Gene flux and acid-imposed selection are the main drivers of antimicrobial resistance in broiler chicks infected with *Salmonella enterica* serovar Heidelberg

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Abstract

Antimicrobial resistance (AR) spread is a worldwide health challenge, stemming in large part, from the ability of microbes to share their genetic material through horizontal gene transfer (HGT). Overuse and misuse of antibiotics in clinical settings and in food production have been linked to this increased prevalence and spread of AR. Consequently, public health and consumer concerns have resulted in a remarkable recent reduction in antibiotics used for food animal production. This is driven by the assumption that removing this selective pressure will favor the recovery of antibiotic susceptible taxa and will limit AR sharing through HGT, allowing the currently available antibiotic arsenal to be effective for a longer period. In this study we used broiler chicks raised antibiotic-free and *Salmonella enterica* serovar Heidelberg (SH), as a model food pathogen, to test this hypothesis. Our results show that neonatal broiler chicks challenged with an antibiotic susceptible SH strain and raised without antibiotics carried susceptible and multidrug resistance SH strains 14 days after challenge. SH infection perturbed the microbiota of broiler chicks and gavaged chicks acquired antibiotic resistant SH at a higher rate. We determined that the acquisition of a plasmid from commensal *Escherichia coli* population conferred multidrug resistance phenotype to SH recipients and carriage of this plasmid increased the fitness of SH under acidic selection pressure. These results suggest that HGT of AR shaped the evolution of SH and that antibiotic use reduction alone is insufficient to limit antibiotic resistance transfer from commensal bacteria to *Salmonella*.

45 ## Importance

46 The reported increase in antibiotic resistant bacteria in humans have resulted in a major
47 shift away from antibiotics use in food animal production. This has been driven by the
48 assumption that removing antibiotics will select for antibiotic susceptible bacterial taxa,
49 and this in turn will allow the currently available antibiotic arsenal to be more effective.
50 This shift in practice has highlighted new questions that need to be answered to assess
51 the effectiveness of antibiotic removal in reducing the spread of antibiotic resistance
52 bacteria. This research demonstrates that antibiotic susceptible *Salmonella* Heidelberg
53 strains can acquire multidrug resistance from commensal bacteria present in the gut of
54 neonatal broiler chicks, even in the absence of antibiotic selection. We demonstrate that
55 exposure to acidic pH drove the horizontal transfer of antimicrobial resistance plasmids
56 and suggests that simply removing antibiotics from food-animal production might not be
57 sufficient to limit the spread of antimicrobial resistance.
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61 # Introduction

62 Antimicrobial resistance (AR) spread is a worldwide health challenge[1,2]. A major aspect
63 of this challenge stems from the ability of microbes to share their genetic material through
64 horizontal gene transfer (HGT)[3]. Exposure to antibiotics can select for a microbiota of
65 commensals and pathogens that can withstand antibiotic selective pressure, creating a
66 potential reservoir of resistance genes that are shared with susceptible taxa allowing for
67 their survival and proliferation[3]. Overuse and misuse of antibiotics in a clinical setting[4]
68 and in food production[5] have been shown to result in such selective environments
69 contributing to the increased prevalence and spread of AR. These public health concerns
70 have resulted in a major shift away from antibiotics use in food production. This was driven
71 by the assumption that removing this selective pressure will favor the recovery of
72 susceptible taxa, and that this in turn will allow the currently available antibiotic arsenal to
73 be effective for a longer period[6-9]. This shift in food production practices has highlighted
74 new questions that need to be answered to understand and assess the effectiveness of
75 antibiotic removal in reducing the spread and prevalence of AR in food borne pathogens.

76 There has been a remarkable reduction in antibiotics sold or distributed in the United
77 States (US) for use in food-producing animals since the publication of the US Food and
78 Drug Administration's guidance for industry #209 (2012) and #213 (2013) and the
79 Veterinary Feed Directive[10]. Chicken production accounted for the largest reduction in
80 medically and nonmedically important antibiotics sold (~47% from 2016 to 2017), also
81 driven by consumer concerns over food safety related to antibiotics use[11]. *Salmonella*
82 *enterica* serovar Heidelberg (SH) strain is one of the prolific serovars causing
83 salmonellosis in the US and poultry is by far the major vector for SH infections[12-14] . One

84 of the largest food-borne outbreaks in US history was associated with the consumption
85 of chicken contaminated with SH[15]. During this outbreak, SH was present in blood
86 samples of case-patients (15%) and resulted in an overall hospitalization rate of 38% (200
87 case-patients). Poultry and clinical strains of SH can be resistant to at least 1 antibiotic in
88 3 or more drug classes. Therefore, we focused on studying antibiotic-free broiler chicken
89 production, the most consumed meat in the US, with a goal to evaluate the prevalence of
90 AR and its potential transfer to SH, an important model pathogen.

91 Our results suggested that *Salmonella* can acquire multidrug resistance from the
92 bacteria of neonatal broiler chicks raised without antibiotics. We determined that *E. coli*
93 strains carrying IncI1 plasmids were the main reservoir of transferable AR in broiler chicks
94 and confirmed that *E. coli* was the donor of IncI1-ST26 (pST26) carrying antibiotic, metal,
95 and disinfectant genes to SH. The presence of pST26 did not impose a fitness cost to the
96 SH host and exposure to acidic pH posed a selection pressure on pST26 plasmid
97 population. Commensals like *E. coli* are a reservoir of mobile genetic elements including
98 plasmids and bacteriophages. Therefore, we conclude that a simple reduction in antibiotic
99 use might not be sufficient to prevent transfer of these mobile elements to a major food-
100 borne pathogen like *Salmonella.*
101

102 # Results

103 **S. Heidelberg abundance in ceca and litter is influenced by the route of challenge.**
104 To investigate if the route of exposure to SH affected intestinal and litter colonization, we
105 challenged 150 one day old broiler chicks with a nalidixic (nal) resistant strain of SH (SH-
106 2813_{nal}^{R}) either by oral gavage (OG), intracloacally (IC) or by the seeder (SB) method
107 (i.e., a few chicks (n =5) were orally challenged and co-mingled with unchallenged chicks
108 (n = 20)) (Fig. 1a). Chicks were grown for 14 days on fresh pine shavings in four separate
109 floor pens (1.8 L ×1.16 W m or 5.9 L×3.8 W ft) including an unchallenged control (CTRL)
110 group (n=25). Chicks were not administered any medication or antibiotics for the duration
111 of the study. The challenge experiments, trial 1 and trial 2, were performed in September
112 2017 and April 2018, respectively and we have published the rate SH colonized the
113 ceca[16]. SH concentration in the ceca of gavaged chicks (SB and OG) was higher than IC
114 for trial 1 (H = 0.94, df =2, P = 0.62) and OG was higher than IC for trial 2 (H = 10.24, df
115 =2, P = 0.006) (Fig. 1b). In the litter, SH levels were higher for IC pens compared to SB
116 and OG in trial 1 and higher in OG pens compared to IC for trial 2 (Fig. 1c). The
117 percentage of chicks that had SH detected in their ceca was 80% for OG and SB and
118 100% for IC during trial 1. For trial 2, 100% of the OG and IC were positive and 40% for
119 SB[15]. The ceca and litter of CTRL were negative for SH by culture; however, one litter
120 sample was positive after 24 h enrichment in buffered peptone water. Litter pH and
121 moisture was higher for trial 1 (pH = 6.86 ± 0.27; moisture = 25± 0.03 %) compared to
122 trial 2 (6.54 ± 0.17; moisture = 21± 0.03 %) (Extended data Fig. 1a and b).

123 **S. Heidelberg perturbed the ceca and litter bacteria in a challenge route-specific**
124 **manner**. Next, we questioned if SH gut colonization perturbed changes in the ceca and

125 litter microbiota. We profiled the microbial community present in the ceca and litter using
126 16S ribosomal RNA gene amplicon sequencing. The bacterial alpha diversity was higher
127 for litter samples compared to the ceca for OG for all alpha diversity measures (Observed,
128 Chao1, Shannon and InvSimpson) examined ($P < 0.01$) (Extended data Fig. 2a). In
129 contrast, ceca from OG had lowest alpha diversity for all measures used compared to
130 CTRL ($P < 0.05$). Observed and Chao1 alpha diversity indices were higher for litter
131 compared to ceca for CTRL and IC ($P = 0.007$). Beta diversity differed between litter and
132 ceca and the route of SH challenge affected the beta diversity of the litter (F = 43.7, df =
133 11, $P = 0.001$) more than the ceca (F = 2.21, df = 29 $P = 0.007$). Cecal samples were
134 more dispersed than litter samples and OG chicks had the highest beta diversity (Fig. 2a).
135 *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Bacteriodetes* were the dominant
136 phyla present in litter (Fig. 2b). Litter from OG and SB carried higher abundance of
137 *Bacteriodetes*, whereas IC and CTRL carried higher abundance of *Firmicutes* than OG
138 and SB. *Firmicutes* dominated the cecal bacteria of broiler chicks and *Ruminococcaceae*
139 and *Lachnospiraceae* (class *Clostridia*) were the major families. *Proteobacteria* was
140 higher in the ceca of CTRL than OG or IC (X^2= 0.6412, P=0.72) (Fig.2b). Amplicon
141 sequence variants (ASV) matching *Subdolinagrum*, *Lactobacillus* and
142 *Family_Lachnospiraceae* were the most abundant ASVs in OG and IC compared to CTRL
143 in the ceca (Extended data Fig.2b). In contrast, *E. coli/Shigella* (*E. coli* and *Shigella* are
144 not distinguishable by 16S rRNA gene sequencing) and *Klebsiella* were the most
145 abundant ASVs in litter. *E. coli* was higher in IC and CTRL compared to OG and SB and
146 *Klebsiella* was higher in CTRL compared to OG, IC and SB (*E. coli*: X^2= 9.9744, P=0.018;
147 *Klebsiella*: X^2=9.6667, P=0.021). This result suggested that SH perturbed the bacterial

148 community present in the litter and ceca of broiler chicks especially the
149 *Enterobacteriaceae*.

150 **S. Heidelberg developed multidrug resistance after IncI1 plasmid acquisition.** We
151 investigated if SH isolates developed AR after 14 days of challenge. We used the national
152 antimicrobial resistance monitoring system sensititre ™ panel for Gram-negative bacteria
153 to conduct antibiotic susceptibility testing (AST) on 250 SH isolates recovered from either
154 the ceca or litter of challenged chicks. SH-2813$_{nal}^{R}$ carries a *gyrA* mutation for nal
155 resistance, therefore all isolates recovered were resistant to nal. SH also carries a
156 chromosome-encoded *fosA7* gene that confers resistance to fosfomycin[17]. In trial 1, only
157 2 % of SH isolates (n = 92) developed resistance to an antibiotic. These two isolates were
158 resistant to gentamicin, streptomycin and sulfisoxazole. For trial 2, 40 % of the isolates
159 developed resistance to at least one antibiotic (Fig. 1d) and > 86 % of them were resistant
160 to 2 or more drug classes like the isolates from trial 1 (Extended data Fig. 3a). However,
161 trial 2 isolates developed AR to tetracyclines instead of sulfisoxazole. A disc diffusion
162 assay on a selected number of multidrug resistant (MDR) isolates (n = 4) revealed that
163 MDR isolates were also resistance to tobramycin, netilmicin and kanamycin (data not
164 shown). Additionally, OG chicks carried a higher percentage (46 %) of MDR isolates than
165 IC (24 %) or SB (8%) chicks (X^2 = 29.2, df = 2, P < 0.001) (Fig. 1e).

166 To find AR determinants responsible for the acquired AR phenotype, we performed
167 whole genome sequencing (WGS) using illumina short read sequencing technology on
168 sixty-nine SH isolates from trial 2 and two isolates from trial 1. We focused our resources
169 on trial 2, for which AR acquisition was higher than trial 1. The isolates sequenced were
170 either susceptible (n = 33) or antibiotic resistant (n = 38). The ancestral SH-2813$_{nal}^{R}$

171 (isolate used for challenge) harbors an IncX1 plasmid and carriage of this plasmid does
172 not confer AR in SH. The IncX1 *inc* region was not detected by plasmidFinder[18] in 42 of
173 the 71 isolates sequenced. Thirty-nine percent of SH isolates acquired Col plasmids, but
174 none carried a known antibiotic resistance gene (ARG). ARGs known to confer resistance
175 to aminoglycosides (*aadA1, aac(3)-Via*) and tetracyclines (*tetA*) were detected in all AR
176 isolates. The AR isolates from trial 1 harbored a sulphonamide resistance gene (*sul1*) in
177 addition to aminoglycoside genes but no *tet* gene. The ARGs were found on a plasmid
178 belonging to *inc* group IncI1 (Fig. 1f) and plasmid Multi Locus Sequence Type 26 (pST26).
179 A change in AR profile was observed in four SB isolates - for these isolates, AST
180 confirmed them to be MDR but after WGS, the AR determinants were not detected. Upon
181 AST re-test, these isolates were found to be susceptible. Acquired ARG was absent in
182 four isolates with resistance to either tetracycline or streptomycin.
183 **HGT and chromosome rearrangement shaped the genome of *S*. Heidelberg**. After
184 introduction of SH to the broiler gut microbiota, we hypothesized that successful
185 colonization will be dependent on gene flux (gain and loss of genes) and mutations. To
186 this end, we reconstructed a maximum-likelihood (ML) tree based on the pan genome
187 and mutations of SH strains recovered after 2 weeks in the gut or litter. The core genome
188 (genes present in ≥ 95% of the strains) and accessory genome (genes present in < 95%
189 of the strains) composed of 3729 and 1531 genes, respectively. A total of 91 new
190 mutations (SNPs and Indels) were on the chromosome of evolved strains and no single
191 mutation was present in every strain. Rather, mutations were unique to individual strains
192 or shared between 2-26 isolates (Supplementary data 1). The accessory genome tree
193 grouped the strains based on AR phenotype, plasmid and ARG carried (Fig. 3a).

194 Contrastingly, the core genome and SNP-based trees did not provide a clear division of
195 strains based on these characteristics and clades had low bootstrap support values
196 (Extended data Fig. 3b and c). Acquisition of IncI (IncI1 and IncI2) plasmid, Col plasmids
197 and the presence/absence of IncX1 plasmids defined the clades seen on the accessory
198 genome tree. Susceptible strains carrying only IncX1 plasmids represented Clade I and
199 MDR strains dominated Clade II. Clade II strains carried either IncI1 only or IncI1 plus a
200 Col plasmid or both were present with an IncX1 plasmid (Fig. 3a).

201 A few susceptible and strains with no plasmid (n = 12) were nested within clade II
202 and the genome of these strains exhibited signs of substantial gene loss (Fig. 3a;
203 Extended data Fig. 4a). Moreover, clade IIa strains had higher number of contigs, higher
204 misassemblies and lower genome size than the rest of the strains sequenced for this
205 study (H = 18.82, df =1, P <0.001; Extended data Fig. 4b-d). To determine if erroneous
206 assembly affected the clustering of this clade, we performed long read sequencing
207 (Pacific Biosciences Inc.) on one strain from this clade and used it in a hybrid approach
208 with Illumina short reads. This resulted in a partially complete chromosome (longest
209 contig - 4.7 Mbp) but 30 non- circular contigs were not scaffolded. An alignment of the
210 ordered contigs with the ancestor showed that the strain did not suffer significant gene
211 loss as suggested through short or long reads only assembly (Supplementary Table 1),
212 however a 3.2 Mbp chromosomal region was inverted (Fig. 3b). This strain also carried
213 an untypeable circular episome that carried uncharacterized proteins and two genes
214 encoding NADH-quinone oxidoreductase (*nuo*) (~22 kbp) (Extended data Fig.4e), and an
215 IncX1 plasmid with divergent protein sequences from the IncX1 present in the ancestor
216 (Extended data Fig.4f). Gene inversions can increase the diversity and virulence of

217 pathogens[19], hence we questioned if chromosomal inversion was present in other
218 evolved strains. To do this, we aligned protein sequences of two complete circular
219 chromosome from trial 1 and 2 with the ancestor. This analysis showed that the isolate
220 from trial 1 harbored a ~2 Mbp chromosomal inversion (Extended data Fig. 5a).

221 Gene inversion changes the leading or lagging strand sequence to its reverse
222 complement, thus altering the GC skew of the affected gene or genome from a positive
223 value to a negative value or negative to a positive[19]. Accordingly, we confirmed that there
224 was a GC skew inversion in these regions (Extended data Fig. 5b and c). Gene inversions
225 occur after rejoining of DNA breaks and inverted genes can introduce sequencing bias
226 and misassembly[20]. Here, deletions affecting the cytochrome c maturation gene cluster
227 (*ccm*) contributed to the inversion of genes in SH strain *og8-05a* (Fig. 3b; Extended data
228 Fig. 5b). Further, the *oriC* of *og8-05a* was reoriented when compared to the ancestor. On
229 the other hand, deletion of a putative YebC/PmpR transcriptional regulator and a deletion
230 between *cysK* and *cysZ* led to the reorientation of *oriC* in strain *ic9b* (Extended data Fig.
231 5a and c). The inverted region in these genomes encoded 34 - 60 % of the 168 virulence
232 genes present in the ancestor including *Salmonella* pathogenicity islands 1 and 2, fimbrial
233 and adherence genes, type 1 and 2 secretion system proteins and prophages
234 (Supplementary data 2; Extended data Fig. 6). Taken together, this result suggested that
235 gene flux and homologous recombination shaped the genome of SH strains after entry
236 into the broiler chick gut.

237 **pST26 differed in their AR genetic context and genome architecture**. To compare
238 pST26 plasmids, we used hybrid assemblies to achieve circular plasmids for two MDR
239 SH isolates recovered from trial 1 and trial 2. The pST26 plasmids from trial 1 (p1ST26)

240 and trial 2 (p2ST26) were determined to be ~ 112 kbp long and ~ 87 % identical (Fig. 1f).
241 Both carried the atypical IncI1 backbone including regions encoding replication, stability,
242 leading and conjugative transfer[21]. To determine the IncI1-complex group, we used the
243 classical *traY* and *excA* protein sequences of plasmid R64 (IncI1) and R621a (IncI1-
244 gamma). A reconstructed tree confirmed that both plasmids belong to the IncI1 group
245 (Fig. 4a). A 19.8 kb variable region encoding transposons and AR was found in both
246 plasmids. For p1ST26, this region encoded *aadA1, aac(3)-Via*), *sul1* and quaternary
247 (quats) ammonium compound resistance *(qacEΔ1*) (Fig.1f; Extended data Fig. 7). In
248 contrast, this region carried *tetA* and *mer* operon in addition to *aadA1* and *aac(3)-Via* in
249 p2ST26 (Fig. 1f) .

250 To investigate whether gene flux influenced IncI1 genome architecture, we used
251 p1ST26 and p2ST26 as reference genomes for the rest of the MDR isolates carrying
252 IncI1. By aligning raw reads to complete pST26 genomes, we were able generate a
253 consensus IncI1 plasmid contig for each MDR isolate. A tree built with these plasmids
254 resulted in two clades represented by p1ST26 and p2ST26 plasmids, respectively (Fig.
255 4b). Multiple alignment of protein sequences revealed that pST26 plasmids differed in
256 number of genes carried and gene alleles. This was pronounced for regions encoding
257 AR, pilus/shufflon assembly proteins (*pil*) and IS66-family of transposases
258 (Supplementary data 3). A pangenome analysis revealed that the pST26 from this study
259 carried 70 core genes (genes present in ≥ 95% of the plasmids) and 90 accessory genes
260 (genes present in < 95% of the plasmids). A tree reconstructed with the core genes and
261 accessory genes divided the pST26 into 5 major groups (Fig. 4d and c). For both trees,
262 the p1ST26 formed one clade and the p2ST26 made up the other clades. No SNPs were

263 found between p2ST26 but p1ST26 differed from p2ST26 with 21 SNPs and 2 insertions
264 (Supplementary data 3; Supplementary Table 2). The insertions were present in only one
265 p1ST26 plasmid. One insertion was found on *tniA* (a DDE-type
266 integrase/transposase/recombinase protein) and the other was between tn3-like
267 transposon and *yadA*. Inserted DNA showed significant homology to *tniA* present in *E.*
268 *coli* plasmid EcPF5 (Genbank: CP054237) and IS5057 present in *E. coli* plasmid
269 pIOMTU792 (Genbank: C542972), respectively.

270 ***E. coli* was the major IncI1 reservoir in broiler chicks**. The application of proximity-
271 ligation method (Hi-C) has improved the assembly of metagenomes and made it possible
272 to detect plasmid-host associations[22]. Therefore, we used Hi-C to determine the bacterial
273 species carrying IncI1 plasmids in the broiler chicken microbiota. Hi-C was done on two
274 cecal samples from chicks challenged with SH via the cloaca (hereafter referred to as Hi-
275 IC-FL1 and Hi-IC-FL2). We chose IC samples since all chicks in this group were
276 successfully colonized with SH compared to OG or SB. Our goal was to determine if Hi-
277 C could find the bacterial host of IncI1 plasmids and detect inoculated SH strains.
278 *Salmonella enterica* was found in Hi-IC-FL1 and Hi-IC-FL2 using metagenome sequence
279 classification bioinformatic tools[23,24] (Fig. 5a). However, serovar determination with
280 cluster contigs (~ 1.4 and 1.2 Mbp total contig size, respectively) was not possible with
281 *Salmonella* serovar prediction tool[25] or after alignment of contigs to SH reference
282 genome. We detected IncI1 plasmid contigs in Hi-IC-FL1 that were linked to *E. coli*
283 clusters (Fig. 5b and c; Supplementary Table 3). In addition, we found contigs (n = 151,
284 mean contig size = 17,607 bp, smallest contig = 1,027 bp, largest contig = 282,389 bp)
285 with protein sequences identical to pST26 in both Hi-IC-FL1 and Hi-IC-FL2

286 (Supplementary data 4). Other bacterial clusters belonging to the *Firmicutes* showed a
287 low level of linkage to IncI1 (~100X less in Hi-IC-FL1). These results suggested that *E.*
288 *coli* was the main reservoir of IncI1 plasmids.

289 Next, we investigated if SH challenge affected *E. coli* and IncI1 plasmid abundance.
290 To answer this, we used qPCR primers targeting the glyceraldehyde-3-phosphate
291 dehydrogenase (*gapA*) gene of *E. coli*, the *inc*RNAi region of IncI1 and selected genes
292 carried on p2ST26 (*intI1*, *aadA* and *tetA*) to determine their concentration in the ceca and
293 litter. Litter carried higher concentration of *E. coli*, IncI1 and p2ST26 genes than ceca on
294 a per gram basis (W = 1, $P < 0.001$). (Fig. 6a; Extended data Fig.8a). In the ceca, IncI1
295 was higher in OG compared to IC or CTRL ($X^2 = 9.17$, df =2, $P = 0.01$), while *aadA* (H =
296 8.25, df = 2, $P = 0.016$) was lower in OG compared to IC or CTRL (Fig. 6a). CTRL chicks
297 carried a higher concentration of *E. coli* ($X^2 = 2.32$, df =2, $P = 0.31$), *tetA* ($X^2 = 5.74$, df
298 =2, $P = 0.05$) and *intI1* ($X^2 = 5.69$, df =2, $P = 0.05$) genes than OG and IC. A principal
299 component analysis with these genes and *Salmonella* tetrathionate reductase (*ttr*)
300 revealed that samples within the control and challenge groups clustered with their
301 respective groups and that the challenge and control groups were separated. (Extended
302 data Fig. 8b). A correlation analysis between *Salmonella* and *E. coli* for challenged chicks
303 showed a significant positive correlation (S = 784, ρ = 0.55, P =0.007) (Fig. 6b). These
304 results suggested that the presence of SH affected *E. coli*, IncI1 and ARG concentrations.

305 **Broiler chicks carried *E. coli* strains harboring pST26 IncI1 plasmids.** To confirm if
306 broiler chicks from this study harbored *E. coli* populations that could serve as a reservoir
307 of pST26 plasmids, we retrospectively screened the cecal contents of the broiler chicks
308 used for Hi-C on CHROMagar™ supplemented with gentamicin and tetracycline (see

309 supplementary methods) and selected five colonies for whole genome sequencing. As
310 expected, we found pST26 in two strains (phylogroup F, MLST 6858), while the other
311 three strains (phylogroup D, MLST 69) carried an IncI1 that harbored no ARG, and an
312 unknown MLST (Table 1). Next, we compared the pST26 in SH to that found in *E. coli*
313 using complete plasmids. The p1ST26 and p2ST6 IncI1 plasmids of SH were determined
314 to be 86.8% and 99.4 % identical to pST26 present in *E. coli*. The main difference was is
315 in the region encoding AR and transposases in p1ST26 (Extended data Fig 9).
316 **pST26 carriage did not pose a fitness cost under selection pressure**. pST26 carried
317 accessory genes for AR, metal resistance or disinfectants (i.e., quaternary ammonium
318 compounds) Furthermore, SH strains carrying pST26 differed by route of exposure e.g.,
319 higher proportion in oral vs. cloacal. To this end, we questioned if any of these factors
320 could exert a selective pressure for pST26. We did not administer antibiotics to broiler
321 chicks throughout study, therefore we did not consider antibiotic selective pressure.
322 However, we detected metals including nickel, chromium, copper, and zinc in the starter
323 feed in parts per million and we found contaminants such as arsenic, lead and cadmium
324 in trace amounts (Supplementary Table 4). Disinfectants including quaternary ammonium
325 compounds were used for the cleaning of broiler houses and equipment, thus we
326 considered disinfectant as another source of selective pressure in this study. To
327 determine their effect on SH fitness, we used phenotype MicroArray™ (PM) 96-well plates
328 supplemented with selected metal chlorides and disinfectants at varying concentrations
329 to compare a pST26 carrying strain to a susceptible evolved strain. There was no
330 significant difference in metabolic activity for SH strain carrying pST26 compared to its
331 susceptible counterpart for metals and disinfectant but pST26 exhibited higher

332 metabolism for benzethonium chloride, chromate, tellurite, and zinc, whereas the
333 susceptible stain showed higher metabolism for dequalinum chloride, chromium, and
334 cesium (X^2= 1.83, df = 1, *P* = 0.1762) (Extended data Fig. 10a and b).

335 Cox and colleagues have suggested that the acidic pH of the upper GI tract was
336 associated with the lower number of chicks positive for *Salmonella* and *Campylobacter*
337 after oral gavage compared to cloacal inoculation[26-28]. Based on this premise, we
338 hypothesized that SH strains carrying pST26 will survive better under exposure to acidic
339 pH. To answer this question, we first compared the survival of the strains under different
340 pH levels (3.5 -10) with or without nitrogen sources and amino acids using PM plates. SH
341 strain carrying pST26 exhibited lower metabolic activity compared to the susceptible
342 strain at pH 3.5 – 4 (V = 3; *P* = 0.5), pH 4.5 with and without nitrogen sources (V = 599;
343 *P* = 3.426e-06) and at pH 9.5-10 with and without sources of nitrogen (V = 714; *P* =
344 6.547e-07) (Fig. 7a). Next, we compared the survival of the susceptible ancestor,
345 susceptible evolved strains without pST26 and antibiotic resistant evolved strains carrying
346 pST26 in pine shaving extract (PSE) adjusted to pH – 2.5. We acclimatized the bacterial
347 population to PSE (pH – 6.5) for 2 h before exposure to acidified PSE (Extended data
348 Fig. 10c). We screened for pST26 carrying populations using the gen^R marker on pST26.
349 After 2h of exposure to acidified PSE, the SH population carrying pST26 (resistant plus
350 susceptible cells) did not suffer a fitness cost, and cells carrying pST26 exhibited higher
351 fitness than their susceptible counterparts (X^2= 4.35, df = 2, *P* = 0.113) (Fig. 7b).
352 Notwithstanding, pST26 SH cells had a lower population size than the ancestor and
353 evolved susceptible strains (X^2 = 6.37, df =3, *P* = 0.095) (Fig. 7c). This result suggested

354 that pST26 carriage does not pose a fitness cost on SH host but benefits the host when
355 exposed to acidic pH.

356 ## Discussion

357 In this study, neonatal Cobb 500 broiler chicks challenged with a susceptible *S*.
358 Heidelberg (SH) strain carried susceptible and multidrug resistance SH population 2-
359 weeks after in their ceca and litter. This resistance phenotype was conferred by the
360 acquisition of IncI1-pST26 plasmids. pST26 plasmids are present in *S*. Heidelberg, *S*.
361 Typhimurium, *S*. Anatum, *S*. Derby, *S*. Schwarzengrund, *S*. Saintpaul and *E. coli* strains
362 isolated from animal and human sources (Extended data Fig. 11). The closest IncI1
363 plasmids to pST26 from this study are pST26 present in *E. coli* (CP018625) and *S*.
364 Typhimurium (CP027409). The plasmids carry aminoglycoside resistance genes with or
365 without tetracycline, sulphonamide, *qac* and *mer* resistance genes, making it a
366 multidrug/disinfectant/metal resistance plasmid. Additionally, pST26 transfer was higher
367 in chicks challenged orally compared to chicks inoculated via the cloaca. This route
368 specific rate of conjugation suggested that the selection for pST26 in our study was
369 associated with the differential pH in the upper GI versus lower GI tract. This was
370 corroborated by in vitro experiments, where acidic pH reduced the metabolism of SH cells
371 carrying pST26 compared to cells with no pST26. Furthermore, carriage of pST26
372 increased the fitness of pST26-carrying cells compared to the susceptible SH population
373 after exposure to acidic pine shaving extract. This type of acid-imposed selection for IncI1
374 alludes to the “negative frequency dependent selection hypothesis” - where the fitness of
375 the population is higher when a plasmid is present at a low frequency in the population[29].

376 This could also explain the higher levels of SH, but lower positivity rate found in the
377 ceca of broiler chicks gavaged compared to cloacally challenged chicks[16] (Fig. 1b). There
378 are multiple barriers that makes it challenging for SH to colonize the ceca of broiler chicks
379 gavaged including the acidity of the upper GI tract and the length of the GI tract to be
380 traveled. When we measured the distance of the cloaca and esophagus to the ceca of a
381 49-day old Cobb-500 broiler chicken (photo not shown), we estimated that the SH inocula
382 will have to travel ~20X farther to get to the ceca of gavaged chicks compared to cloacal
383 inoculated chicks. It is plausible that this longer "residence" time in the upper GI allows
384 more opportunities for SH to make contact and compete with other members of chicken
385 microbiota. In this study, such an event gave rise to SH cells that acquired pST26 from
386 *E. coli*. It is also noteworthy that AR acquisition occurred at a lower rate during trial 1
387 compared to trial 2. This could be due to several factors that warrants further study -
388 including the higher pH and moisture of the litter in trial 1 compared 2, seasonal
389 differences (trial 1 was done in the fall and trial 2 was in the spring) and the
390 antibiotic/disinfectant practices in place at the hatchery or breeder farms at time the
391 experiments were conducted.

392 The IncI1-complex are widespread in *Enterobacteriaceae* and have been reported to
393 be the major driver of AR in *Salmonella* and *E. coli*. Our understanding of their mode of
394 transfer and AR evolution have been limited to studies on their prevalence, mating
395 experiments and in vivo challenge with mice[21,30,31]. Fischer et al[32] showed that *E. coli*
396 populations in broiler chickens acquired β-lactam resistance after 4-days old broiler chicks
397 were gavaged with an *E. coli* strain carrying $bla_{CTX\text{-}M\text{-}1}$ on an IncI1 plasmid. In a twin pair
398 experiment, Hagbo et al[33] revealed that IncI1 carrying $bla_{CMY\text{-}2}$, ColE1 and P1

399 bacteriophage are frequently transferred between microbiota present in pre-term infant
400 gut. Recently, Nyirabahizi,et al[34] used likelihood-ratios to show that *E. coli* AR predicted
401 *Salmonella* resistance to β-lactams in retail meat, and to gentamicin, ceftriaxone, and
402 amoxicillin-clavulanic acid in cecal samples and suggested that the horizontal transfer of
403 bla_{CMY-2} played a role. These studies have demonstrated that IncI1 is the most prevalent
404 plasmid carrying β-lactam in *Enterobacteriaceae* and that they are transferable from
405 *Salmonella* to *E. coli* and vice-versa. IncI1 plasmids can harbor transferable gene
406 cassettes with AR variants for β-lactam (bla_{TEM}, bla_{CMY}, bla_{CTX}), aminoglycoside (*aadA*,
407 *aacA*, *aph*), tetracycline (*tet*), sulphonamide (*sul*), fosfomycin (*fosA*), phenicol (*cmlA*, *floR*)
408 and trimethoprim (*dfrA*) resistance. Furthermore, mobile elements could carry AR genes
409 for disinfectants and metals including ammonium compounds (*qac, sug*), mercury (*mer*
410 operon) and arsenic (*ars* operon).

411 The nutrient-niche hypothesis suggests that SH will only be able to invade a new
412 niche if it can metabolize a growth-limiting resource or if it can outcompete a resident
413 species and a vacant niche arises due to a new component in diet or by eliminating the
414 competitor [35]. Competition for oxygen was shown to be the limiting factor that contributed
415 to the virulence of *S*. Enteritidis towards *E. coli* in neonatal chickens[36]. Competition within
416 and between SH and *E. coli* strains played a key role selecting for antibiotic resistant SH
417 in our study. SH challenge perturbed the bacterial community and ARGs of broiler chicks
418 and SH population was positively correlated with *E. coli*. Consequently, gene flux and
419 genome inversion shaped the evolution of SH in broiler chicks. For SH and *E. coli* to co-
420 evolve and persist in the same new niche, recombination of genes occurred and led to
421 the emergence of new SH strains. In our study, pST26 emerged after conjugation

422 between *E. coli* donors from broiler chicks raised on fresh litter and SH. As expected, SH
423 genomes were grouped into nine core genome MLST clusters with eight clusters
424 representing new strains (Extended data Fig. 12). These species/strains are expected to
425 be fitter in the new niche and persist longer.

426 Materials and Methods

427 **SH inoculum preparation.** We have previously described the characteristics of the SH
428 strain (SH-2813nal^R) used for this study and how the inoculum was prepared[16]. Briefly,
429 the strain belongs to the multilocus sequence type 15, carries a 37-kb conjugative
430 plasmid, and is resistant to erythromycin, tylosin, and fosfomycin. The strain was
431 recovered from broiler chicken carcass in 2013 and made resistant to 200 ppm of nalidixic
432 acid (nal^R) for selective enumeration. The nal^R phenotype is conferred by a serine to
433 tyrosine substitution at position 83 of DNA gyrase subunit A protein (GyrA). The SH
434 inoculum was grown overnight in poultry litter extract, centrifuged, and resuspended in
435 1X phosphate buffered saline (PBS). The resuspended cells were used as inocula. The
436 genome of the ancestor to SH-2813nal^R was sequenced using Illumina and PacBio
437 sequencing to achieve a complete circular chromosome and plasmid (Genbank). SH-
438 2813nal^R carries six mutations (Supplementary data 5) including the mutation affecting
439 *gyrA* that are **not present in the ancestor.**

440 **Challenging broiler chicks with SH**. One-day-old Cobb 500 broiler chicks were
441 purchased from a commercial hatchery in Cleveland, GA, USA. Upon purchase, chicks
442 were placed in plastic crates lined with brown paper and transported to the University of
443 Georgia, Poultry Research Center (33.90693362620281, -83.37871698746522). One
444 hundred chicks were either uninoculated, gavaged or cloacally inoculated with 100 µl-

445 volume of SH inoculum (Fig.1a). We included a seeder-bird (SB) colonization method,
446 whereby five orally gavaged chicks were mingled with twenty uninoculated chicks. Each
447 inoculated chick received ~10^6 colony forming unites (CFU) of SH-2813nal^R. Afterwards,
448 chicks were placed in floor pens at a stocking density of 0.65 m^2/chick on fresh pine
449 shavings litter. Broiler chicks were given water and feed ad libitum and raised antibiotic-
450 free on starter diet for 2-weeks (starter feed was synthesized by the University of
451 Georgia's poultry research center's feed mill). The concentration of metals and priority
452 pollutants in feed was determined by the University of Georgia's feed and environmental
453 water laboratory (Athens, GA, USA) (Supplementary Table 4). Husbandry and
454 management followed commercial broiler chicken industry guidelines. After 2-weeks, ten
455 chicks from OG, IC and control groups and fifteen chickens from SB (five seeder chicks
456 and ten uninoculated pen mate) were sacrificed to determine the extent of SH colonization
457 in ceca. The experiments were done in two trials conducted in September 2017 (Trial 1)
458 and April 2018 (Trial 2). The study was approved by the University of Georgia Office of
459 Animal Care and Use under Animal Use Protocol: A2017 04-028-A2.
460 **Cecal and litter bacteriological analyses**. Ceca were aseptically removed from the
461 eviscera of 2-week-old broiler chicks, placed in a stomacher bag and transported on ice
462 to the US National Poultry Research Center for analysis. Ceca were weighed and
463 buffered peptone water (BPW) (BD Difco, MD, USA) was added 3X volume to the weight
464 (v/w) and stomached for 60s. Serial dilutions were made and plated onto Brilliant Green
465 Sulfa agar (BGS) (BD Difco, MD, USA) containing 200-ppm nal. In addition, a 10 µl
466 inoculating loop was used to streak cecal slurry (cecal contents in BPW) onto Xylose
467 Lysine Tergitol-4 agar (BD Difco, Sparks, MD). supplemented with 4 ppm tetracycline for

468 trial 1 and BGS supplemented 32 ppm ampicillin (amp) or 32 ppm streptomycin for trial
469 2. All antibiotics were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise
470 noted. Plates were incubated along with the cecal slurry for 24 h. All bacterial incubations
471 were carried out at 37°C, unless otherwise noted. After incubation, colonies were
472 manually counted and serial dilution plates with 2 – 100 colonies were used for CFU per
473 gram calculation. If no colonies appeared on serial dilution plates, cecal slurry was
474 streaked onto a new BGS plus nal plate and incubated overnight. After incubation, plates
475 were examined for the presence/absence of *Salmonella* colonies.

476 Broiler chicken litter was collected as grab samples from seven locations (4 corners
477 of the pen and under the waterer) in each pen after chicks were removed. The litter
478 samples were pooled and 30g was processed in duplicates from each pen as previously
479 described[37]. Serial dilutions of the litter slurry were made and plated onto BGS agar
480 containing 200 ppm nal. Plates were incubated overnight, and colonies were manually
481 counted and reported per gram litter dry weight. Litter pH and moisture was determined
482 as described previously[37]. We selected randomly 2 – 6 single colonies from BGS plates
483 supplemented with nal from each ceca and litter sample and archived them in 30% LB
484 glycerol at -80 ^{o}C. In addition, cecal slurry was saved at a 4:1 ratio in Luria Bertani broth
485 (BD Difco, MD, USA) containing 30% glycerol at –80°C, whereas litter samples were
486 stored in vacuum sealed whirl pak bags at -20°C.

487 **Determining SH metabolism under selective pressure.** To determine if exposure to
488 metals, disinfectants or acidic pH poses a selection on SH strains carrying IncI1 plasmids,
489 we used 96-well Phenotype Microarray (PM) ™ MicroPlates (PM10, PM12B, PM13B,
490 PM15B and PM16A) (Biolog, Inc., Hayward CA) to compare the metabolic profile of one

491 gentamicin resistant strain harboring IncI1 and one susceptible strain. PM™ plates uses
492 cell respiration via NADH production to determine cell metabolic activity. If the phenotype
493 is positive in a well, the cells respire actively, reducing a tetrazolium dye and forming a
494 strong color (Biolog, Inc.). Briefly, SH strains were sub-cultured twice in universal growth
495 agar (BUG+B) for 24 h at 37°C. Cells were removed with a sterile swab and transferred
496 to 16 ml IF-0 to achieve a final turbidimetric transmission of 42%T. Afterwards, 15 ml of
497 the 42%T cell suspension was transferred to 75 ml of IF-0 +dye to achieve a final
498 transmission of 85% T before 600 µl of the cell suspension was transferred to 120 ml IF-
499 10+ dye. The suspension was mixed and each well in a microplate was inoculated with
500 100 µl of the suspension. After inoculation, microplates were covered with a sterile plastic
501 film and monitored automatically for color development every 15 min for 48 h at 37 °C
502 using and OmniLog reader for 48 h. To identify phenotypes, the kinetic curves of the
503 gentamicin resistant and susceptible strains were compared using OmniLog PM software.
504 For each PM plate, the respiratory unit for each well at 22 h was extracted and $\log_{10}$
505 transformed. All supplies used were purchased from Biolog, Inc.
506 **Exposing SH to acidic pH.** To determine the fitness of evolved SH strains that acquired
507 antibiotic resistance compared to evolved susceptible strains after exposure to acidic pH,
508 we exposed gentamicin resistant (n = 3) and susceptible (n =3) strains recovered from
509 the litter of chicks gavaged with SH to acidified filter sterilized pine shaving extract. Pine
510 shaving extract (PSE) was prepared as described for poultry litter extract[37], using fresh
511 pine shavings collected from the University of Georgia, Poultry Research Center, Athens,
512 GA, USA. Three single colonies of each strain including the ancestral SH-2813nal^R were
513 selected from overnight cultures grown on sheep blood agar and transferred to a

514 microcentrifuge tube containing 900 µl of PSE (pH = 6.52) i.e., one tube per strain. After
515 transfer, tubes were vortexed, covered with a gas permeable paper strip and incubated
516 at 41°C under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2) for 2 h. After
517 incubation, tubes were vortexed and 100 µl of the suspension was transferred to a
518 microcentrifuge tube containing 900 µl of PSE (pH of PSE was adjusted to 2.54 using 1M
519 HCl (Spectrum Chemical Mfg. Corp., CA, USA) and 1M NaOH (Fisher Chemical, NJ,
520 USA) Afterwards, tubes were vortexed, and incubation continued for another 2 h as was
521 done for PSE at pH 6.5. To determine SH population in PSE, one replicate tube per strain
522 was removed and serially diluted in 1X PBS at timepoints 0 and 2 h for pH 6.5 and 0.5, 1
523 and 2 h for pH 2.5. Serial dilutions were plated onto BGS agar plates supplemented with
524 or without 16 ppm gentamicin. SH colonies were counted 18 - 24 h after incubation using
525 a calibrated automated colony counter.

526 The fitness of each evolved SH population relative to the SH-2813nal^R population
527 was determined after 2h at pH 2.54 as described by San Millan et al[38]:

528
$$W_{strain} = \frac{log_e \frac{N_{final,evol}}{N_{initial,evol}}}{log_e \frac{N_{final,anc}}{N_{initial,anc}}}$$

529 where W_{strain} is the fitness of the evolved susceptible (total susceptible) or gentamicin
530 resistant populations (total resistant and gen^R resistant), $N_{initial,\ evol}$ and $N_{final,evol}$ are the
531 numbers of cells (in CFU) of the evolved susceptible or gentamicin resistant population
532 at timepoint 0 and 2 h after exposure to PSE, and $N_{initial,anc}$ and $N_{final,anc}$ are the numbers
533 of cells of SH-2813nal^R population at timepoint 0 and 2 h.

534 **Antimicrobial susceptibility testing**. We performed antimicrobial susceptibility testing
535 (AST) on two hundred and fifty SH isolates recovered from the ceca and litter of broiler

536 chicks following National Antimicrobial Resistance Monitoring System (NARMS) protocol
537 for Gram-Negative bacteria. Minimum inhibitory concentration for isolates were
538 determined by broth microdilution using the Sensititre semiautomated antimicrobial
539 susceptibility system (Thermo Fisher Scientific, Inc., MA, USA) and interpreted according
540 to clinical and laboratory standards institute guidelines when available; otherwise,
541 breakpoints established by NARMS were used. AST was also done on *E. coli* isolates (n
542 = 5) recovered from the ceca of two broiler chicks challenged intracloacally with SH. The
543 Kirby-Bauer disc diffusion assay for tobramycin, kanamycin, neomycin, netilmicin was
544 done on four gentamicin resistant SH isolates as previously described[37].

545 **DNA extraction.** DNA was extracted and purified from bacterial colonies using FastDNA
546 spin Kit (MP Biomedicals, LLC, CA, USA), whereas 250 mg of cecal and litter were
547 extracted with Qiagen DNeasy Power-Soil DNA kit (Hilden, Germany). The modifications
548 we made to the manufacturers protocol for DNA extraction have been reported [37,39].

549 **Whole genome sequencing and processing.** Illumina short read sequencing was
550 performed on DNA extracted from SH isolates recovered from ceca and litter. In addition,
551 five *E. coli* isolates recovered from two cecal samples were sequenced. Libraries were
552 prepared using either Nextera™ XT or Nextera™ DNA Flex library preparation kits
553 (Illumina, Inc., San Diego, CA) following the manufacturers protocol. Libraries were
554 sequenced on the Illumina MiSeq platform with 150 or 250-bp paired end reads.
555 Additionally, five SH and two *E. coli* isolates were selected for long read sequencing using
556 Sequel II System (PacBio Biosciences Inc.) or MinION (Oxford Nanopore technology).
557 Preparation and sequencing of long read libraries were done by next generation

558 sequencing core centers of University of Georgia and Colorado State University (see
559 supplementary methods for read quality control and demultiplexing).

560 Genome assembly, resistome characterization, and quality assessment of long reads
561 was done using Reads2Resistome pipeline v.1.1.1[40]. Reads2Resistome performed
562 hybrid assemblies using either Illumina reads and PacBio or Illumina and MinION reads,
563 using both Unicycler[41] and SPAdes[42]. In addition, hierarchical genome assembly process
564 (HGAP) assembly was performed using PacBio's SMRT Link v9.0 analysis software suite
565 using default settings. Assembly quality was assessed by QUAST v5.0.2[43], using default
566 settings) and genome annotation was done using Prokka[44], Rapid Annotation using
567 Subsystem Technology (RAST)[45] and BlastKOALA[46]. We confirmed that all SH isolates
568 were *Salmonella enterica* serovar Heidelberg using *Salmonella* In Silico Typing Resource
569 (SISTR)[25], and used ClermonTyping[47] and Multi-Locus Sequence Typing (MLST)[48] for *E.*
570 *coli* strains. For resistome characterization reads2resistome used ARG-ANNOT[49], the
571 Comprehensive Antibiotic Resistance Database[50], MEGARes[51], AMRFinderPlus[52],
572 PlasmidFinder[18], ResFinder[53] and VirulenceFinder database[54]. For plasmid typing and
573 IncI1 clonal complex determination, we used plasmid MLST[18]. PHAST[55] was used to
574 identify prophages present in chromosomal contigs and predicted prophage DNA
575 sequence was annotated with RAST and BlastKOALA. ProgressiveMAUVE v. 1.1.1[56] and
576 MAFFT v. 1.4.0[57] implemented in Geneious Prime® v 2020.0.1 were used for aligning
577 and comparing sequences. In addition, a pan genome analysis of annotated assemblies
578 was conducted with Roary[58]. Phylogenetic trees based on core genome and accessory
579 genome were reconstructed using the maximum likelihood (ML) method implemented in
580 RAxML-NG v 2.0.0[59]. When computationally possible, the best model of sequence

581 evolution predicted by jModelTest[60] was used for tree reconstruction, otherwise the GTR
582 + GAMMMA model was implemented. Lastly, we used the bacterial genomes sequenced
583 in the study to create a BLAST database that can be searched for sequences of interest
584 in Geneious Prime®.

585 Illumina short reads were assembled de novo into contigs using Unicycler v.0.4.7 and
586 characterized with bioinformatic tools described for long reads. Illumina reads were used
587 to determine single nucleotide polymorphisms (SNPs) and indels present in SH isolates.
588 Alignment of raw FASTQ reads to the genome of SH-ancestor was done using Burrows-
589 Wheeler Aligner (BWA)[61] and SNPs/indels were called using Genome Analysis Toolkit[62]
590 as described previously[39]. Variant call format (VCF) files of identified SNPs/indels and the
591 Linux/Unix shell script used have been deposited in Dryad Digital
592 Repository: https://doi.org/10.5061/dryad.4tmpg4f8d. SNP based ML tree was
593 reconstructed by converting merged VCF files to PHYLogeny Inference Package format
594 using PGDSpider[63]. Afterwards, isolates with duplicated SNPs/indels were removed, and
595 a ML tree was drawn using the Jukes-Cantor model of nucleotide substitution and
596 GAMMA model of rate heterogeneity. For other bioinformatic analyses performed
597 including pST26-IncI1 consensus determination through FASTQ alignment, see
598 supplementary methods. All raw FASTQ reads for sequenced bacterial genomes are
599 publicly available under NCBI accession numbers:

600 **Hi-C and cecal metagenome library preparation and analysis** A shotgun and Hi-C
601 DNA library of two cecal samples was created using Nextera™ XT library preparation kit
602 and Phase Genomics (Seattle, WA) ProxiMeta Hi-C Microbiome Kit following the
603 manufacturer's instructions. Library sequencing was performed by Novogene corporation

604 (Sacramento, CA, USA) on the Illumina HiSeq platform using 150bp paired end reads.
605 Two libraries were sequenced per HiSeq flowcell lane resulting in a total of ~125 million
606 shotgun reads, and ~206 million Hi-C reads per sample. Metagenomic FASTQ files were
607 uploaded to the Phase Genomics cloud-based bioinformatics portal for subsequent
608 analysis. Shotgun reads were filtered and trimmed for quality using bbduk[64] and
609 normalized using bbtools[64] and then assembled with metaSPAdes[65] using default
610 options. Hi-C reads were then aligned to the assembly following the Hi-C kit
611 manufacturer's recommendations (https://phasegenomics.github.io/2019/09/19/hic-
612 alignment-and-qc.html). Briefly, reads were aligned using BWA-MEM with the -5SP and
613 -t 8 options specified, and all other options default. SAMBLASTER[66] was used to flag
614 PCR duplicates. Alignments were then filtered with samtools[67] using the -F 2304 filtering
615 flag to remove non-primary and secondary alignments. Lastly, Hi-C read alignments to
616 the assembly were filtered using Matlock (https://github.com/phasegenomics/matlock)
617 with default options (removing all alignments with MAPQ<20, edit distance greater than
618 5, and read duplicates). Metagenome deconvolution was performed with ProxiMeta[68,69],
619 resulting in the creation of putative genome and genome fragment clusters.

620 Clusters were assessed for quality using CheckM[70] and assigned preliminary
621 taxonomic classifications with Mash[71] and Kraken2 (v2.0.8-beta)[23]. To search the
622 metagenome for ARG and plasmids of interest, we used Geneious Prime® to create a
623 BLAST metagenome database. Metagenome contigs with significant homology (E value
624 < 0.05 and at least 1000 bp of aligned sequence) were considered contigs of the
625 respective plasmid or ARG. Hi-C data were then used to link identified sequences to host
626 genomes and genome fragments within ProxiMeta[22]. Phylogenetic visualizations of Hi-C

627 linkages used the placement of clusters in a large prokaryotic phylogeny as estimated by
628 CheckM[70]. Shotgun and Hi-C reads are publicly available under NCBI accession
629 numbers:

630 **16S rRNA gene sequence processing and analysis.** Cecal and litter DNA were used
631 for bacterial community analysis through the sequencing of the V4 hypervariable region
632 of the 16S rRNA gene of bacterial genomes. Sequencing was done using the paired-end
633 (250 × 2) method on the Illumina MiSeq platform. Raw 16S rRNA gene sequences were
634 processed with dada2 (version 1.14) in the R environment[72]. Briefly, the reads were
635 trimmed where the quality profile dropped below Q30 (250bp for forward and 240bp for
636 reverse reads) and all reads with more than 2 expected errors were removed entirely.
637 The remaining reads were used to learn the error rates and the samples were pooled
638 prior to sample inference. After merging of forward and reverse reads with the default
639 parameters, chimeric sequences were removed with the removeBimeraDenovo
640 command in the consensus method. High quality reads were then classified against the
641 SILVA database version 132[73]. To avoid bias introduced by spurious amplicon sequence
642 variants (ASVs) or from samples with low sequencing depth, any ASVs with less than 5
643 reads and samples with less than 5000 reads were removed from the dataset before
644 further analysis. Statistical analysis of microbial communities was performed in the R
645 environment using the packages “phyloseq”, “Ampvis2”, “vegan”, and “MaAsLin2”. Alpha
646 diversity indices were calculated with a dataset rarefied to the minimum sample size
647 (8740 sequences). After assessing normal distribution by qqplots, histograms, and the
648 Shapiro-Wilk normality test, the not normal distributed groups were compared using the
649 Wilcoxon Signed Rank test. Beta diversity was calculated with no initial data

650 transformation and ASVs that were not present in more than 0.1% relative abundance in
651 any sample have been removed. The principal coordinates analysis was based on Bray-
652 Curtis distances. The effect of the route of inoculation on individual members of the
653 bacterial community was computed with MaAsLin2 and a minimum prevalence threshold
654 of 0.1 and a minimum relative abundance threshold 0.01.Benjamini-Hochberg procedure
655 was applied as a correction method for computing the q-values. All raw FASTQ reads for
656 16S rRNA gene sequences have been deposited under NCBI accession number:

657 **Real-time qPCR.** Real-time qPCR amplification was performed on DNA extracted from
658 the ceca and litter of broiler chicks as described previously[37] using a CFX96 Touch Real-
659 Time PCR Detection System (Bio-Rad Inc., Hercules, CA). Reaction mixtures (20 µl) for
660 all assays contained 1X SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Inc.,
661 Hercules, CA), 600 nM (each) primers (Supplementary Table 5), and 2 µl of cecal/litter
662 DNA. Calibration curves used for converting qPCR cycle threshold values to gene copies
663 per gram were determined using genomic DNA of *E. coli* or *Salmonella* strains harboring
664 the region or gene of interest

665 **Statistical analyses.** Continuous variables did not meet the assumption of a normal
666 distribution; therefore, non-parametric testing for direct comparisons was performed using
667 Wilcoxon rank sum and signed rank tests, and the Kruskal-Wallis rank sum test was used
668 for one-way analysis of variance tests. Furthermore, continuous variables were log-
669 transformed before any statistical tests were performed. Lastly, principal component
670 analysis was performed on litter and ceca samples using the copy number of genes
671 quantified by qPCR as features. The samples were then projected onto the first two
672 principal components and colored by route of SH challenge to visualize each route's

673 contribution to the data variance. Pearson correlation coefficients were calculated to
674 examine the correlation between *Salmonella ttr* and *E. coli gapA* gene copies present in
675 ceca and litter. Statistical analyses were performed using R (v 3.4.1).

676 **Data availability.** All raw FASTQ reads including short and long reads for sequenced
677 bacterial genomes are publicly available under NCBI accession numbers: PRJNA683658,
678 PRJNA684578 and PRJNA684580. Shotgun and Hi-C reads are publicly available under
679 NCBI accession number: PRJNA688069 and 16S rRNA gene sequences under NCBI
680 accession number: PRJNA669215. The whole genome assembly for *S*. Heidelberg
681 strains SH-2813-ancestor and ic9b harboring p1ST26 have been made available under
682 NCBI biosample number: SAMN12082795 and DDBJ/ENA/GenBank accession numbers
683 JAEMHU000000000, respectively. The whole genome assembly for *E. coli* strain Ec-FL1-
684 1X and Ec-FL1-2X carrying IncI1 are available under GenBank accession numbers
685 CP066836 and JAFCXR000000000, respectively. Variant call format (VCF) files of
686 identified SNPs/indels and the Linux/Unix shell script used have been deposited in Dryad
687 Digital Repository: https://doi.org/10.5061/dryad.4tmpg4f8d.

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

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709 **Author's contributions.** A.O., Z.A., K.C. and N.A.C. designed the study. A.O., G.Z.,
710 D.E.C. and C.R. performed live-broiler chicken studies. A.O., J.L., G.Z., S.H. and D.C.
711 performed bacteriological analyses, antibiotic susceptibility testing, DNA extraction and
712 qPCR. J.L., G.Z. and A.O. performed Illumina whole genome sequencing. A.O. performed
713 cecal shotgun and Hi-C library preparation. T.L. made 16S rRNA gene libraries and
714 sequencing. B.Z. performed 16S rRNA bacterial community analysis and interpretation.
715 K.I., A.H., and A.O. performed phenotype microarray analyses with the help of J.G. and
716 E.L. A.O., Z.A., M.O.P., J.C.T., S.M.L., R.W., J.L., D.C. and L.L performed bioinformatic
717 analyses and data curation. K.H. and A.O performed *Salmonella* Heidelberg fitness

718 testing and analysis. A.O., J.L. and D.C. isolated multidrug resistant *E. coli* donors from
719 cecal contents with help from M.J.R. A.O., B.Z. and M.E.B. performed statistical analyses.
720 A.O., Z.A., N.A.C., B.Z., R.W., M.P., S.M.L., K.I., G.Z., J.L., K.H. and L.L. drafted the
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722 M.W. supervised the study.

723

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965

966 **Table 1: Multidrug resistant *E. coli* genomes found in the ceca of broiler chicks**

Isolate ID	Resistance phenotype†	Antibiotic resistance genes‡	Plasmids§	Phylogroup	MLST
Ec-FL1-1X	**Amp Gen Str Fis Tet**	***aac(3)-Iid aph(3')-Ia aph(6)-Id aph(3'')-Ib blaTEM-1B tet (B) sul2***	IncF:B:A-, IncI1	D	69
Ec-FL2-2X	**Amp Gen Str Fis Tet**	***aac(3)-Iid aph(3')-Ia aph(6)-Id aph(3'')-Ib blaTEM-1B tet (B) sul2***	IncF:B:A-, IncI1	D	69
Ec-FL2-5X	**Amp Gen Str Fis Tet**	***aac(3)-Iid aph(3')-Ia aph(6)-Id aph(3'')-Ib blaTEM-1B tet (B) sul2***	IncF:B:A-, IncI1	D	69
Ec-FL1-2X*	Gen Str Tet	*aadA1 aac(3)-Via tet(A)*	IncF:B:A- IncI1 IncI2 Col8282 ColRNAI	F	6858
Ec-FL1-5X*	Gen Str Tet	*aadA1 aac(3)-Via tet(A)*	IncF:B:A- IncI1 IncI2 Col8282 ColRNAI	F	6858

967 * *E. coli* isolates carrying IncI1-pST26 with >99% identical DNA sequence with p2ST26 acquired by SH.
968 † Amp – ampicillin, Gen – gentamicin, Str – streptomycin, Tet – tetracycline, Fis – sulfisoxazole.
969 ‡ Boldness denotes antibiotic resistance genes encoded on the chromosome.
970 § Illumina short reads and PacBio long reads were combined to assemble the plasmids of E. coli strains Ec-FL1-1X and Ec-FL1-2X.
971

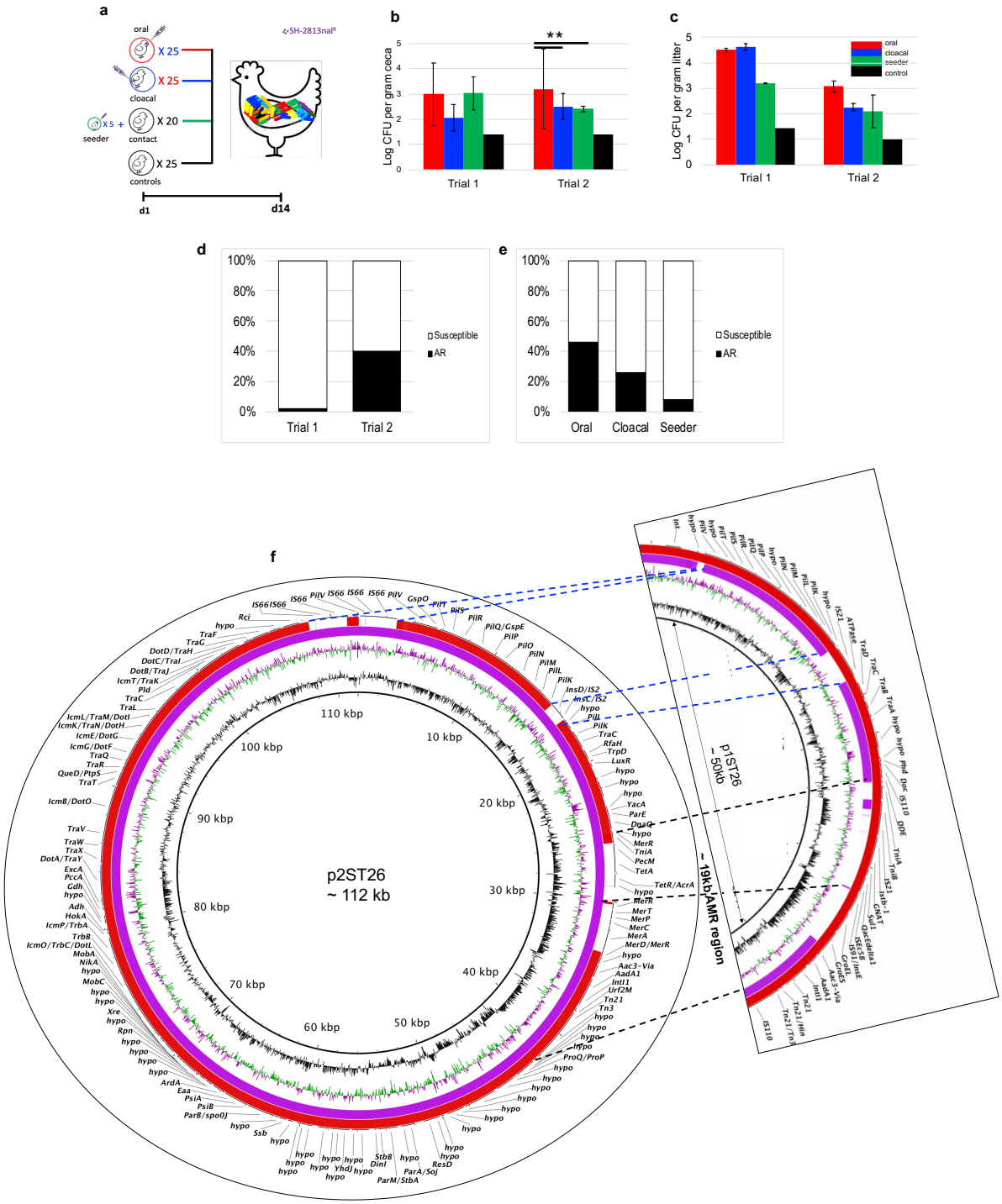


Figure 1. **Broiler chicks challenged with an antibiotic susceptible strain of SH carried antibiotic resistance SH strains 2-weeks after challenge**. **a**, Experimental design for trial 1 and trial 2 conducted in September 2017 and April 2018, respectively. **b,c** SH load in the ceca (n = 10 per trial) and litter (n = 2 per trial) 2 weeks after challenging 1-day broiler chicks (100 chicks/trial) with 10^6 colony forming units (CFU) of SH by oral gavage, cloacal or through the seeder method (*P < 0.01 (Kruskal-Wallis test, multiple comparison correction; error bar = Standard deviation; limit of quantification = Log 1.3 CFU/g litter). **d**, Percentage of SH isolates that acquired antibiotic resistance in trial 1 (n = 92) and trial 2 (n = 158) **e**, Percentage of SH isolates in trial 2 that acquired antibiotic resistance grouped by the route the broiler chicks got challenged (n = 54 62, and 42 for oral, cloaca and seeder, respectively. **f,** BLASTn alignment of the IncI1-complex plasmid acquired during trial 1 (p1ST26) and trial 2 (p2ST26) – dashed black lines highlights mobile region encoding antimicrobial resistance genes while blue dashed lines shows other regions that are different between p1ST26 and p2ST26.

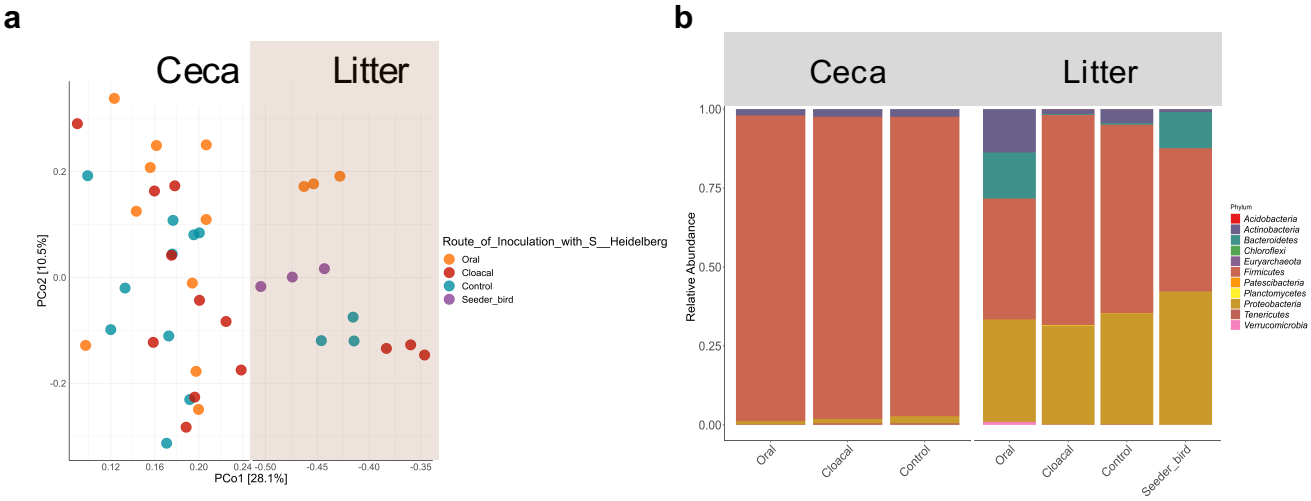
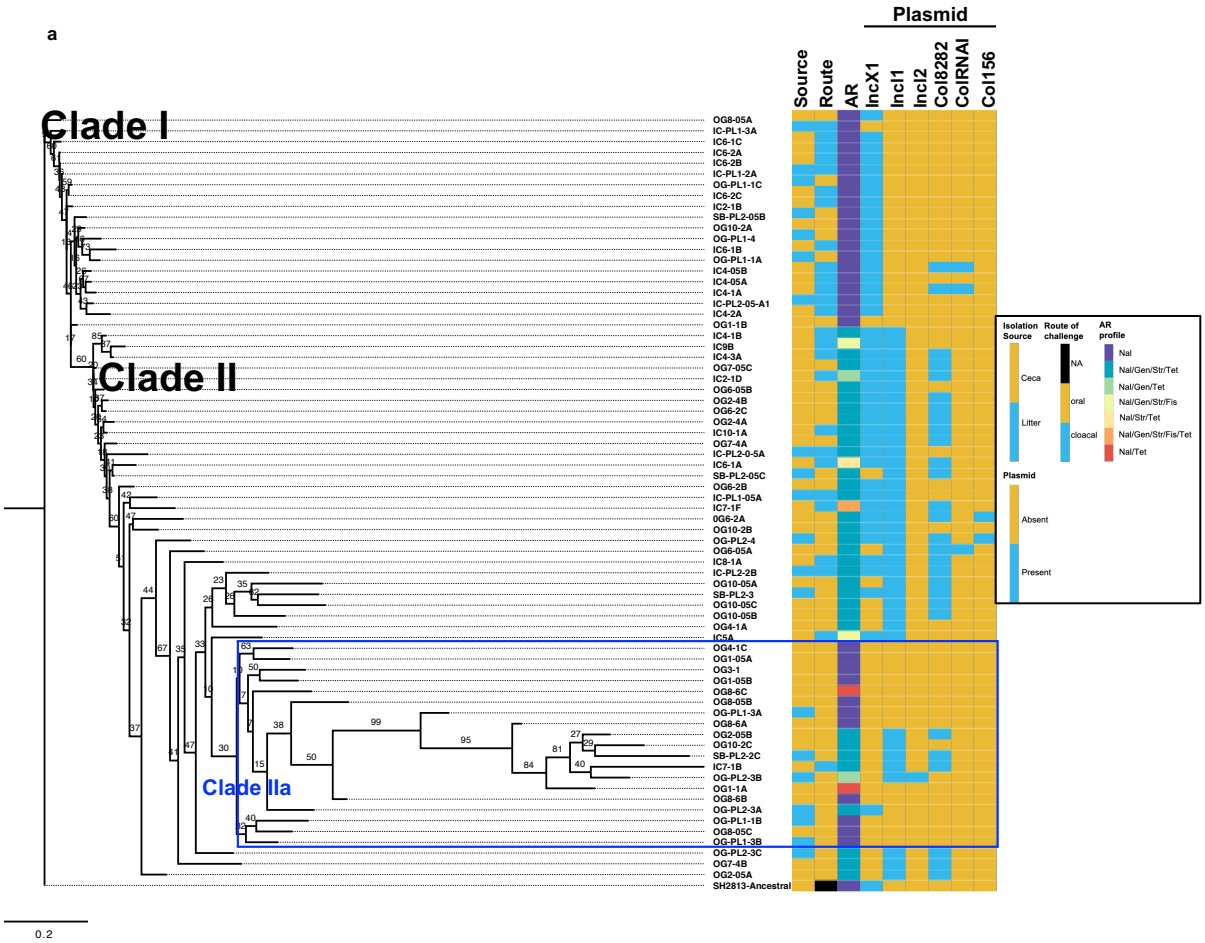


Figure 2. SH infection perturbed the bacterial community present in ceca and litter of broiler chickens. a, Principal coordinates analysis of Bray-Curtis distances based on 16S rRNA gene libraries obtained from ceca and litter samples. Each point represents values from individual libraries with colors expressing the route of inoculation of SH in for respective samples. b, Phylum-level classification of 16S rRNA gene sequence reads in ceca and litter grouped by route of SH infection. Data represent average of amplicon sequence variant (ASV) counts from replicate libraries for each category.



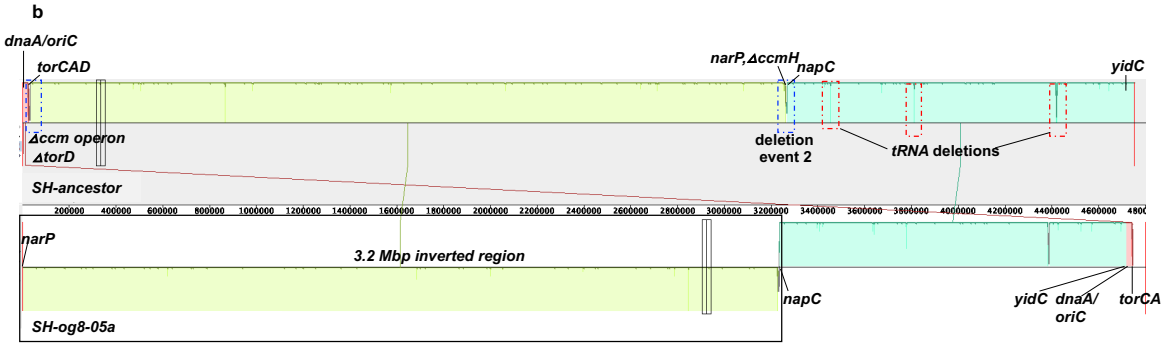


Figure 3. Horizontal gene transfer and genome inversion shaped the fitness of SH in broiler chicks. a, Maximum likelihood tree constructed using accessory genes present in SH isolates (n=72) recovered from the ceca and litter of broiler chicks colonized with SH. The GTR model of nucleotide substitution and the GAMMA model of rate heterogeneity were 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. Tree was rooted with the ancestral susceptible SH strain. Clade numbers were arbitrary assigned to facilitate discussion on isolates that acquired antibiotic resistance. Blue rectangular box highlights the clade with susceptible strains nested within antibiotic resistance strains due to sequencing bias and misassembly. (Legend: Nal – nalidixic acid, Gen – gentamicin, Str – streptomycin, Tet – tetracycline, Fis – sulfisoxazole, NA – Not applicable) **b,** Mauve visualization of the inverted genome of SH strain og8-05a. The chromosomal contigs of og8-05a was aligned and ordered to the complete chromosome of SH ancestor. A colored similarity plot is shown for each genome, the height of which is proportional to the level of sequence identity in that region. When the similarity plot points downward it indicates an alignment to the reverse strand of the SH ancestor genome i.e., inversion. Segment highlighted with solid black rectangular box shows the inverted region in og8-05a, while dashed blue and red rectangular boxes denotes segments with relevant mutations.

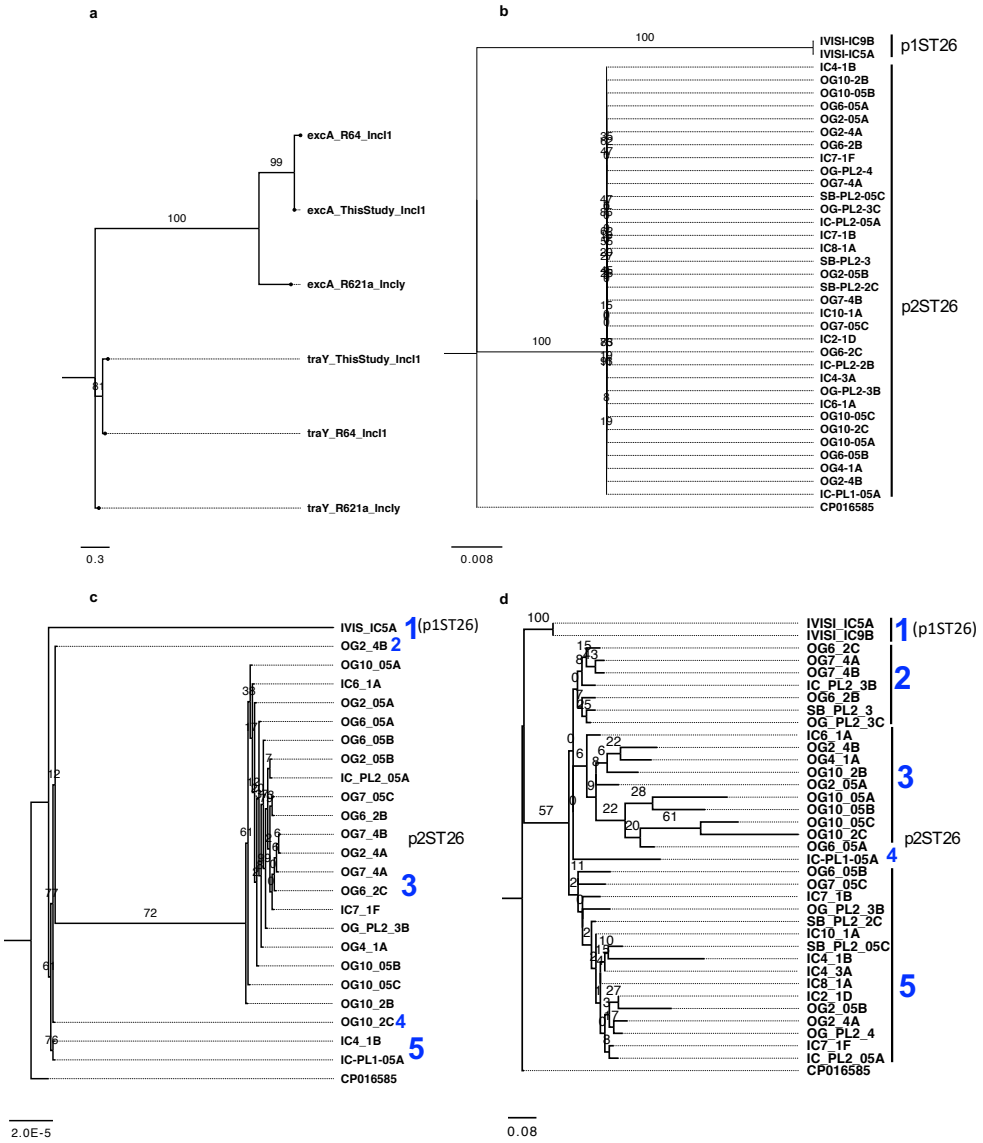


Figure 4. Gene flux contributed to the diversity of IncI1-pMLST26 plasmids. **a,** Maximum likelihood tree constructed using TraY and ExcA protein sequences from a representative IncI1 plasmid from this study, R64 (IncI1) and R621a (IncI1-gamma). The GTR model of nucleotide substitution and the GAMMA model of rate heterogeneity were used for sequence evolution prediction. Tree was rooted with traY sequence of R621a. **b - d,** Maximum likelihood tree of pST26 IncI1 plasmids from this study (n = 36) constructed using complete plasmid DNA sequences, core genes (**c**) and accessory genes (**d**). The GTR+I, GTR and JC+I model of nucleotide substitution and the GAMMA model of rate heterogeneity were used for sequence evolution prediction for b, c, and d, respectively (Tree was rooted with the closest relative (GenBank: CP016585) found through NCBI BLASTn)). Clade numbering were arbitrary assigned to show number of clades found and not order of evolution. Numbers shown next to the branches represent the percentage of replicate trees where associated isolates cluster together based on ~100 bootstrap replicates.

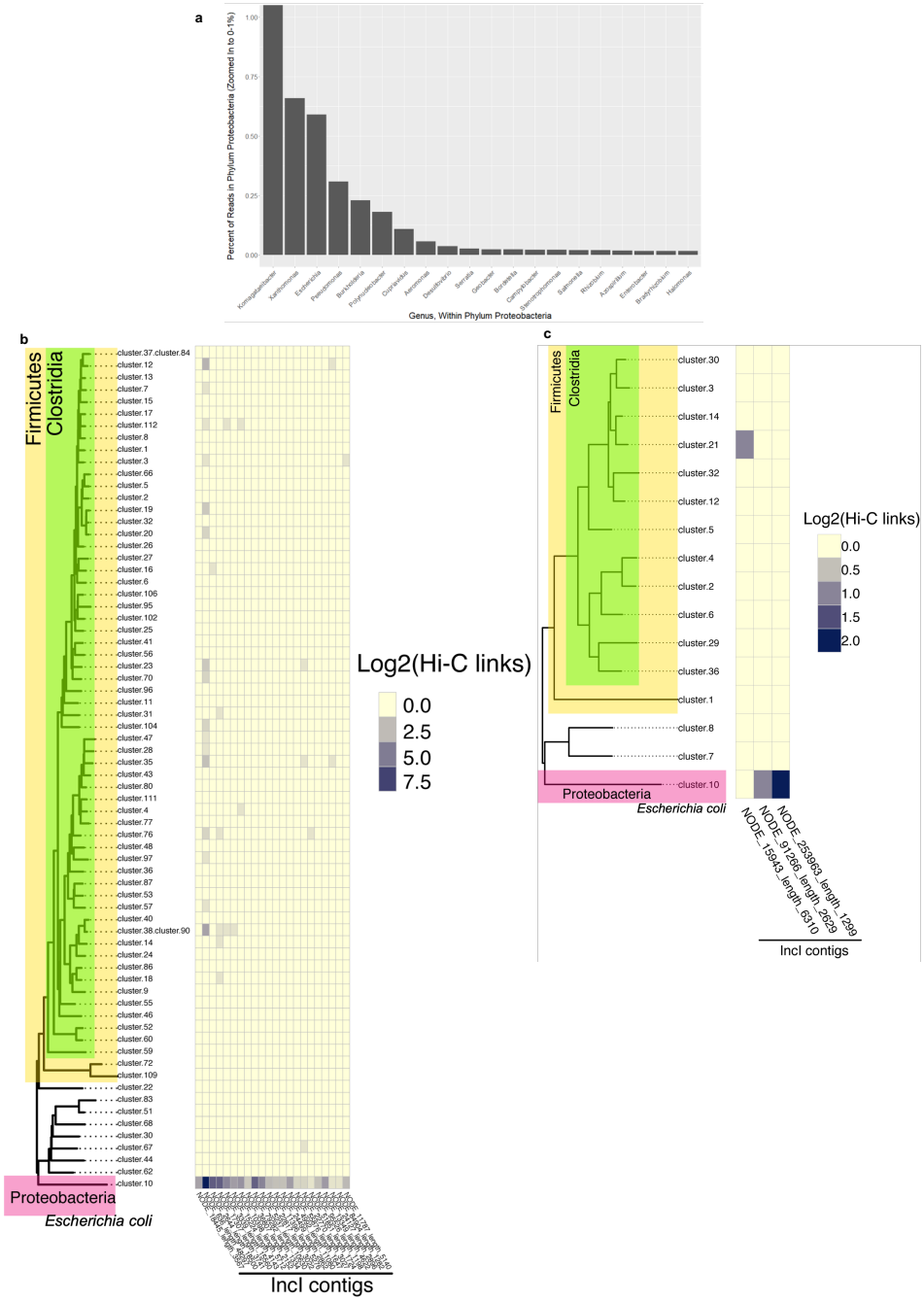


Figure 5. *Proteobacteria* was the reservoir of IncI1-plasmids in broiler chickens. a, Percentage of metagenomic reads assigned to the top 20 bacterial genera of the phylum Proteobacteria present in samples Hi-IC-FL1 and Hi-IC-FL2. Only reads assigned to phylum *Proteobacteria* (70.9 % of the metagenomic reads) were used and zoomed in to show 0-1% of total reads within phylum Proteobacteria (96.7% of reads within phylum *Proteobacteria* were assigned to *Komagataeibacter*). **b and c,** IncI1 contigs hosts found by Hi-C contacts. IncI1-derived contigs (horizontal axis of heatmap) show specific Hi-C associations with metagenome-assembled genomes (MAGs) present in samples Hi-IC-FL1 (**b**) and Hi-IC-FL2 (**c**) (vertical axis of heatmap). MAGs are derived from Hi-C deconvolution of the metagenome assembly and placed into a bacterial phylogeny using CheckM. Cluster.10 in each sample is an *Escherichia coli* genome. In Hi-IC-FL1, one MAG (cluster.82) representing an extremely fragmented and partially contaminated *Escherichia coli* genome is omitted for clarity (due to its contamination, this cluster was placed in *Clostridia* by CheckM). Heatmap values indicate transformed counts of Hi-C read contacts (indicating intracellular physical proximity of IncI1 contigs to those genomes. Heatmap values were pseudocounted to facilitate plotting of log-transformed data including zeroes.

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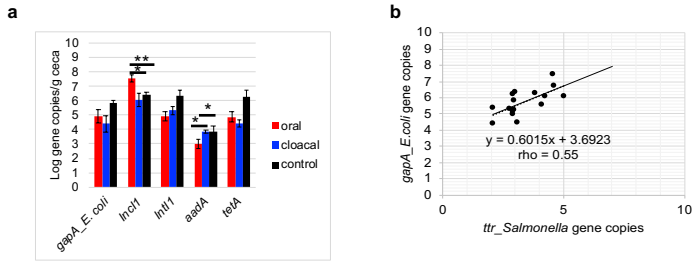

1027 **Figure 6. SH challenge affected the relative abundance of antibiotic resistance genes present in the ceca and litter of broiler chickens.** a, Concentration of selected antibiotic resistance determinants,
1028 IncI1 and *E. coli* present in the ceca of broiler chickens. Gene copies were determined using qPCR. Primers targeting the housekeeping gene (*gapA*) and *inc*RNAI was used for *E. coli* and IncI1 quantification,
1029 respectively. b, Pearson correlation between *Salmonella ttr* and *E. coli gapA* gene copies present in ceca and litter (n =15).

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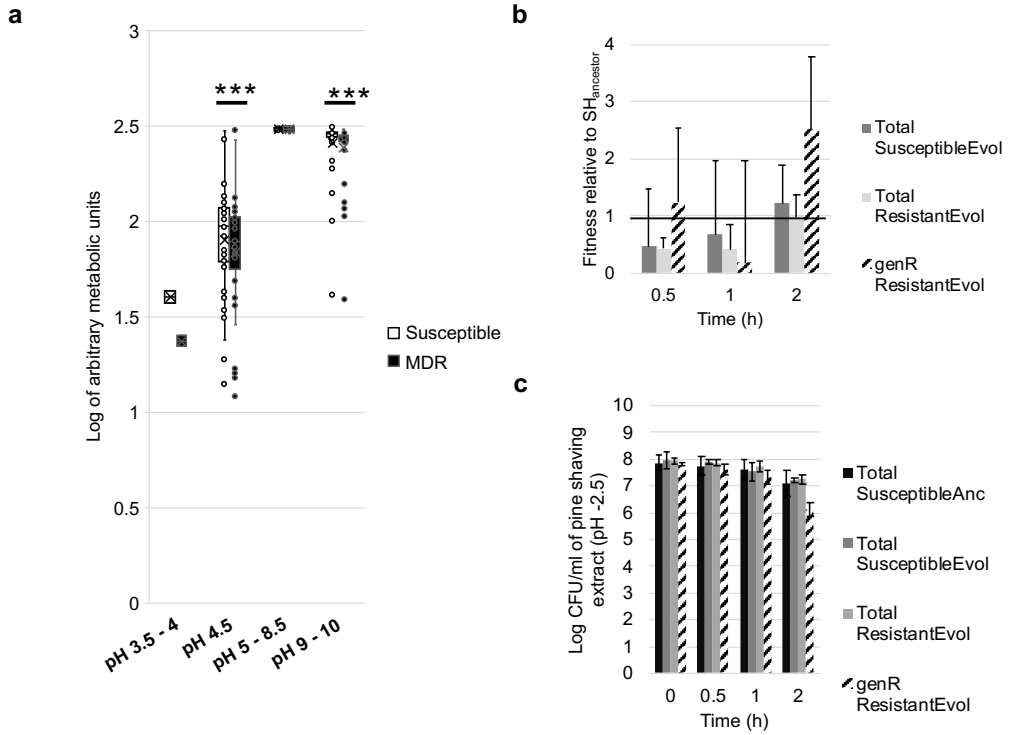

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1042 **Figure 7. SH exposure to acidic pH imposes negative frequency dependent selection for IncI1 (pST26). a** Box plot comparing the metabolic activity of one evolved susceptible (no IncI1) and one multidrug
1043 (MDR carrying pST26) SH strain using phenotype microarray (PM) plates (Biolog Inc). Pre-configured PM plates are composed of microtiter plates with one negative control well and 95 wells pre-filled with or without
1044 nitrogen and at pH of 3.5 – 10 (n = 1 plate per strain; no of wells per plate for pH 3.5-4, 4.5, 5-8.5 and 9-10 was 2, 6, 37, and 38 respectively; ***P < 0.001 (Wilcoxon signed rank test). **b and c,** Fitness and
1045 abundance of evolved susceptible and MDR SH strains when exposed to pine shaving extract of pH 2.5. Each bar represents the average fitness or abundance (colony forming units per ml of pine shaving extract)
1046 of three individual population that was established from three single bacterial colonies from three different strains (Error bar = Standard deviation). The horizontal line in **b** represents the fitness of three SH-Ancestor
1047 population established from three single bacterial colonies. (Anc = Ancestor, Evol – Evolved i.e., SH isolate recovered during *in vivo* experiment, genR – population resistant to gentamicin).

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