *S*. Heidelberg
125 concentration in the ceca (n=10 for oral and cloacal, n=15 for seeder) was not
126 significantly different between chicks raised on fresh litter compared to reused litter (W-
127 statistic, *P*. value; oral = 41, 0.15; cloacal= 41, 0.96; seeder = 5, 1.00) (Fig.1a).
128 However, the ceca of chicks raised on fresh litter were more likely to be positive for nal^R
129 *S*. Heidelberg (X_2= 16.07, df = 2, *P* = 0.0003), than chicks on reused litter. The
130 percentage of cecal samples positive for nal^R *S*. Heidelberg was 100%, 100% and 50%,
131 respectively for oral, cloacal and seeder chicks raised on fresh litter, compared to 80%,
132 100% and 33.3 %, for chicks raised on reused litter. For the seeder treatment on fresh
133 litter, one seeder was lost due to premature mortality and three of the four seeders were
134 positive for nal^R *S*. Heidelberg, while the ceca of four of the ten uninoculated contact
135 chicks were positive. In contrast, the ceca of the five orally challenged seeders raised
136 on reused litter were positive for nal^R *S*. Heidelberg, but all contact chicks were negative
137 (n = 10).

For the litter, oral and seeder treatments carried higher levels of nal^R *S*. Heidelberg in fresh litter compared to reused litter, while cloacally inoculated chicks had higher levels in reused litter compared to fresh litter (Fig.1b). All litter samples from challenged chicks were positive for nal^R *S*. Heidelberg by direct culture or after enriching in buffered peptone water (BPW), except for one reused litter sample from the seeder treatment that was negative. For unchallenged chicks used as controls, one litter sample from fresh and reused litter tested positive after enrichment in BPW. These data suggests that litter age/type did not have a significant effect on the abundance of nal^R *S*. Heidelberg in the ceca, but chicks raised on reused litter (66%) had a lower *Salmonella* positivity rate compared to chicks on fresh litter (79%).

Broiler litter age/type affected the horizontal transfer of AMR.

Next, we questioned if the litter plays a role in the HGT of AMR. First, we performed antibiotic susceptibility testing (AST) on *S*. Heidelberg isolates recovered from the ceca and litter of broiler chicks raised on fresh litter (n=158) and reused litter (n=141). As expected, all *S*. Heidelberg isolates were resistant to nalidixic acid (Fig. 2). Thirty-one percent of isolates from the ceca (37/118) and 37.5 % from the litter (15/40) of chicks on fresh litter acquired resistance to gentamicin, tetracycline, and streptomycin [7] (Fig. 2a). Contrastingly, 3% of *S*. Heidelberg isolates from the ceca of chicks on reused litter acquired resistance to either cefoxitin (1/114) or tetracycline (2/114), while ~18% of isolates from reused litter acquired resistance to either ciprofloxacin (1/27), tetracycline (2/27) or streptomycin (2/27) (Fig. 2a). The percentage of *S*. Heidelberg isolates that acquired AMR in fresh litter differed by the route of inoculation [7]. For instance, 40% of isolates from orally challenged chicks acquired AMR compared to 24% for cloacal [7].

161 We determined if *S*. Heidelberg isolates recovered from chicks raised on fresh or
162 reused litter differed in their core genome or if they acquired new mobile elements like
163 plasmids. To answer this question, we performed whole genome sequence (WGS)
164 analysis on *S*. Heidelberg isolates (n=29) recovered from the ceca and litter of chicks
165 that were cloacally challenged. We chose cloacal samples because only this route of
166 challenge resulted in 100% colonization of the ceca of chicks raised on fresh and
167 reused litter. There was no difference in the core genome multilocus sequence types
168 (cgMLST) of evolved *S*. Heidelberg isolates from fresh and reused litter, however, 28 of
169 the 29 evolved isolates had a different cgMLST from the ancestral nal^R *S*. Heidelberg
170 (Fig. 2b). We used the genomes sequenced to construct a maximum likelihood (ML)
171 tree based on the pangenome and mutations of *S*. Heidelberg strains recovered. The
172 core genome (genes present in ≥ 95% of the strains) and accessory genome (genes
173 present in < 95% of the strains) was composed of 4,373 and 356 genes, respectively.
174 After removing 10 isolates with identical mutations, a total of 118 informative sites
175 (SNPs and indels) was used for ML SNP tree construction.

176 The three constructed ML trees did not group isolates by litter age/type (fresh
177 versus reused) (Fig. 2b, Fig. S1a and b), but *S*. Heidelberg isolates that acquired IncI1
178 plasmids (plasmid MLST 26; hereafter referred to as IncI1-pST26) (n=5) formed a
179 separate clade on the accessory genome tree (Fig. 2b). The IncI1-pST26 plasmid
180 harbored the aminoglycoside (*aadA1*), tetracycline (*tetA*) and mercury (*mer* operon)
181 resistance genes [7] (Fig. S2). Isolates from the ceca (n=3) and litter (n=1) of chicks on
182 fresh litter that harbored IncI1-pST26 displayed the antibiotic resistance phenotype
183 predicted by WGS (Fig. 2b). Contrastingly, one isolate from reused litter carried IncI1-

184 pST26 but was found to be susceptible to all antibiotics tested except for the ancestral
185 nalidixic resistance. These results indicated that chicks grown on reused litter are less
186 likely to carry *S*. Heidelberg isolates harboring AMR compared to chicks on fresh litter.

187 **The microbiome of neonatal chicks differed for fresh and reused litter.**

188 We used 16S rRNA gene sequencing to examine the differences in the microbiome
189 between neonatal chicks raised on fresh and reused litter. The beta diversity of the
190 cecal (n = 59) and litter (n = 22) microbiome of chicks was significantly different
191 between fresh and reused litter (ceca, $P = 0.0002$; litter, $P = 0.0002$) (Fig. 3a).
192 Furthermore, the route of inoculation used for *S*. Heidelberg affected the beta diversity
193 of the ceca and litter for fresh and reused litter ($P < 0.05$). For example, chicks
194 challenged orally (n = 10) or through the cloaca (n =10) and raised on fresh litter
195 harbored a significantly different beta diversity than uninoculated chicks (n = 10) in the
196 ceca ($P < 0.002$) (Fig. 3a). Likewise, the beta diversity of the ceca of chicks challenged
197 orally (n = 10) differed from cloacal (n = 9) and uninoculated controls (n = 10) for reused
198 litter ($P < 0.05$). In the litter, pair wise comparisons between routes of inoculation were
199 not significant ($P > 0.05$) for beta diversity. Nevertheless, fresh litter samples (n = 3)
200 from orally challenged chicks clustered next to litter samples (n = 3) from seeder
201 treatments, while litter samples (n = 2) from cloacally inoculated chicks were different
202 from oral and seeder treatments (Fig. 3a). For reused litter samples, litter from orally (n
203 = 3) and cloacally (n = 3) challenged chicks clustered together, while litter (n = 2) from
204 seeder treatments was similar to litter samples (n = 3) from control chicks. Lastly, the
205 bacterial community structure (assessed using beta dispersion) of reused litter was less
206 variable than fresh litter ($P = 0.001$), however there was no difference in variability for

207 the ceca (P = 0.15). Moreover, the route of challenge affected the bacterial community
208 beta dispersion in the ceca and litter of chicks on fresh and reused litter (P = 0.001).
209 The alpha diversity (i.e., the number of amplicon sequence variants (ASVs)) of the
210 microbiome present in the ceca and litter of chicks on reused litter was higher for the
211 Observed species measure of diversity (ceca, $P < 0.001$; litter, P = 0.001) but not for
212 Shannon (ceca, P = 0.25; litter, P = 0.05) and Simpson (ceca, P = 0.97; litter, P = 0.28)
213 indices. The route of *S*. Heidelberg challenge affected the alpha diversity of the cecal
214 and litter microbiome ($P < 0.05$) (Fig. 3b). The alpha diversity of the ceca of challenged
215 and control chicks raised on reused litter was higher than chicks grown on fresh litter for
216 Observed species measure of diversity ($P < 0.01$). Cloacal challenged chicks raised on
217 reused litter had higher alpha diversity in their ceca compared to chicks on fresh litter for
218 Shannon and Simpson indices ($P < 0.05$). Uninoculated control chicks on fresh litter had
219 higher alpha diversity in their ceca than control chicks on reused litter for Simpson
220 index, (Fig. 3b). The litter of cloacal, seeder, and control chicks on reused litter had
221 higher alpha diversity than the litter of chicks on fresh litter for Shannon measure of
222 diversity ($P < 0.05$) (Fig. 3b). In contrast, the litter of orally challenged chicks on fresh
223 litter had higher alpha diversity than the litter of gavaged chicks on reused litter for
224 Shannon index (P = 0.0001). There was no significant difference in alpha diversity
225 between fresh and reused litter for Observed and Simpson indices ($P > 0.05$).
226 On the taxonomic level, orally challenged chicks on reused litter carried higher
227 abundance of Actinobacteria in the ceca compared to chicks on fresh litter (P = 0.003),
228 while uninoculated control chicks on fresh litter had higher relative abundance of
229 Actinobacteria in the ceca compared to control chicks on reused litter (P = 0.004) (Fig.

230 3c). Proteobacteria was higher in the ceca of oral, cloacal and control chicks on reused
231 litter compared to chicks on fresh litter ($P < 0.01$). Firmicutes had higher relative
232 abundance in the ceca of chicks on fresh litter compared to reused litter for all
233 treatments ($P < 0.01$) while Bacteroidetes had higher relative abundance in the ceca of
234 cloacal and control chicks on reused litter compared to chicks on fresh litter ($P < 0.05$)
235 (Fig. 3c). For the litter, Actinobacteria was higher in reused litter compared to fresh litter
236 ($P < 0.05$) while Proteobacteria and Bacteroidetes were higher in fresh litter compared
237 to reused litter ($P < 0.05$). There was no significant difference in phyla abundance
238 between routes of inoculation ($P > 0.05$) for the litter. These results shows that the
239 microbiome of chicks raised on fresh litter differed from chicks grown on reused litter,
240 and reused litter cecal and litter samples of challenged chicks were more uniform than
241 fresh litter samples.

242 ***S.* Heidelberg challenge modulated the gut microbiome.**

243 We determined that the core microbiome (i.e., ASVs > 0.1% relative abundance and
244 present in at least 80 % of samples) of fresh litter differs from reused litter (Table S1)
245 and found that *S*. Heidelberg challenge perturbed the cecal and litter microbiome of
246 broiler chicks (Fig. 4). For example, Enterobacteriaceae (*Klebsiella pneumonaie* and
247 *Proteus mirabilis*) were part of the core microbiome in fresh litter, while they were not
248 part of the core microbiome of reused litter (Table S1). Contrastingly, Actinobacteria (23
249 ASVs) was part of the core microbiome of reused litter, while only one ASV classified as
250 Actinobacteria was determined to be a core member of fresh litter (Table S1).
251 Unsurprisingly, the bacterial ASVs that increased or decreased in abundance after *S*.
252 Heidelberg challenge differed between chicks on fresh and reused litter (Fig. 4a and b).

253 To determine the ASVs that were modulated by *S*. Heidelberg challenge (i.e., ASVs
254 that increased or decreased after challenge), we compared the relative abundance of
255 ASVs in the ceca and litter of challenged chicks to uninoculated control chicks (Fig. 4).
256 In the ceca of chicks on fresh litter, the ASVs significantly modulated were assigned to
257 five bacterial families (Fig. 4a). Three ASVs matching family Clostridiales vadinBB6
258 group, nine ASVs classified as Ruminococcaceae and seven ASVs classified as
259 Lachnospiraceae increased in abundance in challenged chicks compared to control
260 chicks. Contrastingly, ASVs classified as Lactobacillaceae, Enterococcaceae,
261 Ruminococcaceae (n=8) and Lachnospiraceae (n=10) decreased in abundance in
262 challenged chicks compared to control chicks (Fig. 4a).

263 For the ceca of chicks on reused litter, the ASVs modulated were assigned to
264 twelve bacterial families. Amplicon sequence variants matching Bifidobacteriaceae,
265 order Clostridiales, Moraxellaceae, Rikenellaceae, Eggerthellaceae, Enterococcaceae,
266 Family XIII, Lactobacillaceae (n=2), Ruminococcaceae (n=2) and Lachnospiraceae
267 (n=2) increased in abundance in challenged chicks compared to control chicks. In
268 contrast, ASVs classified as Enterobacteriaceae, Enterococcaceae, Clostridiales
269 vadinBB6 group (n=2), Ruminococcaceae (n=3) and Lachnospiraceae (n=7) decreased
270 in abundance in challenged chicks compared to control chicks (Fig. 4a).

271 For the litter, the ASVs that were significantly modulated in fresh litter were assigned
272 to twenty-one bacterial families (Fig. 4b). The ASVs that increased in abundance in
273 fresh litter of challenged chicks belonged to Caulobacteraceae, Dysgonomonadaceae,
274 Burkholderiaceae, Flavobacteriaceae , Cellvibrionaceae, Enterobacteriaceae,
275 Enterococcaceae (n=2), Lactobacillaceae (n=2), Sphingobacteriaceae (n=2),

276 Pseudomonadaceae (n=3), Ruminococcaceae (n=3), Moraxellaceae (n=4), Clostridiales
277 vadinBB6 group (n=4), and Lachnospiraceae (n=4). Conversely, ASVs assigned to
278 Staphylococcaceae, Brevibacteriaceae, order Micrococcales, Corynebacteriaceae,
279 Xanthomonadaceae, Weeksellaceae, Bacillaceae (n=2), Ruminococcaceae (n=6) and
280 Lachnospiraceae (n=6) decreased in abundance in the litter of challenged chicks
281 compared to control chicks (Fig 4b).

282 Amplicon sequence variants modulated in reused litter after challenge were grouped
283 into twenty-four bacterial families. The ASVs that increased in abundance in reused
284 litter of challenged chicks were assigned to Lactobacillaceae, Saprospiraceae,
285 Bifidobacteriaceae, Rikenellaceae, Pseudomonadaceae, Sphingobacteriaceae,
286 Leuconostocaceae, Glycomycetaceae, Streptococcaceae, Enterobacteriaceae,
287 Wohlfahrtiimonadaceae, Bacteroides (n=2), Ruminococcaceae (n=3), Planococcaceae
288 (n=3), Lachnospiraceae (n=3) and Clostridiaceae 1 (n=5). The ASVs that decreased in
289 the reused litter of challenged chicks were classified as Burkholderiaceae,
290 Microbacteriaceae, Micrococcaceae, Moraxellaceae (n=2), Wohlfahrtiimonadaceae
291 (n=2), Aeromonadaceae (n=3), Clostridiales vadinBB6 group (n=3), Ruminococcaceae
292 (n=4), Lachnospiraceae (n=4) and Bacillaceae (n=6),

293 Not all bacterial families were modulated by the cloacal route of *S*. Heidelberg
294 challenge. For instance, Enterococcaceae was only modulated by oral challenge in the
295 ceca of chicks grown on fresh and reused litter (Fig. 4a). Similarly, Pseudomonadaceae
296 was only modulated in the litter of chicks that were orally challenged (Fig. 4b). Together,
297 these results show that the litter microbiome modulated by *S*. Heidelberg challenge

298 differed between chicks on fresh litter and reused litter, and the route of challenge
299 affected the ASVs that increased or decreased in abundance.

300 **Enterobacteriaceae and Clostridiales were the major bacterial hosts for AMR**.

301 We used Hi-C metagenomics to explore the bacterial reservoir of AMR in the cecal
302 microbiome of 2 cecal samples each from cloacally challenged chicks on fresh and
303 reused litter. We previously used this approach to identify *E. coli* as the main bacterial
304 reservoir of transferable plasmids in fresh litter [7]. Here, we extended our analysis to
305 cecal samples from reused litter. The average assembly size and number of contigs for
306 the four samples was 1,238,395,273±167,820 bp and 1,797,423±281,253, respectively.
307 The number of metagenome assembled genomes (MAGs) found in cecal samples from
308 fresh and reused litter was 456±422. We searched the MAGs for sequences matching
309 plasmid incompatibility (*inc*) groups and replicons available on the PlasmidFinder
310 database. The *inc* groups found matched common Enterobacteriaceae plasmids
311 including IncF, IncI, IncX, IncH, IncY, IncB/O/K/Z, p0111 and Col-like plasmids (Table
312 1). Three plasmid replicons were found for Gram-positive bacteria including *rep2*,
313 *rep18b* and *repA* (Table 1). The low representation of plasmids is expected, since the
314 PlasmidFinder database is much more comprehensive for Enterobacteriaceae than
315 other bacterial species, and within the well-studied Proteobacteria most plasmids
316 cannot be typed [31].

317 Next, we used proximity-ligation to find the bacterial host of ARGs and to determine
318 if they are encoded on plasmids or the chromosome. One hundred ARGs were found in
319 the MAGs and the majority (~ 50%) were harbored by family Enterobacteriaceae (Fig.
320 5). *Escherichia coli* was the only Enterobacteriaceae found to be the putative host for

321 these ARGs in fresh and reused litter. Members of the order Clostridiales were the
322 putative hosts for 27% of ARGs, while unclassified bacterial species (n = 6) and
323 members of the phyla Firmicutes (n = 9), Bacteriodetes (n = 3) and Actinobacteria (n =
324 1) were the hosts for the remaining ARGs. Fifty-four percent of the ARGs were found to
325 be encoded on the chromosome while 46% were found on plasmids. One cecal sample
326 from fresh litter (Fig. 5) harbored *E. coli* MAGs that encoded silver and copper
327 resistance genes (*silABCFRS* and *pcoABCDRS*) on the chromosome, while virulence
328 genes (*iroBCDEN*) were found on plasmids. A cecal sample from a chick raised on
329 reused litter harbored *E. coli* MAGs that encoded ampicillin (bla_{TEM1B}), tetracycline
330 (*tetA*), and mercury resistance (*merCPRT*) genes on plasmids (Fig. 5). This result
331 suggests that the microbiome of chicks on fresh and reused litter harbored AMR, and
332 Enterobacteriaceae and Clostridiales were the putative bacterial hosts of the ARGs.

333 ***E. coli* isolates harbored AMR on plasmids and genomic islands.**

334 In our earlier work, we found that *E. coli* MAGs were the primary hosts of IncI1 by
335 retrospectively screening the cecal contents of chicks on fresh litter for *E. coli* isolates
336 [7]. Using this approach, we confirmed that the IncI1-pST26 plasmid acquired by *S*.
337 Heidelberg was identical to the IncI1-pST26 plasmid present in *E. coli* strains (Fig. S2).
338 Here, we performed AST and WGS on additional *E. coli* isolates recovered from the
339 ceca of chicks used for Hi-C metagenomics (Table S2) and a pangenome analysis was
340 done on nineteen of them. The isolates were randomly selected from either
341 CHROMagar™ plates supplemented with or without gentamicin and tetracycline.

342 The inferred phylogeny of the *E. coli* strains using their core (n = 3385) and
343 accessory genes (n = 6024) partially grouped the isolates by the litter used for raising

344 the chicken host (Fig. 6). The ML trees grouped the isolates into three main clades
345 (Clade I - III). One *E. coli* isolate from the ceca of chicks on reused litter made up Clade
346 I on the core and accessory gene tree, while the number of isolates in Clade II and III
347 strains differed between core (Fig. 6a) and accessory gene tree (Fig. 6b). Clade II
348 isolates were clonal and recovered from chicks on fresh litter. Clade III was divided into
349 two subclades represented by isolates from fresh (subclade IIIa) and reused litter
350 (subclade IIIb) and subclade IIIb isolates were more clonal compared to subclade IIIa.
351 *E. coli* Isolates classified as phylogroup A, D and F were more likely to be found in
352 chicks on fresh litter, while phylogroup B1 was found in the ceca of chicks from reused
353 litter (Fig. 6, Table S2). Likewise, cgMLST classification showed that ST69, ST2705 and
354 ST6858 were associated with *E. coli* strains originating from chicks on fresh litter, while
355 ST1403 was found in reused litter. These results suggest that the *E. coli* strains from
356 this study have evolved to persist in either fresh or reused litter.

357 Some *E. coli* strains from fresh litter harbored ARGs on the chromosome and
358 plasmids. *E. coli* strains classified as pandemic lineage of extraintestinal pathogenic
359 ST69 from fresh litter harbored a ~128 kb genomic island encoding virulence genes
360 (pyelonephritis-associated pili (*pap)* operon) and ARGs for aminoglycoside (*strAB* and
361 *aph(3')-Ia*) tetracycline (*tetB*), sulfonamide (*sul2*) and metal resistance (silver (*sil*) and
362 copper (*pco)* operons) (Table S2; Fig. S3). The presence of a genomic island encoding
363 AMR suggests that the ARGs are mobile. We found DNA regions encoding identical
364 metal resistance genes (10 - 40 kb) as seen in the genomic island in p0111/IncH1B
365 plasmids harbored by *E. coli* strains from this study and in *E. coli* genomes found in the
366 NCBI database (Fig. S3). In addition, ST69 *E. coli* strains harbored a multireplicon IncF

367 plasmid encoding *bla*$_{TEM-1B}$, *aac(3)-Ild* and virulence genes (colicin M and catecholate
368 siderophore uptake system (*iroBCDEN*)), and an untypeable IncI1 plasmid. (See Data
369 Set S1 in the supplemental material; Data Set S1 to S5 are available in the Dryad
370 repository online at https://doi.org/10.5061/dryad.c866t1g6c).

371 As previously reported [7], *E. coli* ST6858 from fresh litter carried an identical *S*.
372 Heidelberg IncI1-pST26 plasmid, and harbored IncI2, IncF, and cryptic Col-type
373 plasmids (Data Set S2). Antibiotic susceptible ST2705 *E. coli* isolates from fresh litter
374 harbored no plasmid encoding antibiotic resistance genes but carried a phage-like
375 plasmid classified as p0111 and a multireplicon IncF plasmid encoding metal resistance
376 and virulence genes (Data Set S3). The common ARG carried by ST1403 *E. coli* strains
377 from reused litter was *bla*$_{TEM}$-1B. The gene was encoded on an IncX1 plasmid (Data Set
378 S4), and it conferred ampicillin resistance. Additionally, ST1403 *E. coli* strains harbored
379 IncFIB, IncI2 and ColRNAi plasmids. One *E. coli* isolate (ST93) from reused litter
380 harbored an IncH1B/p0111 plasmid encoding ARGs for aminoglycoside (*aadA1, aac(3)-*
381 *Vla*), tetracycline (*tetB*), sulfonamide (*sul1*) and mercury resistance (*mer* operon) and an
382 IncF plasmid encoding colicin M and *iroBCDEN* genes (Data Set S5).

383 **Bacterial hosts from reused litter maintained IncI1 plasmid at low copies.**

384 The low rate of AMR acquisition by *S*. Heidelberg isolates from reused litter made us
385 hypothesize that IncI1-pST26 plasmids are present at lower copies in reused litter. To
386 test this hypothesis, we used qPCR assays targeting the region upstream of the *repA* of
387 IncI1 plasmid including the *inc*RNAI to determine their copy number in the ceca of
388 chicks on fresh and reused litter. We normalized the abundance of IncI1 against
389 glyceraldehyde-3-phosphate dehydrogenase A (*gapA*) housekeeping gene encoded on

390 the chromosome of several enteric bacteria [32] including *Salmonella* and *E. coli.* We
391 targeted Enterobacteriaceae with *gapA* because *E. coli* was determined to be the hosts
392 for AMR by metagenomics and WGS in this study. The relative abundance of
393 Enterobacteriaceae bacterial population carrying IncI1 was higher in the ceca of orally
394 challenged chicks on fresh litter compared to chicks on reused litter (W = 85, *P* =
395 0.00041) and was not significantly different for cloacally inoculated (W = 23, *P* = 0.24)
396 and uninoculated control chicks (W = 63, *P* = 0.16) (Fig. 7).

397 To further explore the effect of low copy IncI1 plasmids on AMR transfer, we
398 selected the *S*. Heidelberg isolate from reused litter harboring IncI1-pST26 that was
399 unexpectedly susceptible to antibiotics predicted by ARGs encoded on the plasmid. The
400 isolate (SH-16-0-5B) was confirmed twice by broth microdilution to be susceptible to
401 gentamicin, streptomycin, and tetracycline and once on Mueller Hinton agar
402 supplemented with varying concentrations (0, 2, 4, 8 and 16 mg/l) of gentamicin (see
403 supplementary methods). To ensure that the plasmid contig identified as IncI1-pST26 in
404 this strain was not an artifact of short read sequencing, we performed qPCR on plasmid
405 DNA and targeted four coding DNA sequence on IncI1-pST26 including its *inc*RNAi,
406 class-1 integron (*intI1*), aminoglycoside (*aadA1*) and tetracycline resistance (*tetA*)
407 genes (Table S3). This confirmed that the strain carried IncI1-pST26 (qPCR cycle
408 threshold ranged from 20.2 – 27.9 for the four targets). Afterwards, we used WGS
409 (depth of coverage of IncI1-pST26 contig/depth of coverage of the largest chromosome
410 contig) to compare the copy number of IncI1-pST26 in SH-16-0-5B to the copy number
411 of IncI1-pST26 in four *S*. Heidelberg isolates (hereafter referred to as SH-IncI1-FL)
412 recovered from the ceca of cloacally inoculated chicks on fresh litter. This revealed that

413 SH-16-0-5B maintained IncI1-pST26 at ~0.2 copies/cell (i.e., 1 of 5 cells harbored
414 IncI1) compared to 2.8±0.2 copies/cell in SH-IncI1-FL from fresh litter.

415 To investigate if the copy number difference determined using WGS exist in relevant
416 environmental conditions, we first acclimatized the isolates in pre-reduced cecal extract
417 (pH 6.5) for 2 h before exposing them to pre-reduced cecal extract at pH 2.5 under
418 microaerophilic conditions. We reasoned that the low pH of the upper gastrointestinal
419 tract (GIT) of the chicken host will interfere with the copy number of plasmids kept in a
420 bacterial cell, which would have a direct effect on the rate of HGT. The copy number of
421 IncI1-pST26 was significantly lower (0.00005 - 0.21 copies /per cell i.e., 1 in 20,000 to 1
422 in 5 cells carried IncI1) in SH-16-0-5B compared to SH-IncI1-FL (4.94 - 6.65 copies /per
423 cell) for pH 6.5 (W = 9, P = 0.1) and pH 2.5 (W = 81, P = 4.114e-05) (Table 2). These
424 results show that *E. coli* and *S*. Heidelberg populations in reused litter harbored lower
425 copies of IncI1-pST26 than the populations in fresh litter.

426 **Discussion**

427 Litter is commonly used as a bedding material for raising poultry and it is ingested
428 by chickens during pecking activities. Therefore, the microbiome of the litter is one of
429 the first inoculum that colonizes the GIT of broiler chicks. Litter can be managed as
430 single use i.e., complete removal of litter from a broiler house after each flock or it can
431 be reused over multiple flocks. The benefits of litter reuse include lower cost for growing
432 chickens and the sustainable management of litter waste [33]. It has been shown that
433 chickens raised on reused litter harbor a different microbiome than chicks grown on
434 fresh litter [17, 34] and that reused litter stimulates higher humoral and cell-mediated
435 immune responses than fresh litter in chickens [35]. Muniz et al. [23] and Roll et al. [26]

showed that the number of litter samples positive for *Salmonella* significantly decreases as the number of litter reuses increased compared with the first use of the litter.

In this study, we corroborate these findings by showing that neonatal chicks challenged with *S*. Heidelberg and raised on reused litter were more resistant to S. Heidelberg colonization i.e., lower positivity rate compared to chicks on fresh litter. In addition, we found that chicks raised on reused litter were less likely to be colonized with *S*. Heidelberg isolates harboring plasmids encoding AMR compared to chicks on fresh litter. We determined that the major difference between chicks grown on fresh and reused litter was the bacterial community harbored in the litter and ceca.

Pathogen induced gut microbiome modulation can create an imbalance in the abundance of bacterial species i.e., some species outgrow known resident bacteria, leading to a dysbiosis of the gut microbiome and successful *Salmonella* colonization and AMR transfer [36]. However, gut conditions that allow the maintenance of a diverse and beneficial microbiome are likely to succeed in establishing homeostasis and limit the transfer of AMR [37, 38]. In this study, we found that the microbiome of reused litter was associated with a lower HGT of AMR to *S*. Heidelberg populations in neonatal chicks. The microbiome data led us to draw one main conclusion that the litter used for growing chicks and the route of *Salmonella* challenge affected which ASVs increased or decreased in abundance in the ceca and litter.

This was the case for the ASV assigned to *Bifidobacterium* that increased in the ceca and litter of orally challenged chicks raised on reused litter but was not modulated in cloacally inoculated chicks or in chicks on fresh litter. Consequently, *Bifidobacterium* was not part of the core microbiome of the ceca or litter in this study (Table S1) and

459 most likely evolved with neonatal chicks. *Bifidobacterium* are strict anaerobic
460 Actinobacteria and are among the first microbes to colonize the GIT of vertebrates [39].
461 Additionally, *Bifidobacterium* has been shown to affect the abundance of AMR and
462 virulence genes in the GIT of infants [40-42]. Casaburi et al [42] reported that infants fed
463 a probiotic strain of *B. longum* harbored lower levels of ARG compared to non-fed
464 controls. Alignment of shotgun metagenomic reads generated from the ceca of broiler
465 chicks (n = 4) to Kraken database [43] revealed that 21% and 11% of the reads
466 mapping to the genus *Bifidobacterium*, were aligned to *B. longum* and *B. catenulatum,*
467 respectively (Fig. S4). Their increase in abundance for only orally challenged chicks on
468 reused litter suggests that the route *S*. Heidelberg used for gaining entry into the GIT
469 and the litter microbiome affected how *Bifidobacterium* was perturbed. Therefore, it is
470 conceivable that reused litter microbiome created conditions that allowed
471 *Bifidobacterium* to flourish and proliferate, while the microbiome in fresh litter led to a
472 reduction or elimination of *Bifidobacterium* species.

473 We used metagenomics and WGS to show that *E. coli* was the main reservoir of
474 AMR in the cecal microbiome of fresh and reused litter. Importantly, we found the
475 identical IncI1-pST26 plasmid, acquired by *S*. Heidelberg, to be present in *E. coli*,
476 suggesting that the plasmid was transferred in vivo within chicks raised on fresh litter.
477 The possible sources of the *E. coli* strains in this study include the chicken host and the
478 broiler house environment. Although, we did not establish if the meconium harbored *E.*
479 *coli*, it is plausible that post-hatch chicks were colonized with *E.coli* from the hatchery
480 [44]. The evolution of these ancestral *E. coli* strains would be shaped by selective
481 pressures such as exposure to antibiotics and metals, and competition with resident

482 microbiota in the environment. For chicks placed on fresh litter, the bacterial community
483 of the first fecal droppings will compete with mostly pine shaving/plant associated
484 microbiome. In contrast, chicks placed on reused litter will compete with microbes and
485 metabolites deposited from earlier flocks including resident *E. coli* and *Salmonella*
486 strains that are well-adapted to the broiler house environment. Also, the physio-
487 chemical properties of the litter will affect the litter structure, the native microbiome and
488 the survival of the invading pathogen. The difference in litter physio-chemical
489 parameters in this study was inferred using pH and moisture. The pH of reused litter
490 was higher (7.12 ± 0.26) than fresh litter (6.54 ± 0.17) (P = 0.028), while fresh litter had
491 higher moisture (20.83 ± 3.54 %) than reused litter (14.65± 3.51 %) (P = 0.11). Litter
492 moisture and pH are known factors that affect pathogen survival and microbial diversity
493 [45, 46].

494 We did not administer antibiotics to chicks in this study and have shown that metals
495 did not significantly affect the metabolism of *S*. Heidelberg [7]. Hence, the litter
496 microbiome and physio-chemical properties are the major selection pressure that could
497 explain the evolutionary trajectory of *E. coli* in this study. This hypothesis was further
498 supported after a functional protein pathway analysis was performed on the cecal
499 metagenome of chicks raised on fresh and reused litter. Pathways related to the
500 biosynthesis of organic and antimicrobial molecules were enriched to a greater degree
501 in the ceca of chicks on reused litter than chicks on fresh litter (Fig. S5). Of note was the
502 enrichment of biosynthetic pathways for antimicrobials and secondary metabolites in
503 reused litter (e.g., macrolides, tetracyclines, carbapenems, vancomycin and

504 polyketides), suggesting that reused litter harbored a higher abundance of microbial
505 species with antimicrobial properties than fresh litter.

506 Contrastingly, pathways associated with the biosynthesis of secondary metabolites
507 of plants including flavonoids, indole-alkaloids and stilbenoid, diarylheptanoid and
508 gingerol were enriched in samples from fresh litter (Fig. S5). Though we measured only
509 two cross-sectional timepoints for reused and fresh litter, the relative abundance of
510 plant-metabolic related pathways in fresh litter compared to the enrichment of
511 antimicrobial molecules in reused litter suggests that a selective pressure gradient
512 exists over time for the colonizing microbiome in reused litter. Thus, the microbiome of
513 reused litter has the antimicrobial capability to competitively exclude invading pathogens
514 including strains carrying AMR encoding plasmids.

515 Plasmid carriage is expected to impose a fitness cost on the host, but plasmid
516 bearing microbes are pervasive in nature [47]. We showed previously that carriage of
517 IncI1-pST26 by *S*. Heidelberg isolates from fresh litter presented a variable fitness cost
518 to the host [7]. We found here that Enterobacteriaceae populations in the ceca of
519 chicks on reused litter harbored lower copies of the IncI1 plasmid per cell compared to
520 chicks on fresh litter. In addition, we did not isolate any *E. coli* isolate carrying IncI1 from
521 the ceca of chicks raised on reused litter. The limited number of Enterobacteriaceae
522 IncI1 donor population would affect the rate of transfer to *S*. Heidelberg recipients as
523 direct contact is a requirement for conjugation [48]. Nevertheless, we found one *S*.
524 Heidelberg isolate from the ceca of one chick on reused litter that harbored IncI1-
525 pST26, however, only a fraction of its population harbored IncI1 plasmid. This isolate
526 did not exhibit the resistance phenotype predicted by the ARGs encoded on the IncI1-

527 pST26 plasmid. These results suggest that IncI1 plasmid carriage in reused litter posed
528 a fitness cost on the bacterial host, but this cost was ameliorated by maintaining IncI1 at
529 low copies in our study.

530 It is important to mention that the litter used for this study is not representative of
531 all broiler litter. Litter composition may differ between farms and there are multiple
532 physio-chemical factors that can affect the litter microbiome, e.g., moisture and pH,
533 antibiotic usage, and feed additives. Furthermore, the age of the litter i.e., numbers of
534 flocks that have been grown on the litter, and the length of the downtime between flocks
535 are examples of management practices that may influence microbiome succession.
536 Nonetheless, our study showed that the litter microbiome significantly affects the
537 bacterial diversity of broiler chicks and the HGT of AMR.

538 **Materials and Methods**

539 Details of methods used for preparing the *S*. Heidelberg inocula, challenging neonatal
540 chicks, bacterial and DNA analyses, whole genome, and metagenome sequencing and
541 bioinformatics have been described before for neonatal chicks raised on fresh litter [7] .
542 We briefly redescribe these methods and present others below.

543 **Determining if post-hatch chicks were *Salmonella*-free.**

544 Chick pad that conveyed chicks from the hatchery were pre-enriched in 500 ml of
545 Buffered Peptone Water (BPW) for 18-24h at 37° C. Two different enrichments broths
546 were used to isolate *Salmonella* from the BPW pre-enrichment broths: Tetrathionate
547 (TT; Becton-Dickinson, Sparks, MD) broth and Rappaport-Vassiliadis (RV; Becton
548 Dickinson) media. After overnight incubation at 42°C in both enrichment broths, 10 µl
549 aliquots from each enrichment broth was spread on two different differential media:

550 Brilliant Green Sulfa (BGS; Becton Dickinson) agar and xylose lysine tergitol-4 (XLT-4;
551 Becton Dickinson) agar and incubated for 18-24 h at 37° C. Isolated colonies
552 characteristic of *Salmonella* were stabbed and streaked onto triple sugar iron agar (TSI;
553 Becton-Dickinson) and lysine iron agar fermentation (LIA; Becton-Dickinson) and
554 incubated for 18-24 h at 37° C for biochemical confirmation.

555 ***S*. Heidelberg inoculum preparation.**

556 The *S*. Heidelberg strain was made resistant to 200 ppm of nalidixic acid (nal^R *S*.
557 Heidelberg) for selective enumeration and was grown overnight in poultry litter extract,
558 centrifuged, and resuspended in 1X phosphate buffered saline (PBS). The resuspended
559 cells were used as inocula. The complete genome of the ancestor to nal^R *S*. Heidelberg
560 is available under GenBank accession number : CP066851.

561 **Challenging broiler chicks with nal^R *S*.*S*. Heidelberg.**

562 One-day-old Cobb 500 broiler chicks were either uninoculated (n =25), gavaged (n =25)
563 or (n =25) cloacally inoculated with ~10^6 colony forming units (CFU) of nal^R *S*.
564 Heidelberg. We also included a seeder-bird colonization method, whereby five chicks
565 were gavaged and mingled with twenty uninoculated chicks. Afterwards, chicks were
566 placed in floor pens at a stocking density of 0.65 m^2/chick on fresh pine shavings (fresh
567 litter, n =100) or reused litter (n =100). The reused litter was previously used to raise
568 three flocks of broiler chicken's antibiotic-free under simulated commercial poultry
569 production conditions and was top dressed with 0.5 cm of fresh pine shavings before
570 the placement of each flock. Broiler chicks on fresh and reused litter were housed
571 separately and were raised for 14 days on antibiotic-free starter diet and water. At 14
572 days, forty-five chicks from each fresh and reused litter (ten chicks from gavaved,

573 cloacal and uninoculated groups and fifteen chickens from the seeder method (5 seeder
574 and 10 contact chicks) were euthanized to determine the extent of nal^R *S*. Heidelberg
575 colonization in ceca. Additionally, litter samples were collected as grab samples from
576 each pen after chicks were euthanized. The experiments were performed in April 2018.
577 The study was approved by the University of Georgia's Office of Animal Care and Use
578 under Animal Use Protocol: A2017 04-028-A2.

579 **Determination of nal^R *S*. Heidelberg concentration in the ceca and litter.**

580 Ceca were removed from the eviscera of broiler chicks and stomached for 60 seconds
581 after the addition of 3X volume to the weight (v/w) of BPW. Litter was collected as grab
582 samples from seven locations (4 corners of the pen and 3 locations under the waterer)
583 in each pen after chicks were removed. The litter samples were pooled and 30g was
584 subsampled in duplicates from each pen as previously described [29]. Serial dilutions of
585 the cecal and litter slurry were plated onto BGS containing 200-ppm nalidixic acid and
586 plates were incubated for 24 h at 37°C.

587 After incubation, colonies were counted and calculated per gram of ceca or litter dry
588 weight. When no colonies appeared, pre-enriched cecal and litter slurry was streaked
589 onto BGS agar supplemented with nalidixic acid and incubated overnight. These plates
590 were then examined for the presence/absence of *Salmonella* colonies. Two to six single
591 colonies were randomly selected from each plate and archived in 30% LB glycerol at -
592 80 ^{o}C. In addition, cecal slurry was saved at a 4:1 ratio in Luria Bertani (LB) broth (BD
593 Difco, MD, USA) containing 30% glycerol at –80°C, while litter samples were stored in
594 vacuum sealed whirl pack bags at -20°C. Litter pH and moisture was determined as
595 described previously [29].

596 **Antibiotic resistance phenotype determination.**

597 Antibiotic susceptibility testing was done on *S*. Heidelberg and *E. coli* isolates by
598 following the National Antimicrobial Resistance Monitoring System (NARMS) protocol
599 for Gram-Negative bacteria. Results were interpreted according to clinical and
600 laboratory standards institute guidelines and breakpoints established by NARMS. In
601 addition, we used agar dilution to determine the minimum inhibitory concentration of
602 gentamicin for one *S*. Heidelberg isolate (SH-16-0.5B) (see supplementary methods).

603 **Genomic, environmental and plasmid DNA extraction.**

604 Unless otherwise noted, DNA was extracted and purified from bacterial cultures using
605 FastDNA™ spin Kit for Soil (MP Biomedicals, LLC, CA, USA), while 250 mg of cecal
606 slurry (previously saved in LB broth containing glycerol) and litter were extracted with
607 Qiagen DNeasy Power-Soil DNA kit (Hilden, Germany). Extracted DNA were used for
608 qPCR, 16S rRNA gene sequencing, shotgun metagenomics and whole genome
609 sequencing. Plasmid DNA was extracted from two *S*. Heidelberg isolates that harbored
610 IncI1. Isolates were cultured overnight on sheep blood agar and plasmid DNA was
611 extracted using Qiagen Plasmid Midi kit (Qiagen Inc, Germantown, MD) as per
612 manufacturer's instructions. Plasmid DNA was used to construct a calibration curve for
613 IncI1 plasmid copy number determination.

614 **Real-time quantitative PCR.**

615 Real-time qPCR amplification was performed as described previously (45) using a
616 CFX96 Touch Real-Time PCR Detection System (Bio-Rad Inc., Hercules, CA). Reaction
617 mixtures contained 1X SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Inc.,
618 Hercules, CA), 600 nM (each) primers) and 2 µl of DNA. Primers used in this study are

619 shown in Table S3. Unless otherwise stated, primers were designed with beacon
620 designer (Premier Biosoft, Palo Alto, CA) and synthesized by Integrated DNA
621 Technologies (Coralville, IA). Calibration curves used for converting qPCR cycle
622 threshold values to gene copies per gram of ceca was determined using the genomic
623 DNA from an *E. coli* strain that harbored IncI1-pST26 plasmid. To convert qPCR cycle
624 threshold values to IncI1 copies per cell of *S*. Heidelberg, plasmid DNA of a relevant *S*.
625 Heidelberg strain harboring IncI1-pST26 was used for calibration curve construction
626 (Table S3). Two primer sets specific to the *gapA* gene and the region upstream of the
627 *repA* of IncI1 including the *inc*RNAI were used to determine the gene copies of IncI1 in
628 the ceca. The *gapA* is a housekeeping gene encoded on the chromosome of enteric
629 bacteria including *E. coli*, *Klebsiella*, *Citrobacter* and *Salmonella* [32]. The gene copies
630 of IncI1 per cell was determined as the copy ratio of *inc*RNAi to *gapA* [49]

631 **Whole genome sequencing and analysis.**

632 Whole genome sequencing libraries were prepared using either Nextera™ XT or
633 Nextera™ DNA Flex library preparation kits (Illumina, Inc., CA, USA) following the
634 manufacturers protocol. Libraries were sequenced on the Illumina MiSeq platform with
635 150 or 250-bp paired end reads. Additionally, five *E. coli* isolates were selected for long
636 read sequencing using Sequel II System (PacBio Biosciences Inc.) or MinION device
637 (Oxford Nanopore Technology) (File S1). Preparation and sequencing of long read
638 libraries were done by sequencing core centers of University of Georgia and Colorado
639 State University. The method used for read quality control and demultiplexing has been
640 reported [7].

641 Genome assembly, resistome characterization, and quality assessment of
642 assemblies was done using Reads2Resistome pipeline v.1.1.1 [50]. For tools used for
643 genome annotation, bacteria and plasmid typing, see supplementary methods. MAFFT
644 v. 1.4.0 [51] implemented in Geneious Prime® v 2020.0.1, was used to align and
645 compare sequences. Roary [52] was used for pan genome analysis and phylogenetic
646 trees were constructed using the maximum likelihood method implemented in RAxML-
647 NG v 1.0.0 [53]. IslandViewer [54] was used to predict genomic islands.

648 **Identification of single nucleotide polymorphism (SNP) and Indels present in *S*.**
649 **Heidelberg isolates**.

650 Alignment of raw FASTQ reads to *S*. Heidelberg (Genbank accession number:
651 CP016573) was done using BWA (v0.7.17) [55] and SNPs/indels were called using
652 Genome Analysis Toolkit [56]. Variant call format (VCF) files of SNPs/Indels and the
653 script used are available in Dryad Digital
654 Repository: https://doi.org/10.5061/dryad.c866t1g6c.

655 **16S rRNA gene community analysis.**

656 The V4 hypervariable region of the 16S rRNA gene was sequenced using the paired-
657 end (250 × 2) method on the Illumina MiSeq platform. The cecal (n = 59; 30 and 29
658 chicks from fresh litter and reused litter, respectively) and litter (n = 22, 11 samples each
659 from fresh and reused litter) samples were part of a larger sequencing run and
660 processed together with other samples. Thus, detailed sequence processing
661 parameters are described in [7]. To avoid bias introduced by spurious amplicon
662 sequence variants (ASVs) or from samples with low sequencing depth, any ASVs with

663 less than 5 reads and samples with less than 5000 reads were removed from the
664 dataset before further analysis.

665 Statistical analysis of microbial communities was performed in the R environment
666 using the packages “phyloseq”, “Ampvis2”, and “vegan”. Alpha diversity indices were
667 calculated with a dataset rarefied to the minimum sample size (8740 sequences) and
668 compared using the Wilcoxon Signed Rank test. Principal coordinates analysis (PCoA)
669 based on Bray-Curtis distances was performed with no initial data transformation and
670 after removing ASVs present with relative abundance less than 0.1% in any sample.
671 The core microbiome was determined as ASVs 0.1% relative abundance and present
672 in at least 80 % of samples.

673 **Cecal metagenome sequencing.**

674 Two hundred and fifty milligrams of cecal slurry from chicks cloacally challenged with *S*.
675 Heidelberg and raised on fresh (n = 2) or reused litter (n = 2) were used for generating
676 shotgun and Hi-C DNA library. For shotgun library, cecal DNA was extracted with
677 Qiagen DNeasy Power-Soil DNA kit (Hilden, Germany) and Nextera™ XT library
678 preparation kit was used to make the library. Hi-C library was made using Phase
679 Genomics (Seattle, WA, USA) ProxiMeta Hi-C Microbiome Kit following the
680 manufacturer's instructions. Libraries were sequenced by Novogene corporation
681 (Sacramento, CA, USA) on the Illumina HiSeq platform using 150bp paired end reads.
682 Two libraries were sequenced per HiSeq flowcell lane resulting in a total of ~111 million
683 shotgun reads, and ~213 million Hi-C reads per sample. Quality controlled shotgun
684 reads were classified using Kraken2 (v2.0.8 beta) [43] to create a count profile of the

685 metagenome. We used cumulative sum scaling implemented in metagenomeSeq to
686 normalize read counts [57].

687 **Metagenome assembled genomes and associated plasmids and ARGs**

688 The details of the methods used for proximity-guided metagenome assembly and
689 deconvolution have been described before [7]. Briefly, shotgun metagenomics
690 sequence reads were filtered and trimmed for quality and normalized before assembly
691 with metaSPAdes [58] using default options. Hi-C sequence reads were then aligned to
692 the assembly following the ProxiMeta Hi-C kit manufacturer's recommendations
693 (https://phasegenomics.github.io/2019/09/19/hic-alignment-and-qc.html). Metagenome
694 deconvolution was performed with ProxiMeta [59], resulting in the creation of putative
695 genome and genome fragment clusters. Hi-C clusters were assessed for quality using
696 CheckM [60] and assigned taxonomic classifications with Mash v2.1 [61] resulting in the
697 metagenome assembled genomes (MAGs). We used PlasmidFinder (v2.1.1) [62] to
698 identify plasmids present in MAGs.

699 To view AMR gene connections to hosts, AMR genes in the metagenomic
700 assembly were annotated using NCBI's AMRFinderPlus software (version: 3.10.5) with
701 the --plus option specified, and all other options set to defaults. The AMR assembly
702 annotations were then used in combination with the MAG taxonomic annotations and
703 the mobile element to host association matrices generated by ProxiMeta to annotate
704 which AMR genes are present in each MAG, and specify whether the gene originated
705 from a genomic, viral or plasmid contig. This was done by making a NxM matrix C,
706 where $N = |AMRa|$ and $M = |MAG|$, where $MAG = \{$set of individual metagenomic bins$\}$

707 and $AMRa = \{x \subset AMR : x \in \text{Assembly}\}$, where

708 $AMR = \{\text{set of all AMR genes in database}\}$. The matrix ($C$) was then filled as:

709 For i in $|AMRa|$:

710 For j in $|MAG|$:

711 $$C_{i,j} = \begin{cases} 0 & \text{if } AMR_i \notin MAG_j \\ S & \text{if } AMR_i \in MAG_j \end{cases}, \text{ where } S = \begin{cases} 1 & \text{if } AMR_i \in AMRg \\ 2 & \text{if } AMR_i \in AMRv \\ 3 & \text{if } AMR_i \in AMRp \end{cases}$$

712 and where, $AMRg = \{x \subset AMRa : x \in \text{Genomic Contigs}\}$, $AMRv = \{x \subset AMRa : x \in$

713 Viral Contigs$\}$, $AMRp = \{x \subset AMRa : x \in \text{Plasmid Contigs}\}$. The matrix, C, was then

714 filtered to remove rows and columns with only 0 values and plotted using a color

715 encoding system indicating whether the AMR gene was of genomic (cyan) or plasmid

716 (gold) origin.

717 **Functional Pathway Analysis**.

718 Both shotgun sequences and ProxiMeta-deconvoluted genomes were also utilized to

719 assess enrichment of functional pathways. Shotgun metagenomic sequences were

720 aligned to the Hi-C genomic contigs using BWA-MEM algorithm as implemented in the

721 BWA (v0.7.17) [55]. The Hi-C genomic contigs were then annotated using Prokka

722 (v1.13) [63]. For each annotated region on the Hi-C genomic contigs, sequences

723 overlapping the annotated region in the sequence alignments were counted using a

724 custom Python script to produce a count matrix of genes for all samples. Gene counts

725 were normalized by gene length and using total sum scaling normalization to control for

726 differences in sequencing depth [64]. Enzyme commission identifiers were then mapped

727 to KEGG functional pathways according to the KEGG ontology. For each pathway, an

728 adjacency graph was reconstructed from the KEGG XML files, and connected

729 components were determined using depth-first search [65]. Z-scores were calculated for
730 pathways and connected components for each sample and statistical testing was then
731 performed by comparing scores to the normal distribution. Details of this analysis is
732 described in the supplementary methods.

733 ***E. coli* isolation from ceca.**

734 The method used for screening frozen cecal contents has been reported [7]. Briefly, two
735 cecal slurry from challenged chicks raised on fresh litter and reused litter were spread
736 plated onto CHROMagar™ plates supplemented with or without gentamicin (8 ppm) and
737 tetracycline (8 ppm) or ampicillin (16 ppm) and cefoxitin (16 ppm) (Thermo Fisher,
738 Waltham, MA). After 24 h incubation, blue green and blue-cream colonies were counted
739 as presumptive *E. coli* and 4 - 5 colonies from each spread plate were picked and
740 restruck for colony purification. No colonies grew on CHROMagar™ plates
741 supplemented with ampicillin and cefoxitin. Colonies were preserved in LB broth
742 containing glycerol and frozen before they were used for including solid agar
743 competition experiment.

744 **Determination of plasmid copy number of IncI1.**

745 To compare the copy number of IncI1 harbored by *S*. Heidelberg strain (SH-16-0-5B)
746 from reused litter to the copy number of IncI1 in three SH-IncI1-FL isolates from fresh
747 litter, we exposed each isolate to acidified filter sterilized cecal extract. Cecal extract
748 (CE) was prepared from the cecal content of 2-weeks old broiler chickens (see
749 supplementary methods). Cecal extracts were pre-reduced by covering tubes with gas
750 permeable paper strip and incubating overnight under microaerophilic conditions (5%
751 O_2, 10% CO_2, and 85% N2) at 42°C. Three single colonies of each strain were selected

752 from overnight cultures grown on sheep blood agar and transferred to a microcentrifuge
753 tube containing 900 µl of pre-reduced CE (pH = 6.52), i.e., one tube per for each SH-
754 IncI1-FL strain and three tubes for SH-16-0-5B.

755 After transfer, tubes were vortexed, covered with a gas permeable paper strip and
756 incubated at 41°C under microaerophilic conditions for 2 h. After incubation, tubes were
757 vortexed and 100 µl of the suspension was transferred to a microcentrifuge tube
758 containing 900 µl of CE (pH of CE was adjusted to 2.52 using 1M HCl (Spectrum
759 Chemical Mfg. Corp., CA, USA) and 1M NaOH (Fisher Chemical, NJ, USA) Afterwards,
760 tubes were vortexed, and incubation continued for another 24 h. To determine the copy
761 number of IncI1, one replicate tube per strain was removed at timepoints 2 h for pH 6.5
762 and 0.5, 4 and 24 h for pH 2.5 and used for DNA extraction. DNA was extracted from
763 500 - 800 µl of CE samples using FastDNA™ SPIN Kit for Soil. Calibration curves for
764 *gapA* and *inc*RNAI were generated using genomic and plasmid DNA extracted from
765 ancestral *S*. Heidelberg (GenBank accession number : CP066851) and one SH-IncI1-
766 FL isolate, respectively. Plasmid copy number of IncI1 was determined as the copy ratio
767 of *inc*RNAi to *gapA* [49]

768 **Statistical analyses.**

769 Continuous variables were log-transformed before any statistical tests were performed.
770 Moreover, continuous variables did not meet the assumption of a normal distribution;
771 therefore, non-parametric testing for direct comparisons was performed using Wilcoxon
772 rank sum and signed rank tests, and the Kruskal-Wallis rank sum test was used for one-
773 way analysis of variance tests. Additionally, a generalized linear model with a binomial
774 distributed outcome and a log link function was performed as described earlier [66] to

determine if there are significant differences in *Salmonella* prevalence (presence/absence) and the litter used for raising broiler chicks. The significance of the model was established using a likelihood ratio test (R function ANOVA with argument test set to “Chisq”). Multiple comparisons of means, i.e., regression coefficients for route of inoculation and litter age/type were done using the multcomp package in R. The mcp function was used to specify linear hypotheses and the glht function was used to make Tukey contrasts. Statistical analyses were performed using R (v4.0.3).

Ethics statement.

All animal experiments were approved by the University of Georgia Office of Animal Care and Use under Animal Use Protocol: A2017 04-028-A2.

Data availability.

All raw short and long FASTQ reads for *S*. Heidelberg are publicly available under NCBI accession number: PRJNA683658, while *E. coli* FASTQ reads are available under NCBI accession number: PRJNA684578. Shotgun and Hi-C reads from fresh litter and reused litter cecal samples are publicly available under NCBI accession number: PRJNA688069 and 16S rRNA gene sequences under NCBI accession number: PRJNA669215. The complete genome assemblies for *E. coli* strains Ec-FL1-1X and Ec-FL1-2X were previously published and are available under GenBank accession numbers CP066836 and JAFCXR000000000. Complete genome of *E. coli* strain Ec-RL2-1X has been made available under GenBank accession numbers CP066839. Variant call format (VCF) files of identified SNPs/indels and the Linux/Unix shell script used have been deposited in Dryad Digital Repository: https://doi.org/10.5061/dryad.c866t1g6c.

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Author's contributions.

A.O., and Z.A. designed the study. A.O., G.Z., D.E.C. and C.R. performed live-broiler chicken studies. M.J.R contributed to the design of the solid agar mating experiment. A.O., J.L., G.Z., and D.C. performed bacteriological analyses, antibiotic susceptibility testing, DNA extraction and qPCR. G.Z. and A.O. performed Illumina whole genome

821 sequencing. A.O. performed cecal shotgun and Hi-C library preparation. T.L. made 16S
822 rRNA gene libraries and sequencing. B.Z. performed 16S rRNA bacterial community
823 analysis and interpretation. A.O., Z.A., M.O.P., I.L., J.R.G., C.W., J.C.T., S.M.L., and
824 R.W. performed bioinformatic analyses and data curation. A.O., J.L. and D.C.
825 performed solid agar mating experiment. A.O., and B.Z. performed statistical analyses.
826 A.O., Z.A., N.A.C., B.Z., R.W., M.O.P., J.R.G., S.M.L., G.Z., and J.L. drafted the
827 manuscript, which was reviewed and edited by all authors. A.O., Z.A., and S.A.A.
828 supervised the study.

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850 Table 1. Plasmid incompatibility groups and replicons found in cecal metagenome assembled genomes
851 using PlasmidFinder[a].
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	Hi-C-FL1	Hi-C-FL2	Hi-C-RL1	Hi-C-RL2
Gram-negative	Col(MG828)	Col(MG828)	Col(MG828)	Col(MG828)
	Col156	ColpVC	Col(pHAD28)	Col156
	ColpVC	ColRNAI	Col156	IncFIB
	ColRNAI	IncB/O/K/Z	ColRNAI	IncFII(pCoo)
	IncFIB	IncFIB	IncFIB	IncHI1B(pNDM-CIT)
	IncFIC(FII)	IncFII(pSFO)	IncFII(pSFO)	IncI1-I(Alpha)
	IncFII(pRSB107)	IncI2	IncI1-I(Alpha)	

	IncHI1B(pNDM-CIT) IncI1-I(Alpha) IncI2(Delta) IncX1 IncX2 p0111	IncX1 IncX2	IncI2 IncX1 IncX2 IncY	
Gram-positive	rep2 repA(pB82)	None	rep18b	repA(pB82)

853 [a] Sample IDs; FL = Fresh litter, RL = Reused litter.
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867 Table 2. IncI1 copy number in *S*. Heidelberg isolates recovered from fresh and reused litter.

S. Heidelberg Strain ID	Time (h)	pH of cecal extract	IncI1 copy number/cell[a](S.D)	Coefficient of variation (%)
SH-16-0-5B-RL	2	6.52	0.001±0.001	155.8
SH-IncI1-FL	2	6.52	5.594±0.582	10.4
SH-16-0-5B-RL	0.5	2.52	0.001±0.0003	47.9
	4	2.52	0.141±0.064	46.0
	24	2.52	0.084±0.038	46.4

SH-IncI1-FL	0.5	2.52	6.304±0.165	2.6
	4	2.52	6.301±0.343	5.4
	24	2.52	5.892±0.378	6.4

868 [a]Three individual *S*. Heidelberg isolates from fresh litter (FL) were used to determine the copy number of
869 IncI1 for SH-IncI1-FL, while IncI1 copy number in *S*. Heidelberg strain SH-16-0-5B from reused litter (RL)
870 was determined using three replicates.
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888 **Figure Captions**

889 **Fig 1. *S*. Heidelberg concentration in the ceca and litter of broiler chicks**. Box plot
890 of nal^R *S*. Heidelberg concentration in the (**a**) ceca (n=10 for oral and cloacal, n=15 for
891 seeder) (**b**) and litter (n = 2 subsamples from pooled litter) of chicks raised on fresh or
892 reused litter ($P > 0.05$; Wilcoxon signed-rank test). Cecal and litter samples were

893 collected 14 days after challenging chicks with nal^R *S*. Heidelberg through oral gavage,
894 cloacal inoculation or through the seeder method.
895
896 **Fig 2. Broiler chicks raised on reused litter carried lower levels of antimicrobial**
897 **resistant *S*. Heidelberg.** (**a**) Distribution of antibiotic resistance acquired by nal^R S.
898 Heidelberg isolates from the ceca and litter of chicks on fresh (n=158) and reused litter
899 (n=141). (**b**) Maximum likelihood tree constructed using accessory genes present in *S*.
900 Heidelberg isolates ($n = 30$) recovered from the ceca and litter of cloacally inoculated
901 chicks raised on fresh and reused litter. The GTR model of nucleotide substitution and
902 the GAMMA model of rate heterogeneity were used for sequence evolution prediction.
903 Numbers shown next to the branches represent the percentage of replicate trees where
904 associated isolates cluster together based on ~100 bootstrap replicates. All *S*.
905 Heidelberg strains were assembled using Illumina short reads except SH4-3A and SH-
906 ancestral that were assembled by combining Illumina short reads with PacBio or
907 MinION long reads. The tree was rooted with the ancestral *S*. Heidelberg strain (SH-
908 ancestral, Genbank accession no: CP066851). Green colored subclade shows strains
909 that harbored IncI1-pST26. All isolates are expected to be resistant to Nalidixic acid.
910 (Nal, nalidixic acid; Gen, gentamicin; Str, streptomycin; Tet, tetracycline; Fox, Cefoxitin;
911 Cip, Ciprofloxacin)
912
913 **Fig 3. Reused litter samples harbored a uniform and diverse microbiome than**
914 **fresh litter samples.** (**a**) Principal coordinates analysis of Bray-Curtis distances based
915 on 16S rRNA gene libraries obtained from ceca and litter samples. Each point is values
916 from individual libraries with colors denoting the route used for *S*. Heidelberg challenge
917 for respective samples. (b) Average alpha diversity indices of rarefied ceca and litter
918 samples grouped by the litter age/type and the route of *S*. Heidelberg challenge. Boxes
919 indicate the interquartile range (75th to 25th) of the data. Whiskers extend to the most
920 extreme value within 1.5 * interquartile range and dots represent outliers beyond that
921 range. Black bar represents the median. (**c)** Phylum-level classification of 16S rRNA
922 gene sequence reads in each ceca and litter sample grouped by the route of *S*.
923 Heidelberg challenge. 16S rRNA gene sequencing was performed on the ceca (59
924 chicks from fresh litter (n = 30) and reused litter (n = 29)) and litter (n = 22 (11 samples
925 each from fresh and reused litter)).
926
927 **Fig 4. *S*. Heidelberg challenge modulated the microbiome of the ceca and litter of**
928 **broiler chicks.** Plots of amplicon sequence variants (ASVs) that were significantly
929 differentially abundant ($p_{adj} < .05$) in the (**a**) ceca and (**b**) litter of challenged chicks on
930 fresh and reused litter compared to uninoculated controls. Significant ASVs are plotted
931 individually and colored according to their family-level classification. The route of *S*.
932 Heidelberg challenge is shown on the edge of each plot as colored squares.
933
934 **Fig 5. Enterobacteriaceae and Clostridiales were the bacterial hosts of AMR in**
935 **broiler chicks**. AMR gene hosts found by Hi-C contacts. AMR genes (horizontal axis of
936 heatmap) associated with metagenome-assembled genomes (MAGs) present in cecal
937 samples from chicks on fresh litter (Hi-C-FL1 and Hi-C-FL2) and reused litter (Hi-C-RL1
938 and Hi-C-RL2). MAGs and AMR gene sources were derived from Hi-C based

deconvolution of the metagenome assembly and placed into a bacterial phylogeny using Mash and CheckM by the ProxiMeta platform. The MAG assigned to family Enterobacteriaceae in each sample matches most closely to an *Escherichia coli* genome. (Legend: Gold - AMR genes that are associated with plasmid sequences on the same contigs, Cyan – AMR genes that are likely to be genomically integrated, Black – no contact above statistical thresholds)

Fig 6. *Escherichia coli* was the main reservoir of plasmids and AMR in broiler chicks. Pan genome analysis was performed on *E. coli* strains (n =19) recovered from the ceca of broiler chicks challenged with *S*. Heidelberg and raised on fresh (FL) (n = 2) or reused litter (RL) (n=2). Maximum likelihood tree constructed using the core (**b**) and accessory **(c**) genes of the *E. coli* strains. Illumina short reads were combined with either PacBio or MinION long reads to assemble the genomes of *E. coli* strains Ec-FL1-1X, Ec-FL1-2X, Ec-FL1-3, Ec-RL2-1 and Ec-RL2-1X. GTR model of nucleotide substitution and GAMMA model of rate heterogeneity was used for sequence evolution prediction. Numbers shown next to the branches represent the percentage of replicate trees where associated isolates cluster together based on ~100 bootstrap replicates. Clade numbers were assigned arbitrarily to ease discussion on the differences in phylogeny between isolates. Tree was rooted with Ec-RL2-1X. (Note: cgMLST – core genome Multi Locus Sequence Type). Black rectangular box shows the *E. coli* strain carrying identical IncI1-pST26 as *S*. Heidelberg.

Fig 7. Relative abundance of IncI1 in Enterobacteriaceae bacterial population of the ceca. Box plot of the ratio of IncI1 gene copies/gram of ceca to gene copies of *gapA* /gram of ceca for chicks raised on fresh (oral = 9, cloacal = 8, control = 9) and reused (oral = 10, cloacal = 9, control = 10) litter. (***$P < 0.001$; Wilcoxon signed-rank test).

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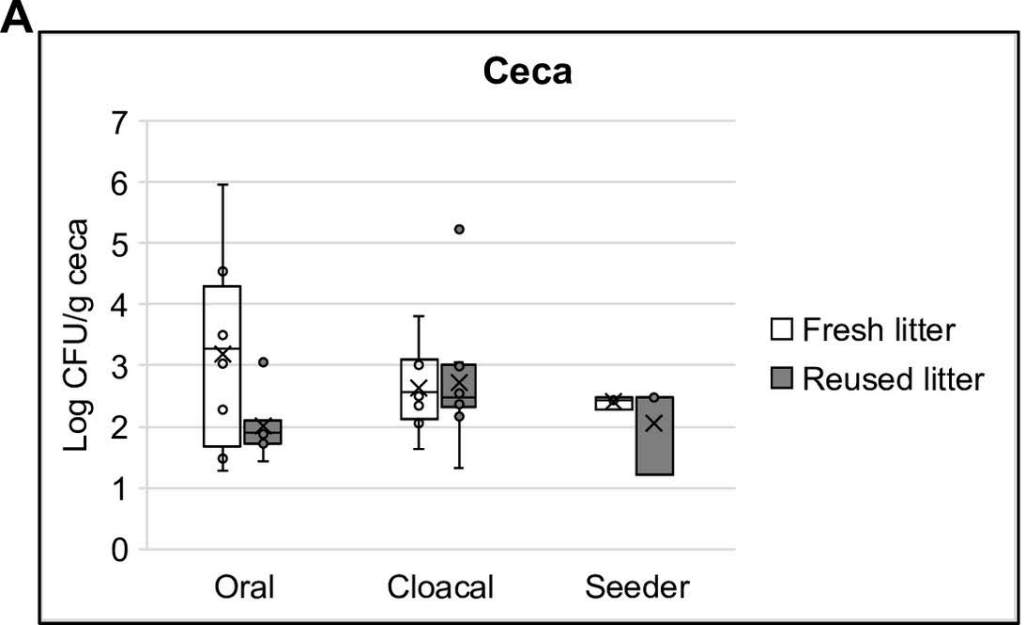
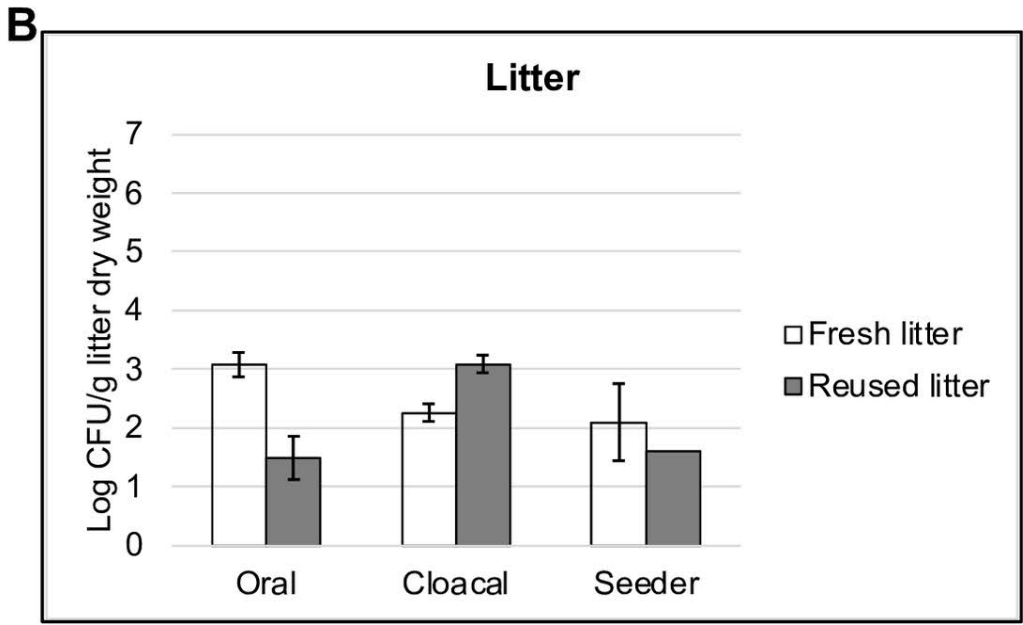
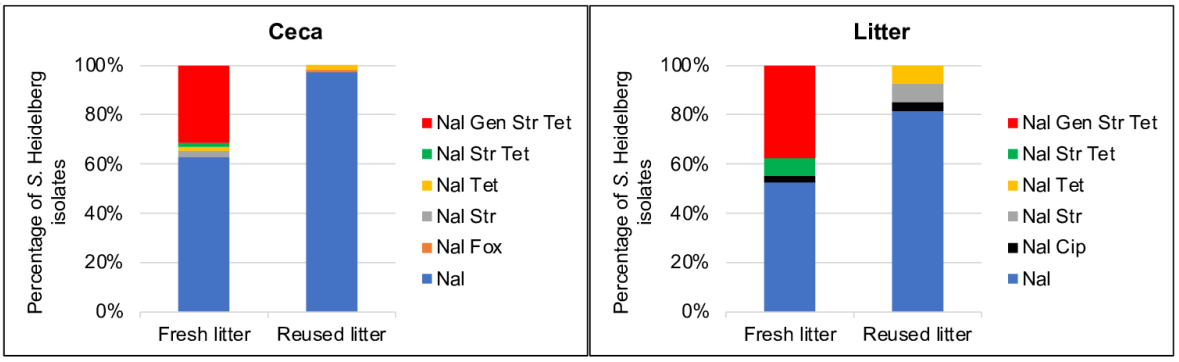
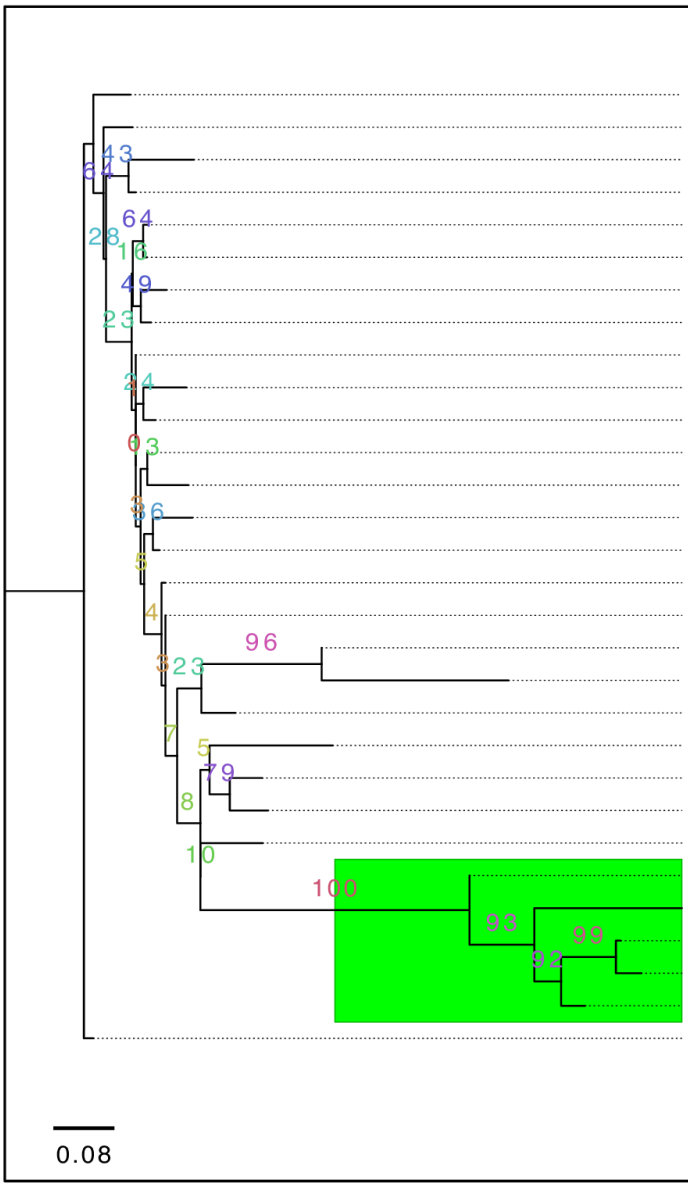

A
Ceca
Log CFU/g ceca
7
6
5
4
3
2
1
0
Oral
Cloacal
Seeder
Fresh litter
Reused litter


B
Litter
Log CFU/g litter dry weight
7
6
5
4
3
2
1
0
Oral
Cloacal
Seeder
Fresh litter
Reused litter

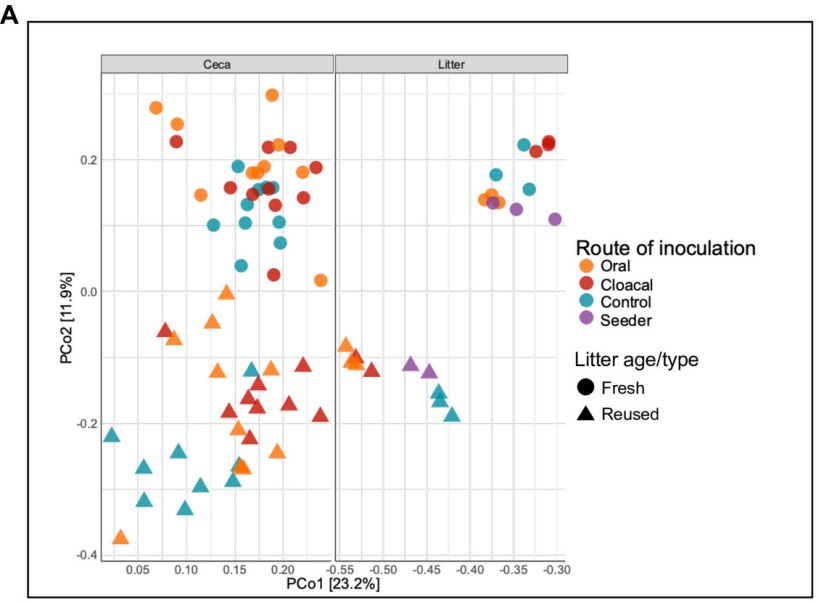
A

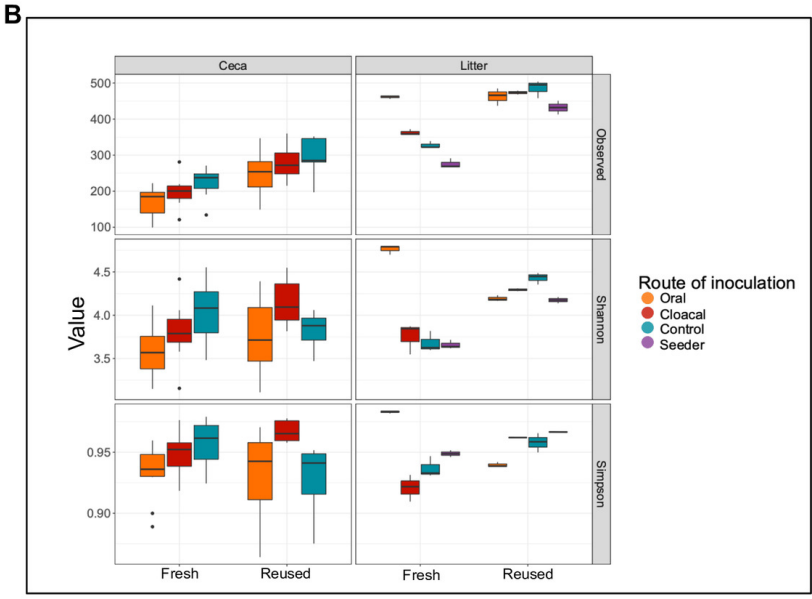


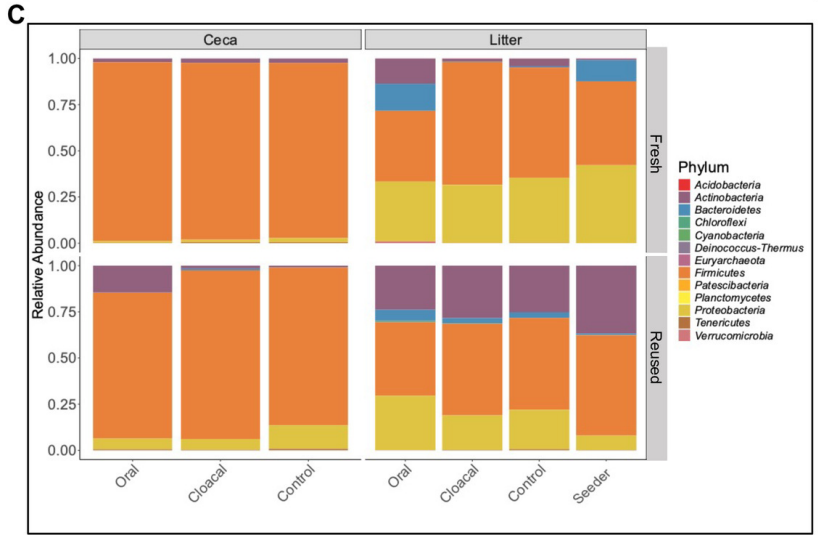
B



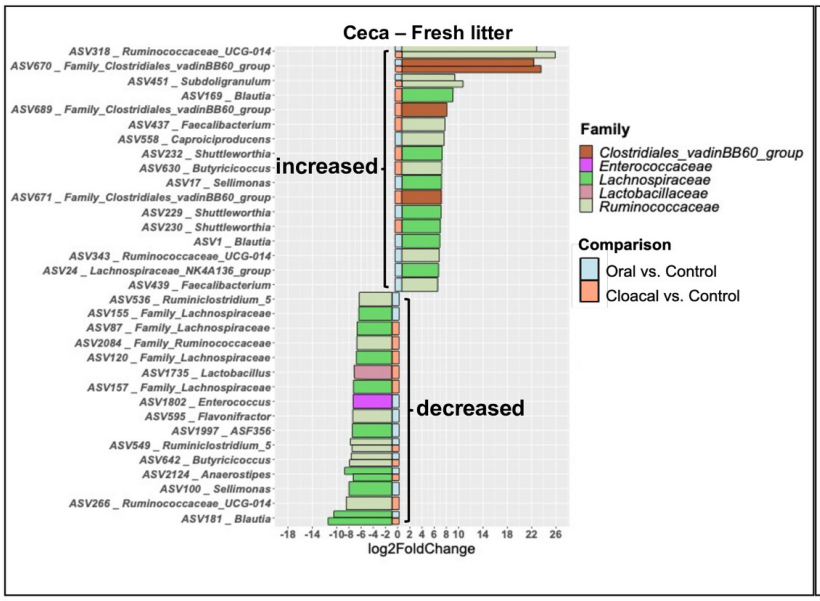
	Litter age/type	Isolation Source	AMR phenotype	Acquired ARG	Plasmids	cgMLST
SH4-2A	Fresh	Ceca	Nal	None	IncX1	186980
SH-FL2-0-5A	Fresh	Litter	Nal	None	IncX1	186980
SH20-4A	Reused	Ceca	Nal	None	IncX1	186980
SH-FL1-3A	Fresh	Litter	Nal	None	IncX1	186980
SH-FL1-2A	Fresh	Litter	Nal	None	IncX1	186980
SH6-2B	Fresh	Ceca	Nal	None	IncX1	186980
SH6-1C	Fresh	Ceca	Nal	None	IncX1	186980
SH6-2A	Fresh	Ceca	Nal	None	IncX1	186980
SH16-1B	Reused	Ceca	Nal	None	IncX1	186980
SH16-1A	Reused	Ceca	Nal	None	IncX1	186980
SH16-0-5C	Reused	Ceca	Nal	None	IncX1	186980
SH20-0-5C	Reused	Ceca	Nal	None	IncX1	186980
SH20-4C	Reused	Ceca	Nal	None	IncX1	186980
SH-RL1-0-5A	Reused	Litter	Nal, Str	None	IncX1	186980
SH-RL2-4A	Reused	Litter	Nal	None	IncX1	186980
SH-RL2-0-5A	Reused	Litter	Nal	None	IncX1	186980
SH6-2C	Fresh	Ceca	Nal	None	IncX1	186980
SH16-0-5A	Reused	Ceca	Nal	None	IncX1	186980
SH20-0-5A	Reused	Ceca	Nal	None	IncX1	186980
SH6-1B	Fresh	Ceca	Nal	None	IncX1	186980
SH20-0-5B	Reused	Ceca	Nal	None	IncX1,Col8282, ColRNAI	186980
SH4-0-5A	Fresh	Ceca	Nal	None	IncX1,Col8282, ColRNAI	186980
SH4-1A	Fresh	Ceca	Nal	None	IncX1, Col8282, ColRNAI	186980
SH4-0-5B	Fresh	Ceca	Nal	None	IncX1,Col8282, ColRNAI	171575
SH-FL1-0-5A	Fresh	Litter	Nal, Gen, Str, Tet	aac(3)-Via, aadA1, tetA	IncX1, IncI1	186980
SH16--0-5B	Reused	Ceca	Nal	aac(3)-Via, aadA1, tetA	IncX1, IncI1, Col8282	186980
SH4-3A	Fresh	Ceca	Nal, Gen, Str, Tet	aac(3)-Via, aadA1, tetA	IncX1, IncI1, Col8282	186980
SH4-1B	Fresh	Ceca	Nal, Gen, Str, Tet	aac(3)-Via, aadA1, tetA	IncX1,IncI1	186980
SH6-1A	Fresh	Ceca	Nal, Gen, Str, Tet	aac(3)-Via, aadA1, tetA	IncX1, IncI1, Col8282	186980
SH-ancestral	NA	NA	Nal	None	IncX1	171516

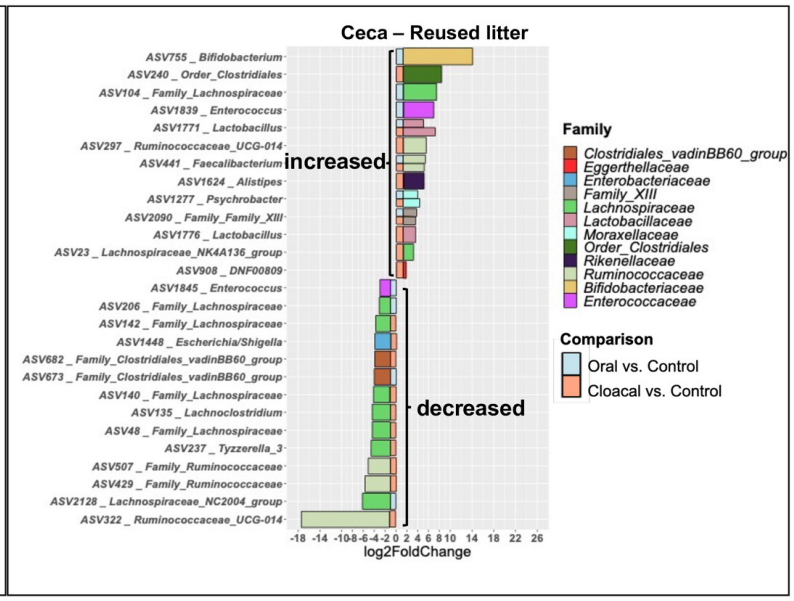

A
Ceca
Litter
PCo2 [11.9%]
PCo1 [23.2%]
Route of inoculation
Oral
Cloacal
Control
Seeder
Litter age/type
Fresh
Reused


B
Ceca
Litter
Observed
Shannon
Simpson
Value
Fresh
Reused
Route of inoculation
Oral
Cloacal
Control
Seeder

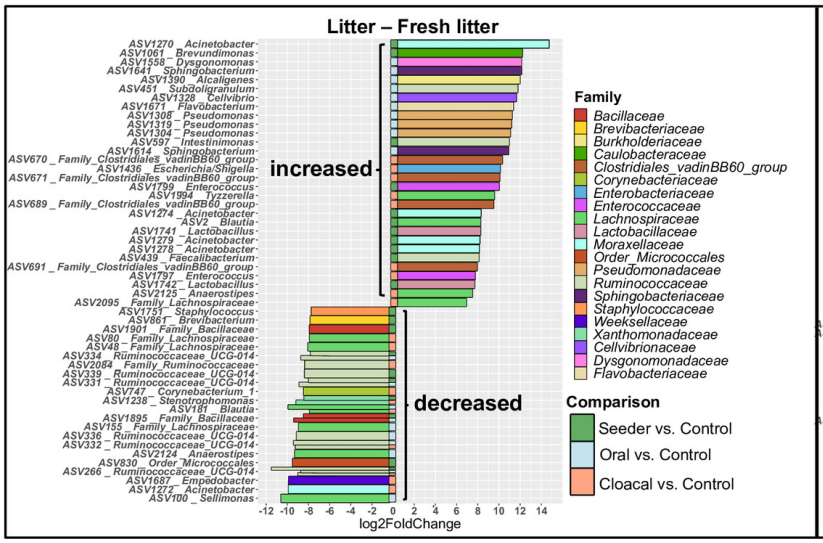

C
Ceca
Litter
Fresh
Reused
Relative Abundance
Phylum
Acidobacteria
Actinobacteria
Bacteroidetes
Chloroflexi
Cyanobacteria
Deinococcus-Thermus
Euryarchaeota
Firmicutes
Patescibacteria
Planctomycetes
Proteobacteria
Tenericutes
Verrucomicrobia
Oral
Cloacal
Control
Seeder

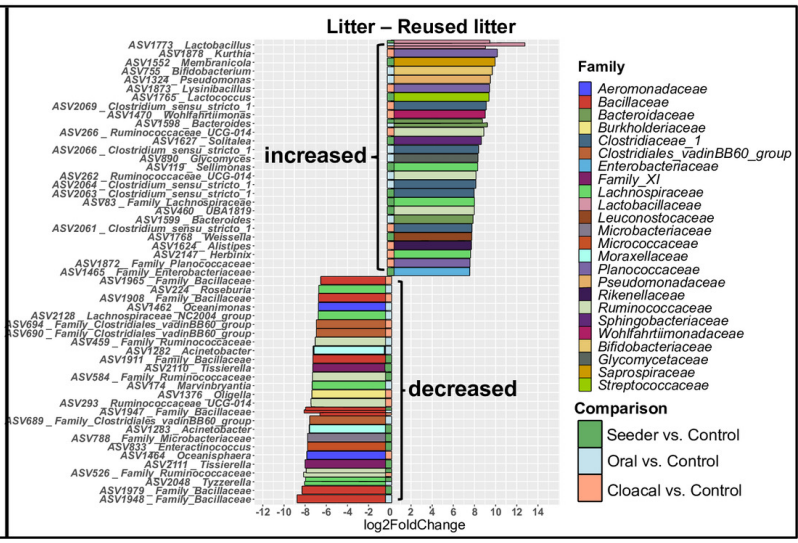
A

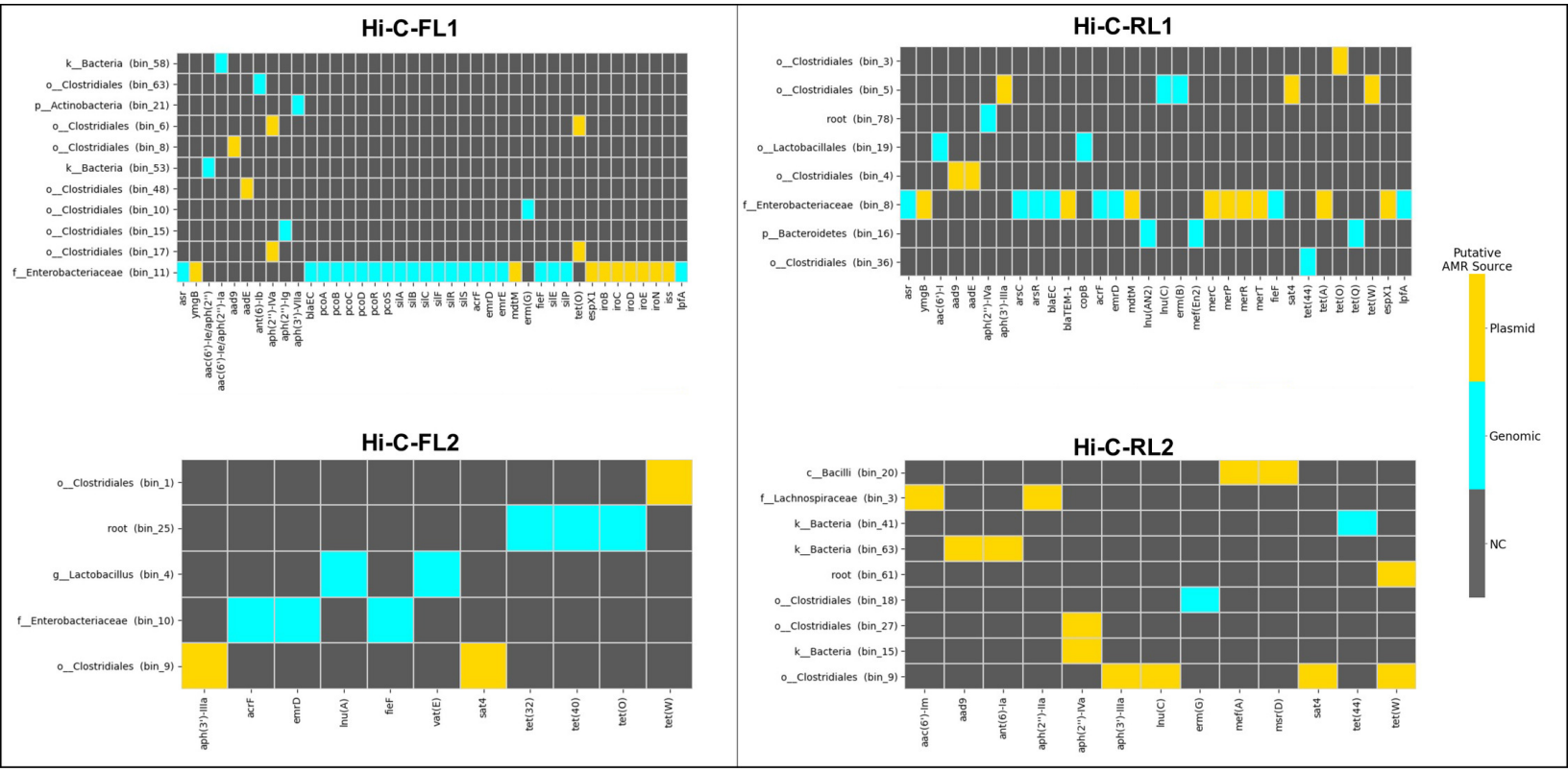

Ceca – Fresh litter
ASV318 _ Ruminococcaceae_UCG-014
ASV670 _ Family_Clostridiales_vadinBB60_group
ASV451 _ Subdoligranulum
ASV169 _ Blautia
ASV689 _ Family_Clostridiales_vadinBB60_group
ASV437 _ Faecalibacterium
ASV558 _ Caproicipriodcens
ASV232 _ Shuttleworthia
ASV630 _ Butyricicoccus
ASV17 _ Sellimonas
ASV671 _ Family_Clostridiales_vadinBB60_group
ASV229 _ Shuttleworthia
ASV230 _ Shuttleworthia
ASV1 _ Blautia
ASV343 _ Ruminococcaceae_UCG-014
ASV24 _ Lachnospiraceae_NK4A136_group
ASV439 _ Faecalibacterium
ASV536 _ Ruminiclostridium_5
ASV155 _ Family_Lachnospiraceae
ASV87 _ Family_Lachnospiraceae
ASV2084 _ Family_Ruminococcaceae
ASV120 _ Family_Lachnospiraceae
ASV1735 _ Lactobacillus
ASV157 _ Family_Lachnospiraceae
ASV1802 _ Enterococcus
ASV595 _ Flavonifractor
ASV1997 _ ASF356
ASV549 _ Ruminiclostridium_5
ASV642 _ Butyricicoccus
ASV2124 _ Anaerostipes
ASV100 _ Sellimonas
ASV266 _ Ruminococcaceae_UCG-014
ASV181 _ Blautia
increased
decreased
-18 -14 -10 -8 -6 -4 -2 0 2 4 6 8 10 14 18 22 26
log2FoldChange
Family
Clostridiales_vadinBB60_group
Enterococcaceae
Lachnospiraceae
Lactobacillaceae
Ruminococcaceae
Comparison
Oral vs. Control
Cloacal vs. Control

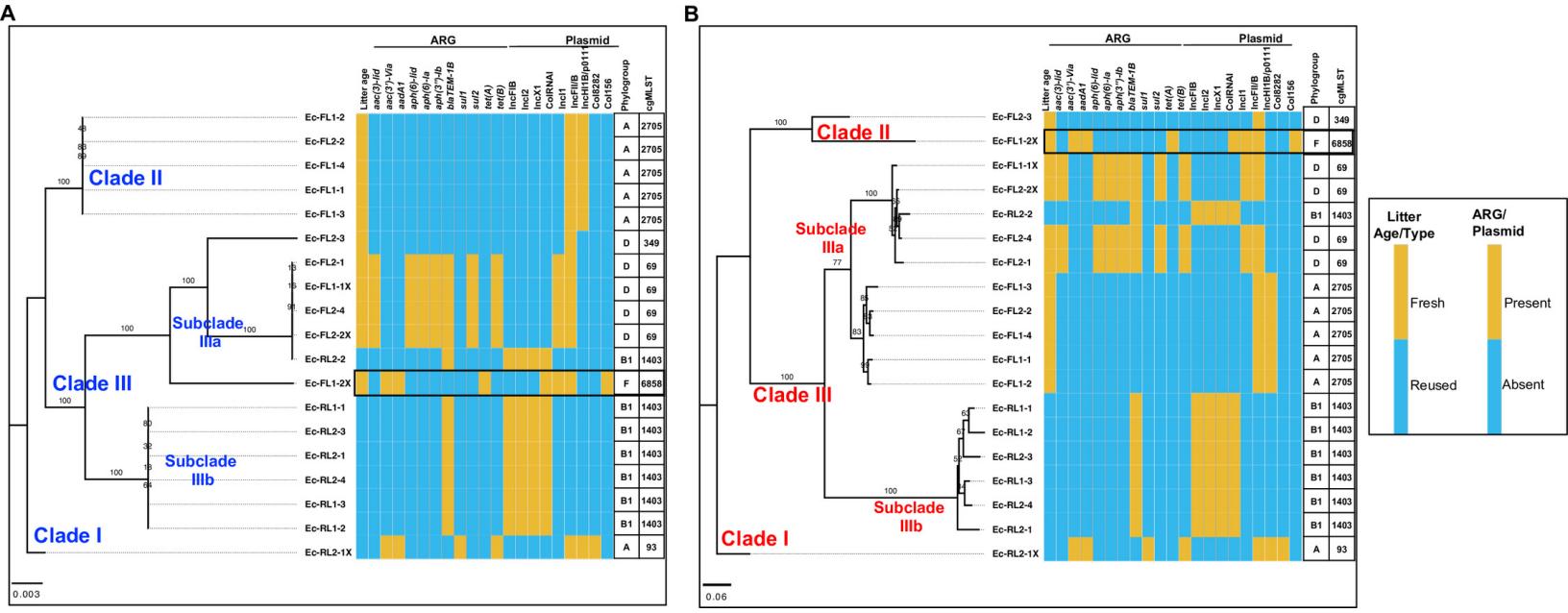

Ceca – Reused litter
ASV755 _ Bifidobacterium
ASV240 _ Order_Clostridiales
ASV104 _ Family_Lachnospiraceae
ASV1839 _ Enterococcus
ASV1771 _ Lactobacillus
ASV297 _ Ruminococcaceae_UCG-014
ASV441 _ Faecalibacterium
ASV1624 _ Alistipes
ASV1277 _ Psychrobacter
ASV2090 _ Family_Family_XIII
ASV1776 _ Lactobacillus
ASV23 _ Lachnospiraceae_NK4A136_group
ASV908 _ DNF00809
ASV1845 _ Enterococcus
ASV206 _ Family_Lachnospiraceae
ASV142 _ Family_Lachnospiraceae
ASV1448 _ Escherichia/Shigella
ASV682 _ Family_Clostridiales_vadinBB60_group
ASV673 _ Family_Clostridiales_vadinBB60_group
ASV140 _ Family_Lachnospiraceae
ASV135 _ Lachnoclostridium
ASV48 _ Family_Lachnospiraceae
ASV237 _ Tyzzerella_3
ASV507 _ Family_Ruminococcaceae
ASV429 _ Family_Ruminococcaceae
ASV2128 _ Lachnospiraceae_NC2004_group
ASV322 _ Ruminococcaceae_UCG-014
increased
decreased
-18 -14 -10 -8 -6 -4 -2 0 2 4 6 8 10 14 18 22 26
log2FoldChange
Family
Clostridiales_vadinBB60_group
Eggerthellaceae
Enterobacteriaceae
Family_XIII
Lachnospiraceae
Lactobacillaceae
Moraxellaceae
Order_Clostridiales
Rikenellaceae
Ruminococcaceae
Bifidobacteriaceae
Enterococcaceae
Comparison
Oral vs. Control
Cloacal vs. Control

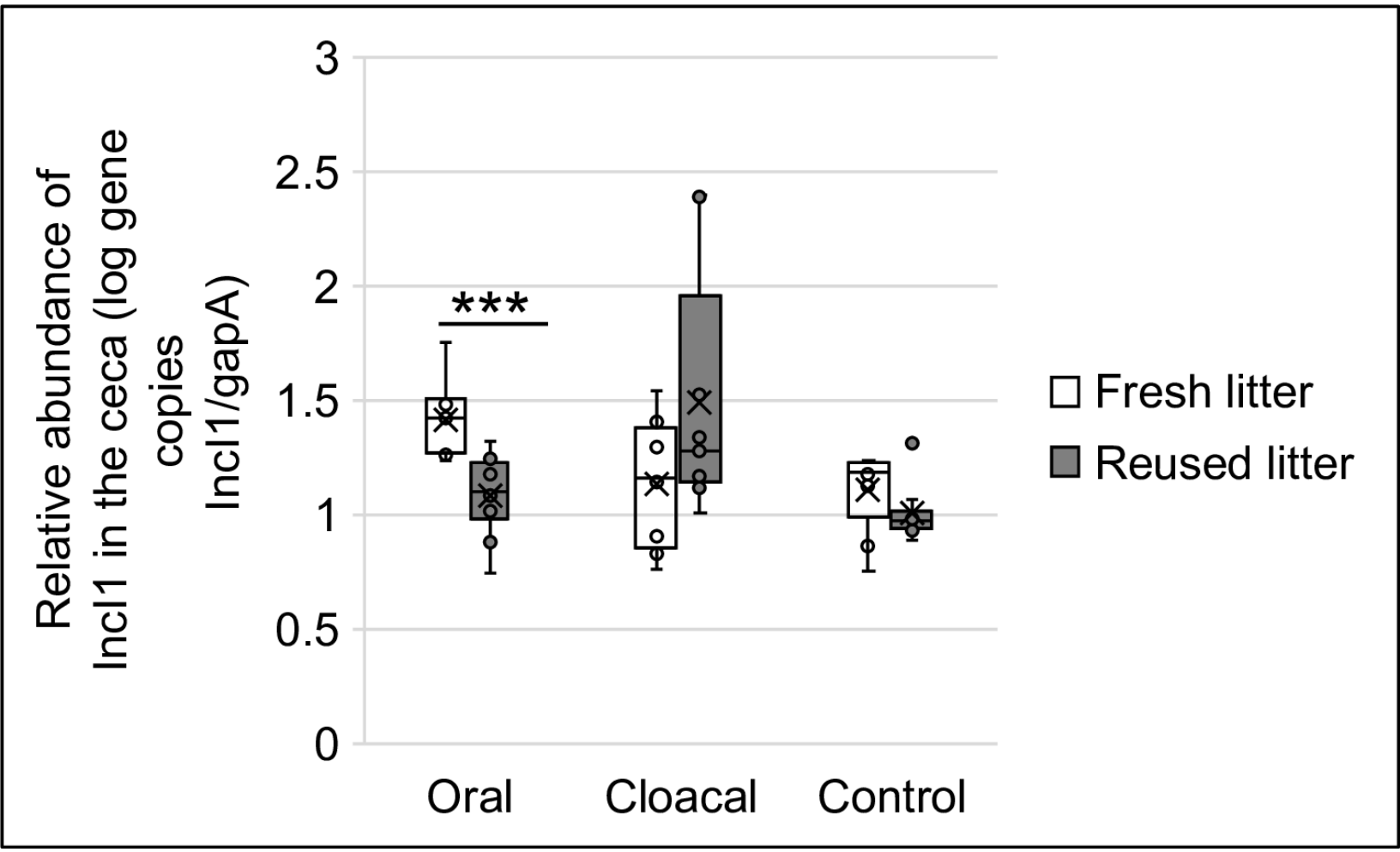
B


Litter – Fresh litter
ASV1270 _ Acinetobacter
ASV1061 _ Brevundimonas
ASV1558 _ Dysgonomonas
ASV1641 _ Sphingobacterium
ASV1390 _ Alcaligenes
ASV451 _ Subdoligranulum
ASV1328 _ Selivibrio
ASV1671 _ Flavobacterium
ASV1308 _ Pseudomonas
ASV1319 _ Pseudomonas
ASV1304 _ Pseudomonas
ASV597 _ Intestinimonas
ASV1614 _ Sphingobacterium
ASV670 _ Family_Clostridiales_vadinBB60_group
ASV1436 _ Escherichia/Shigella
ASV671 _ Family_Clostridiales_vadinBB60_group
ASV1799 _ Enterococcus
ASV1994 _ Tyzzerella
ASV689 _ Family_Clostridiales_vadinBB60_group
ASV1274 _ Acinetobacter
ASV2 _ Blautia
ASV1741 _ Lactobacillus
ASV1279 _ Acinetobacter
ASV1278 _ Acinetobacter
ASV439 _ Faecalibacterium
ASV691 _ Family_Clostridiales_vadinBB60_group
ASV1797 _ Enterococcus
ASV1742 _ Lactobacillus
ASV2125 _ Anaerostipes
ASV2095 _ Family_Lachnospiraceae
ASV1751 _ Staphylococcus
ASV861 _ Brevibacterium
ASV1901 _ Family_Bacillaceae
ASV80 _ Family_Lachnospiraceae
ASV48 _ Family_Lachnospiraceae
ASV334 _ Ruminococcaceae_UCG-014
ASV2064 _ Family_Ruminococcaceae
ASV339 _ Ruminococcaceae_UCG-014
ASV331 _ Ruminococcaceae_UCG-014
ASV747 _ Corynebacterium_1
ASV1238 _ Stenotrophomonas
ASV181 _ Blautia
ASV1895 _ Family_Bacillaceae
ASV155 _ Family_Lachnospiraceae
ASV336 _ Ruminococcaceae_UCG-014
ASV332 _ Ruminococcaceae_UCG-014
ASV2124 _ Anaerostipes
ASV830 _ Order_Micrococcales
ASV266 _ Ruminococcaceae_UCG-014
ASV1687 _ Empedobacter
ASV1272 _ Acinetobacter
ASV100 _ Sellimonas
increased
decreased
-12 -10 -8 -6 -4 -2 0 2 4 6 8 10 12 14
log2FoldChange
Family
Bacillaceae
Brevibacteriaceae
Burkholderiaceae
Caulobacteraceae
Clostridiales_vadinBB60_group
Corynebacteriaceae
Enterobacteriaceae
Enterococcaceae
Lachnospiraceae
Lactobacillaceae
Moraxellaceae
Order_Micrococcales
Pseudomonadaceae
Ruminococcaceae
Sphingobacteriaceae
Staphylococcaceae
Weeksellaceae
Xanthomonadaceae
Cellvibrionaceae
Dysgonomonadaceae
Flavobacteriaceae
Comparison
Seeder vs. Control
Oral vs. Control
Cloacal vs. Control


Litter – Reused litter
ASV1773 _ Lactobacillus
ASV1878 _ Kurthia
ASV1552 _ Membranicola
ASV755 _ Bifidobacterium
ASV1324 _ Pseudomonas
ASV1873 _ Lysinibacillus
ASV1765 _ Lactococcus
ASV2069 _ Clostridium_sensu_stricto_1
ASV1479 _ Wohlfahrtiimonas
ASV1598 _ Bacteroides
ASV266 _ Ruminococcaceae_UCG-014
ASV1627 _ Solitalea
ASV2066 _ Clostridium_sensu_stricto_1
ASV890 _ Glycomyces
ASV119 _ Sellimonas
ASV262 _ Ruminococcaceae_UCG-014
ASV2064 _ Clostridium_sensu_stricto_1
ASV2063 _ Clostridium_sensu_stricto_1
ASV83 _ Family_Lachnospiraceae
ASV460 _ UBA1819
ASV1599 _ Bacteroides
ASV2061 _ Clostridium_sensu_stricto_1
ASV1768 _ Weissella
ASV1624 _ Alistipes
ASV2147 _ Herbinix
ASV1872 _ Family_Planococcaceae
ASV1465 _ Family_Enterobacteriaceae
ASV1965 _ Family_Bacillaceae
ASV224 _ Roseburia
ASV1908 _ Family_Bacillaceae
ASV1462 _ Oceanimonas
ASV2128 _ Lachnospiraceae_NC2004_group
ASV694 _ Family_Clostridiales_vadinBB60_group
ASV690 _ Family_Clostridiales_vadinBB60_group
ASV459 _ Family_Ruminococcaceae
ASV1282 _ Acinetobacter
ASV1911 _ Family_Bacillaceae
ASV2110 _ Tissierella
ASV584 _ Family_Ruminococcaceae
ASV174 _ Marvinbryantia
ASV1376 _ Oligella
ASV293 _ Ruminococcaceae_UCG-014
ASV1947 _ Family_Bacillaceae
ASV689 _ Family_Clostridiales_vadinBB60_group
ASV1283 _ Acinetobacter
ASV788 _ Family_Microbacteriaceae
ASV833 _ Enteractinococcus
ASV1464 _ Oceanisphaera
ASV2111 _ Tissierella
ASV526 _ Family_Ruminococcaceae
ASV2048 _ Tyzzerella
ASV1979 _ Family_Bacillaceae
ASV1948 _ Family_Bacillaceae
increased
decreased
-12 -10 -8 -6 -4 -2 0 2 4 6 8 10 12 14
log2FoldChange
Family
Aeromonadaceae
Bacillaceae
Bacteroidaceae
Burkholderiaceae
Clostridiaceae_1
Clostridiales_vadinBB60_group
Enterobacteriaceae
Family_XI
Lachnospiraceae
Lactobacillaceae
Leuconostocaceae
Microbacteriaceae
Micrococcaceae
Moraxellaceae
Planococcaceae
Pseudomonadaceae
Rikenellaceae
Ruminococcaceae
Sphingobacteriaceae
Wohlfahrtiimonadaceae
Bifidobacteriaceae
Glycomycetaceae
Saprospiraceae
Streptococcaceae
Comparison
Seeder vs. Control
Oral vs. Control
Cloacal vs. Control


Hi-C-FL1
k__Bacteria (bin_58)
o__Clostridiales (bin_63)
p__Actinobacteria (bin_21)
o__Clostridiales (bin_6)
o__Clostridiales (bin_8)
k__Bacteria (bin_53)
o__Clostridiales (bin_48)
o__Clostridiales (bin_10)
o__Clostridiales (bin_15)
o__Clostridiales (bin_17)
f__Enterobacteriaceae (bin_11)
asr
ymgB
aac(6')-Ie/aph(2'')
aac(6')-Ie/aph(2'')-Ia
aad9
aadE
ant(6)-Ib
aph(2'')-IVa
aph(2'')-Ig
aph(3'')-VIIa
blaEC
pcoA
pcoB
pcoC
pcoD
pcoR
pcoS
silA
silB
silC
silF
silR
silS
acrF
emrD
emrE
mdtM
erm(G)
fieF
silE
silP
tet(O)
espX1
iroB
iroC
iroD
iroE
iroN
iss
lpfA
Hi-C-RL1
o__Clostridiales (bin_3)
o__Clostridiales (bin_5)
root (bin_78)
o__Lactobacillales (bin_19)
o__Clostridiales (bin_4)
f__Enterobacteriaceae (bin_8)
p__Bacteroidetes (bin_16)
o__Clostridiales (bin_36)
asr
ymgB
aac(6')-I
aad9
aadE
aph(2'')-IVa
aph(3')-IIIa
arsC
arsR
blaEC
blaTEM-1
copB
acrF
emrD
mdtM
lnu(AN2)
lnu(C)
erm(B)
mef(En2)
merC
merP
merR
merT
fieF
sat4
tet(44)
tet(A)
tet(O)
tet(Q)
tet(W)
espX1
lpfA
Putative AMR Source
Plasmid
Genomic
NC
Hi-C-FL2
o__Clostridiales (bin_1)
root (bin_25)
g__Lactobacillus (bin_4)
f__Enterobacteriaceae (bin_10)
o__Clostridiales (bin_9)
aph(3')-IIIa
acrF
emrD
lnu(A)
fieF
vat(E)
sat4
tet(32)
tet(40)
tet(O)
tet(W)
Hi-C-RL2
c__Bacilli (bin_20)
f__Lachnospiraceae (bin_3)
k__Bacteria (bin_41)
k__Bacteria (bin_63)
root (bin_61)
o__Clostridiales (bin_18)
o__Clostridiales (bin_27)
k__Bacteria (bin_15)
o__Clostridiales (bin_9)
aac(6')-Im
aad9
ant(6)-Ia
aph(2'')-IIa
aph(2'')-IVa
aph(3')-IIIa
lnu(C)
erm(G)
mef(A)
msr(D)
sat4
tet(44)
tet(W)


A
ARG
Plasmid
Litter age
aac(3)-Iid
aac(3')-VIa
aadA1
aph(6)-Iid
aph(6)-Ia
aph(3")-Ib
blaTEM-1B
sul1
sul2
tet(A)
tet(B)
IncFIB
IncI2
IncX1
ColRNAI
IncI1
IncFII/B
IncHI1B/p0111
Col8282
Col156
Phylogroup
cgMLST
Ec-FL1-2 A 2705
Ec-FL2-2 A 2705
Ec-FL1-4 A 2705
Ec-FL1-1 A 2705
Ec-FL1-3 A 2705
Ec-FL2-3 D 349
Ec-FL2-1 D 69
Ec-FL1-1X D 69
Ec-FL2-4 D 69
Ec-FL2-2X D 69
Ec-RL2-2 B1 1403
Ec-FL1-2X F 6858
Ec-RL1-1 B1 1403
Ec-RL2-3 B1 1403
Ec-RL2-1 B1 1403
Ec-RL2-4 B1 1403
Ec-RL1-3 B1 1403
Ec-RL1-2 B1 1403
Ec-RL2-1X A 93
Clade II
Subclade IIIa
Clade III
Subclade IIIb
Clade I
0.003
B
Clade II
Subclade IIIa
Clade III
Subclade IIIb
Clade I
0.06
Litter Age/Type
Fresh
Reused
ARG/ Plasmid
Present
Absent


Relative abundance of IncI1 in the ceca (log gene copies IncI1/gapA)
3
2.5
2
1.5
1
0.5
0
***
Oral
Cloacal
Control
Fresh litter
Reused litter