Competition is a determinant of multidrug resistant plasmid acquisition in *Salmonella*

Running Title: Commensal bacteria can competitively exclude *Salmonella* Heidelberg

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Abstract

Host microbiome homeostasis ensures that gut conditions are unfavorable to an invading pathogen such as *Salmonella enterica*. Consequently, fostering a "balanced" gut microbiome through the administration of microbes that can competitively exclude pathogens has gained a lot of attention and use in human and animal medicine. However, little is known on how competitive exclusion affects the transfer of antibiotic resistance. To shed more light on this question, we challenged neonatal broiler chicks raised on reused broiler chicken litter – a complex environment comprising of decomposing pine shavings, feces, uric acid, feathers, and feed, with *Salmonella* Heidelberg (S. Heidelberg), a model pathogen. We show that chicks raised on reused litter carried lower abundance of *Salmonella* and harbored a more uniform and diverse microbiome comprising of bacterial species that are known to provide colonization resistance towards *Salmonella* compared to chicks raised on fresh bedding composed of pine shavings. Additionally, these bacterial species were associated with a lower horizontal transfer of multidrug resistance genes to *S*. Heidelberg. Using in vitro competition experiments, we confirmed that conjugation between *S.* Heidelberg and *E. coli* strains from chicks raised on fresh litter resulted in the acquisition of multidrug resistant plasmids. Contrastingly, bacteriophage-mediated recombination between *S*. Heidelberg and *E. coli* strains made the acquisition of plasmid-mediated β-lactamase gene (bla_{CMY-2}) possible. Collectively, this study demonstrates that competitive exclusion can reduce the transfer of antibiotic resistance and provides information on the bacterial species that can be explored for their benefits to reduce antibiotic resistance transfer.

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 antibiotic 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 antimicrobial resistance spreads in the absence of antibiotic selection and the role commensal bacteria play in reducing antibiotic resistance transfer. In this study, we demonstrate that commensal bacteria play a key role in reducing the horizontal transfer of antibiotic resistance to *Salmonella* and provide the identity and characteristics of the bacterial species that performs this function in broiler chickens.

Introduction

Horizontal gene transfer is recognized as the main way bacteria acquire antibiotic resistance (AR) and exposure to antibiotics has been shown to be a driver of antibiotic resistance gene (ARG) transfer (1). Consequently, the rise in AR bacteria in hospital settings has been linked to the overuse of antibiotics in humans and its 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). However, how much reduction in AR transfer is achievable from removal is still poorly understood. We previously showed that neonatal broiler chicks challenged with a susceptible *S*. Heidelberg (SH) strain and raised antibiotic-free on fresh bedding composed of pine shavings acquired multidrug resistant (MDR) SH strains at a high rate (7). We also demonstrated that horizontal transfer of a MDR plasmid from *E. coli* strains to SH conferred the MDR phenotype (7). We selected SH as the model pathogen because of its promiscuity to plasmids carrying AR (8, 9).

There is limited research on if and how competitively exclusion reduces AR in food-borne pathogens (10-16) such as *Salmonella enterica* or the role it plays in AR reduction and transfer. Therefore, the goal of this study was to determine the role competitive exclusion plays in limiting *Salmonella enterica* from acquiring AR and provide information on the bacterial species and mechanism involved. To do this, we compared the dynamics of AR transfer in neonatal broiler chicks raised on fresh bedding composed of pine shavings 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, 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 flock of broiler chickens is a widespread practice in the US and Brazil while Canada (20) and European counterparts (21) recommend fresh litter bedding for every flock. One argument against litter reuse is that it harbors pathogenic bacteria that can be horizontally transferred to the next flock (22). Contrastingly, proponents of litter reuse argue it confers competitive exclusion against pathogens when effectively managed and it is cost-effective (23-26). Beyond broiler chicken production, competitive exclusion by “beneficial” microbes 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

Neonatal broiler chicks raised on reused litter did not acquire antibiotic resistant *S.* Heidelberg

Total *Salmonella* concentration in the ceca of broiler chicks was not significantly different between chicks on fresh litter compared to chicks on reused litter using both culture (p=0.15) and qPCR methods (p = 0.29) (Fig. 1a and b). However, *Salmonella* concentration in the litter was variable and differed between culture and qPCR method. For instance, *Salmonella* concentration was not significantly different between fresh

litter and reused litter using qPCR (p = 0.34) but was higher in reused litter (p = 0.08) using culture method. The number of cecal samples positive for *Salmonella* after enrichment in buffered peptone water (BPW) was 76% (n =34) for fresh litter and 63% for reused litter (n =35). Chicks inoculated via the cloaca were all positive for *Salmonella* after ceca was enriched in BPW regardless of the litter type. A few cecal (n = 5) and litter (n = 2) samples from the uninfected control chicks were positive for *Salmonella* with qPCR and after enrichment in BPW. Lastly, 36 % of SH isolates (n = 158) from the ceca and litter of chicks from fresh litter acquired MDR (i.e., resistance to gentamicin, tetracycline, and streptomycin), compared to 0.7 % of the isolates (n =141) from reused litter (Fig. 1e).

Reused litter carried lower abundance of transferable plasmids than fresh litter

We used qPCR to target selected genes present on the MDR IncI1-pMLST26 plasmid that was acquired by SH (7). This plasmid carried antibiotic resistance genes aminoglycosides (*aadA1* and *aac(3)-via*) and tetracyclines (*tetA*). There was no significant difference in the abundance of IncI1, *aadA* and *tetA* (p>0.2) between fresh and reused litter but reused litter carried lower levels of these genes (Fig. 1c). Class -1 integron gene (*IntI1*) was significantly higher in reused litter compared to fresh litter (p = 0.03). In the ceca, *tetA* (p=0.01) *and aadA* (p=0.04) were higher for chicks on reused litter compared to chicks on fresh litter.

To determine the plasmid and ARG types in the ceca, we aligned metagenome assembled contigs of cecal samples (n = 4) to plasmid and ARG reference sequences available on PlasmidFinder and ResFinder databases (see methods for description). This revealed that chicks from fresh litter carried more plasmids (n = 6±4) than chicks

on reused litter (n = 3±2), including the MDR IncI1 plasmid that was acquired by SH (7) (Fig. 1d). Similarly, the ceca of broiler chicks from reused litter carried fewer ARG contigs (n = 2±2) than chicks on fresh litter (n = 16±3). The majority of the ARG's found confer resistance to various drugs in the aminoglycoside class of antibiotics. Together, this data suggests that reused litter harbored fewer MDR plasmids that could be transferred to *Salmonella* compared to fresh litter.

Neonatal chicks raised on reused litter harbored a diverse population of beneficial microbes and upregulated metabolic pathways

The alpha diversity of the bacterial community present in the ceca and litter of chicks on reused litter was higher for the observed species measure (Ceca $p<0.001$, litter $p=0.001$) but not for Shannon (Ceca $p=0.25$, litter $p=0.05$) and Simpson (Ceca $p=0.969$, litter $p=0.28$) index (Supplementary Fig. 1a). Chicks on reused litter carried a more uniform bacterial community in their ceca and litter compared to chicks on fresh litter and beta diversity was affected by the route chicks were inoculated with SH (Supplementary Fig. 1b and c). Actinobacteria was higher in reused litter compared to fresh litter while Proteobacteria and Bacteroidetes were higher in fresh litter compared to reused litter (Fig. 2a; Supplementary Fig. 2). In the ceca, Actinobacteria and Proteobacteria were higher in the ceca of broiler chicks on reused litter compared to chicks on fresh litter. We determined that members of the genus *Bifidobacterium*, *Brevibacterium*, *Brachybacterium*, *Corynebacterium*, *Faecalibacterium*, *Escherichia/Shigella*, *Halomonas, and Salinococcus* among others contributed significantly to the higher diversity of litter and ceca of chicks raised on reused litter compared to fresh litter (Fig. 2b).

To determine the species associated with the 16S rRNA amplicon sequence variants found in reused litter, we aligned shot gun reads from the ceca of broiler chicks on fresh (n =2) and reused (n = 2) litter (~111 million reads per sample) to Kraken database (30). We identified ten species of *Bifidobacterium* including *B. longum* (21% of the genera), and *B. catenulatum (*11% of the genera*)* that had the most abundant reads in the genus (Fig. 2c; Supplementary File 1). *Escherichia/Shigella* were represented by *E. coli* (~98% of the genera, *E. albertii*, *E. marmotae*, *E. fergusonii*, *S. dysenteriae*, *S. boydii*, *S. flexneri* and *S. sonnei*. *Faecalibacterium* **prausnitzii**, *Salinicoccus halodurans and Jeotgalicoccus saudimassiliensis* were the only species identified using shot gun reads. We identified between 18 – 59 species for the genera *Halomonas*, *Streptococcus*, *Lactobacillus* and *Corynebacterium* (Supplementary File 1).

Next, we used metagenome assembled genomes (MAGs) to assess functional pathways that were enriched in the ceca of chicks from fresh compared to reused litter. The average assembly size and number of contigs was 1,238,395,273±167,820 bp and 1,797,423±281253 for the four samples. The number of MAGs found in reused litter was higher (754±409) than fresh litter (156±98). Pathways related to biosynthesis of organic and antimicrobial molecules were enriched to a greater degree in reused litter samples than in fresh litter samples (Fig. 2d). Of note was the enrichment of antimicrobial biosynthesis pathways in reused litter (macrolides, tetracyclines, and carbapenems), suggesting an increased level of competition between bacterial species as the litter became more used. Increased competition in reused litter was supported by a simultaneous increase in molecules that are targets of antimicrobials, such as folate, which is the target of the sulfonamides. Contrastingly, pathways associated with the

biosynthesis of secondary metabolites of plants including flavonoids and indole-alkaloids were enriched in samples from fresh litter.

Neonatal chicks raised on reused litter carried fewer MDR *E. coli* strains

Microbiota analyses revealed that SH invasion of the chicken gut resulted in a reduction of *E. coli* populations in the ceca of chicks raised on fresh and reused litter, while the cecal metagenome revealed that *E. coli* was the main reservoir of multidrug resistant IncI1 plasmids (7). Furthermore, *E. coli* population was higher for broiler chicks on reused litter compared to chicks on fresh litter (Fig. 3a). Therefore, we retrospectively screened the ceca of broiler chicks from fresh and reused litter for susceptible and multidrug resistance *E. coli* strains and sequenced the genomes of nineteen selected strains.

We observed that the phylogeny of the *E. coli* strains could be explained by the litter used for raising broiler chicks. For instance, phylogroups A, D and F were only found in the ceca of chicks raised on fresh litter, whereas phylogroups B1 were restricted to the ceca of chicks from reused litter, except for one strain that belonged to phylogroup A (Fig. 3b and c). Likewise, multi locus sequence type (MLST) classification showed that ST 69, 2705 and 6858 were associated with *E. coli* strains originating from fresh litter. The only ARG carried by *E. coli* strains from reused litter was $bla_{TEM\text{-}1B}$, except for one MDR isolate. On the other hand, *E. coli* strains from fresh litter harbored multiple ARG on their chromosome and plasmids, including the MDR IncI1 plasmid that was acquired by SH.

Competition experiments between SH and *E. coli* strains resulted in the acquisition of MDR IncI1 plasmids.

To confirm the transfer of MDR plasmids from *E. coli* to SH and to determine the mechanism involved, we performed a competition experiment on solid CHROMagar™ with an antibiotic susceptible SH strain and four *E. coli* strains (n = 2 *E. coli* strains from fresh and reused litter, respectively) that differed in their virulence and AR genes (Fig.4a, Supplementary File 2). To decipher the role of transduction (bacteriophage-mediated transfer) in MDR acquisition, we added either Ca^{2+} to CHROMagar™, a cation required for phage adsorption to bacterial cells, or citrate which chelates Ca^{2+} (31). Additionally, we used synthetic 2-hexadecynoic acid (2-HDA), a potent inhibitor of conjugal plasmid transfer (32) to determine if cell-to-cell contact (conjugation) was needed for MDR plasmid transfer from *E. coli* to SH (Fig. 4a).

Adding citrate to CHROMagar™ reduced the population of SH (X^2=13.03, df =3, P = 0.004) and *E. coli* (X^2=19.83, df =3, P = 0.0002) and SH did not acquire MDR (Fig. 4b and c). However, SH acquired gentamicin (gen^R) and ampicillin (amp^R) resistance in the absence of citrate, 2-HDA and $CaCl_2$. Gentamicin resistant SH population appeared at a lower a rate when 2-HDA was added to CHROMagar™ compared to controls (X^2=15.71, df =3, P = 0.001) (Fig. 4b). We observed a 24 h delay in the appearance of amp^R SH and colony counts did not follow the expected dilution-fold differences or were too numerous to count (n = 9) (Supplementary Fig. 3a). Furthermore, none of the amp^R SH isolates (n = 9) selected from $CaCl_2$ plates were resistant to ampicillin after a second susceptibility screen. For amp^R isolates that passed the second screen, 8 of 35 displayed AmpC β-lactamase resistance phenotype by broth microdilution (these eight ampR isolates are thereafter referred to as amp^R recombinants) while 13 were resistant to cefoxitin. This suggested that amp^R was not stable in SH after acquisition.

To determine the genetic determinants associated with amp^R and gen^R, we sequenced nineteen SH recombinants including susceptible isolates. Amp^R recombinants harbored IncI1-pMLST12 (pST12) encoding *bla*$_{CMY-2}$, whereas gen^R recombinants carried IncI1-pMLST26 encoding multidrug resistance. In addition, SH recombinants that acquired pST12 lost parental resistance to nalidixic acid. It is noteworthy to mention that none of the *E. coli* strains harbored pST12 or *bla*$_{CMY-2}$ and suggests that recombination of genes caused an emergence of pST12.

The acquisition of IncI1 plasmid carrying *bla*$_{CMY-2}$ gene altered the genome of *S.* Heidelberg.

To determine the HGT mechanism involved in the emergence and transfer of pST12, we compared mutations found in gen^R to amp^R recombinants. Amp^R recombinants had parallel/hotspot mutations in 107 loci on the chromosome and 3 loci on IncX1 plasmid (Supplementary File 3). A KEGG orthology analysis examining proteins affected by these mutations suggests they are involved in enzyme metabolism, amino acid and secondary transport, and transcription of DNA to RNA (Supplementary Table 1). In addition, 20% (18 of 87) of proteins affected by mutations are associated with P22 bacteriophage infection (33) (Supplementary File 3). For instance, we found mutations in genes that are part of an O-antigen gene cluster (*rfbBCP)*. The *rfb* cluster genes are either required for or aid in P22 infection (33). Surprisingly, the *gyrA* mutation that conferred nal resistance was reverted in all amp^R recombinants. In comparison, we did not find *gyrA* reversion or hot spot mutations in gen^R or susceptible recombinants, but all recombinants carried 6 mutations in the intergenic region between P4-like prophage DNA-binding transcriptional regulator AlpA (Supplementary Table 2) and virulence

factor PgtE. Furthermore, the MLST (34) of the core genome of amp^R recombinants (cgST=114049) differed from that of the ancestor (cgST=171516) and genR or susceptible recombinants (cgST=186980).

A ML tree with chromosomal SNPs and accessory genes (Fig. 5a and b) grouped amp^R recombinants on the same clade with agar lysate and distantly separated them from gen^R and/or susceptible recombinants. Furthermore, a whole genome alignment with protein sequences of two complete chromosomal contigs showed that inversion occurred in the amp^R recombinant and not in gen^R recombinant (Supplementary Fig. 4). This inversion affected a 700 Kbp region and deletions in the intergenic regions of 5S-23S and 5S-16S rRNA gene contributed to *oriC* reorientation.

The clustered regularly interspaced short palindromic repeats (CRISPRs) and their associated cas immunological system is supposed to supply memory of the foreign genetic elements that have invaded the host bacteria. In SH, there are two CRISPR I-E systems (CRISPR1 and 2) known to perform this function (35). Therefore, we compared the CRISPR-Cas genes in SH recombinants to determine if acquisition of protospacers associated with CRISPR adaptation occurred. We found a 305-bp CRISPR repeat in CRISPR2 for all amp^R recombinants that was absent in gen^R recombinants or the SH ancestor (Supplementary Fig. 5a and d). This included 5 direct-repeats (29 bp each) interspersed by 5 spacers (32 bp) – hereafter referred to as spacer 1 – 5. The extra repeats were inserted between spacer 3 and repeat 4 of the ancestral CRISPR2 (Supplementary Fig. 5a). Furthermore, the extra repeat had higher DNA sequence coverage than the surrounding CRISPR2 DNA sequence (n = 7, V = 28, P = 0.015) (Supplementary Fig. 5b).

To decipher the biogenesis of this repeat, we performed BLAST alignment against the genome of *E. coli* donors. We reasoned that SH could add spacers against non-self-foreign DNA. We found regions of homology in the repeat region of all *E. coli* donors, but no homology was found for the spacers (Supplementary Fig. 5c). To determine the targets of the spacers, we performed searches against curated phage, plasmid and transposon databases using NCBI or CRISPRtarget (36). We found no match for spacer 1, 3 and 5, albeit spacer 2 matched IncFIB plasmids present in *Raoultella Orthinolytica* (RefSeq: NZ_CP012557.1) and *Pantoea* sp. (RefSeq: NZ_CP022519) and spacer 4 matched CRISPR2 spacers in several *S*. Heidelberg and *S*. Typhimurium genomes. The matching spacer 2 was found in a ParB/RepB/Spo0J family plasmid partition protein (SopB) encoded by Inc (F4: A-: B1) (59 % pairwise DNA identity i.e., 19 of 32 bases were 100% identical) and IncI1 plasmids (pST12 and pSTnovel are 52% identical to spacer 2) present in *E. coli* donor strains from fresh litter (Supplementary Fig. 5d and e). These results suggested that acquisition of IncI1 plasmid carrying bla_{CMY-2} gene changed the genome and sequence type of SH recombinants.

Identifying how a bacteriophage facilitated the transfer of IncI1 plasmid carrying bla_{CMY-2} gene

Compared to conjugation, transduction is more likely to lead to a change in bacterial gene content, genetic structure, and mutations. To determine if a bacteriophage mediated pST12 transfer, we sequenced the lysate extracted from citrate treated CHROMagar™ and performed long read sequencing on a gen^R and amp^R recombinant to achieve complete genomes. For amp^R recombinant, complete circular contigs were assembled for IncX1 and ColpVC plasmid, while the longest chromosomal contig

measured 4.7Mb (Supplementary Table 3). The rest of the contigs were determined to be part of pST12 (~101 kb). Contrastingly, the gen^{R} recombinant assembly resulted in a complete chromosome, IncX1 and Col8282 and the remaining contigs were mapped to pMLST26 (~107 kb).

Next, we aligned the sequence reads of the lysate to SH genome and de-novo assembled the unmapped reads. Our hypothesis was that SH transcription would be directed towards transducing phage proteins and therefore would have greater number of sequenced read coverage. After read alignment, we found a ~40 kb CDS region encoding *Salmonella* P22 phage with twice the coverage of the SH chromosome (Supplementary Fig. 6). Additionally, the assembled unmapped reads were pST12, suggesting the lysate carried a transducing particle (Supplementary Table 3). Furthermore, SH strains that acquired pST12 carried mutations affecting P22 including a mutation downstream of *gtrC* (serotype conversion gene) and phage tail (endorhamnosidase) (Supplementary File 3).

To determine how the pST12 plasmid backbone evolved, we performed multiple alignment of all chromosome and plasmid contigs from *E. coli* donors except IncX1 (present in the SH, thus incompatible) and Col plasmids and used pST12 contigs as the reference genome (Fig. 5c). This revealed that pST12 was a chimera of plasmids and chromosomal genes with a pST26 and novel IncI1 (pSTnovel) backbone (pST12 is ~40% identical to pMLST26 and ~30% to pSTnovel). Other plasmids that were determined to be part of pST12 were *inc* groups FIB/FII and IncHI1B/p0111. For example, *inc*FIB/FII present in Ec-RL-1X was the only plasmid harboring the five

uncharacterized genes located between *ccdA* and *cia*, and no plasmid was identified for regions encoding *ISEc9-sugE-blc-amp*C.

After comparing the pST12 from this study with identical plasmids (n = 93), we found only twenty-four genes to make up its core genome. These included genes encoding conjugal transfer (*tra, dot and icm)* and tyrosine recombinase *(xerD)*. (Supplementary Table 4). A ML tree reconstructed with accessory genes revealed that the closest MLST to pST12 comprised of non-typeable plasmids, ST24, ST26 and ST36 (Supplementary Fig. 7). Furthermore, a KEGG orthology analysis of the IncI1 plasmids from this study revealed they share gene modules for secretion system, cytoskeleton, and prokaryotic defense system, but pST26 and pST12 have additional modules for transport and AR (Supplementary Table 5). These results suggest that recombination between plasmids and chromosomal genes present in strains of *E. coli* and SH gave rise to IncI1 plasmid carrying bla_{CMY-2} gene and a P22 bacteriophage aided its transfer to SH.

Identifying recombination events between SH and *E. coli* that gave rise to a transferable bla_{CMY-2} gene.

We found an AmpC region with identical DNA sequences to bla_{CMY-2} on the chromosome of *E. coli* and SH. This region encodes translation elongation factor (*efP*), quaternary ammonium compound-resistance (*sugE),* outer membrane lipoprotein (*blc*), AmpC β-lactamase (*blaMIR)* and fumarase reductase genes (*frdABCD*) in *E. coli*, but in SH a *luxR* regulator and an uncharacterized protein replaced AmpC. A phylogenetic tree built with the AmpC/Frd operon showed that *E. coli* donors (Ec-FL-1X and Ec-FL-2X) were the closest relative (78%) to the bla_{CMY-2} region in pST12 (Supplementary Fig. 8). The *frd* proteins of SH and *E. coli* (*frd* of SH is 87 % identical to *frd* of *E. coli*) are

more closely related compared to *blc-sugE (blc-sugE* of SH is 70% identical to *blc-SugE* of *E. coli)*. The results suggested that AmpC was mobilized from the chromosome to IncI1.

We did not find the *ISEc9* transposon that was co-mobilized with AmpC in *E. coli* donor strains or SH recipient but *ISEc9* was present on the chromosome of agar lysate and amp^R recombinants (Supplementary Fig. 9a). *ISEc9* was inserted into the chromosome of amp^R recombinants, and it truncated an uncharacterized protein (*ynfC*) located downstream of spermidine N (1)-acetyltransferase (*speG*) (Supplementary Fig. 8a). Similarly, *ISEc9* was inserted into a hypothetical protein (*yagA*) in pST12 and in so doing truncated the *yagA* gene (Supplementary Fig. 9b). This suggested that *ISEc9* was first encoded on the chromosome before it was mobilized to IncI1. A BLAST search against the NCBI RefSeq database revealed that multiple chromosomal regions in *Enterobacteriaceae* encode *ISEc9* (n= 50) including *Salmonella* (n = 19) and *E. coli* (n =9). For ~ 60% of the occurrences, bla_{CTX-M} or bla_{CMY-2} was the neighboring gene to *ISEc9* (Supplementary File 4). For example, *S*. Typhimurium strains (Genbank: CP022062 and CP028314) carried *ISEc9* and bla_{CMY-2} on their chromosome and on a IncI1 plasmid.

In *E. coli*, the promoter of *ampC* overlaps *frdD* and serves as a transcriptional terminator of the Frd operon. Therefore, AmpC and Frd operon are tightly coupled and changes in the overlapping region are expected to affect both operons (37). The Frd operon is important for anaerobic respiration and requirement for fumarate can drive the transcription of a cascade of genes (38) and lead to the formation of antibiotic resistant bacterial persisters (39). This selection for fumarate may explain the reports of plasmids

encoding Frd and AmpC operon in *Enterobacteriaceae* species (39-41). We searched the cecal metagenomes for pST12 and found contigs with identical protein sequences (n =119, mean contig size = 7,858 bp, smallest contig = 1,000 bp, largest contig = 103,724 bp) including a ~ 18 kb contig encoding AmpC/Frd operon in chicks from fresh litter (Supplementary Fig. 9a ; Supplementary File 5). Likewise, we found contigs matching pST12 (n =121, mean contig size = 22,643 bp, smallest contig = 1,017 bp, largest contig = 243,272 bp) in the cecal metagenome of chicks from reused litter and one contig (~18 kb) also carried AmpC/Frd operon. We did not find any contig encoding *ISEc9* in the cecal metagenome but when we restricted our BLAST search to viral genomes available on NCBI, we found *ISEc9* on two bacteriophages - *Enterobacter* phage LAU1 and *Salmonella* phage SJ46.

E. coli donor strains carried multiple lambda phages and SJ46 (Supplementary Fig. 11a) and some shared proteins with significant homology with P22 phage in SH. For instance, donor Ec-FL-1X harbored two lambda phages with one encoding lysozyme R (*rrrD*), superinfection exclusion protein B (*sieB*) and recombination proteins (*ninBE*) (Supplementary Fig. 11b). These genes are important for bacterial host lysis, resistance to phage re-infection and phage recombination, and their presence suggests P22 can lysogenize the *E. coli* genomes used in this study and vice versa. Similarly, P22 encodes two regions of homology with pSTnovel, including its coat protein (~9% identical to *parA* in pSTnovel) that is required for P22 packaging and Rha family transcriptional regulator (~12% identical to *repZ* in pSTnovel). Together, our results suggest that selection for fumarate genes encoded by *E. coli* and SH gave rise to

387 recombinants carrying *bla*$_{CMY-2}$ on an IncI1 plasmid, and bacteriophages aided its
388 mobilization from chromosome to plasmid.

389 **Proposing a transduction model for IncI1 plasmid carrying *bla*$_{CMY-2}$ gene**

390 Competition resulted in the lysis of some *E. coli* and SH strains and our model suggests
391 that P22 erroneously acquired part of their genome during procapsid packaging and
392 transduced this DNA after re-infecting SH (Supplementary Fig. 12a). P22 can transduce
393 DNA that are between 2- 5% of the size of its genome i.e., < 750 - 2,000 bp (42).
394 Further, DNA regions in multiple copies and under selection are expected to be
395 transduced at a higher rate than low copy DNA (43, 44). In this study, we determined
396 this region to encode AmpC/Frd operon in *E. coli* donors. We could not identify the
397 origin of *ISEc9* transposon or why it is co-located with *blc-sugE*-AmpC in IncI1, but our
398 results suggest it was formed during transduction. By inserting itself next to *speG*, an
399 indispensable protein for polyamine detoxification, *ISEc9* was maintained in the
400 chromosome and transferred to a plasmid by a bacteriophage.

401 Amp^R recombinants carried a high copy small ColpVC plasmid and an IncX1 with 3
402 hotspot mutations including a 22-bp iteron deletion. When we compared the hotspot
403 mutations of amp^R recombinants to other SH genomes carrying ColpVC, we found that
404 all genomes carried 50 and 3 hotspot mutations on their chromosome and IncX1,
405 respectively (Supplementary File 6; Supplementary Fig. 12b). In our previous work (45),
406 we demonstrated that SH strains that had a deleted iteron carried a higher copy of
407 IncX1, which we speculated was needed for mobilizing ColE1 plasmids. This was also
408 true in this study, Amp^R recombinants carried higher copies of IncX1 (1.81 $\pm$ 0.05 copies

per cell) than gen^R and susceptible recombinants (1.16 ± 0.17 copies per cell) (W = 0, *P* < 0.001).

The non-coding region downstream of the origin of replication of ColpVC (84 bp) is 94% identical to the origin of transfer (*oriT*) of the relaxosome (*nikA*) of pST12 and pSTnovel (~ 110 bp region upstream of *nikA*). In addition, we found the region upstream of *nikA* to have the highest sequence coverage (8X for pST12 and 5X for pSTnovel) when compared to the rest of the IncI1 genomes (Supplementary Fig. 14). Likewise, ColpVC had the highest genome coverage (~13,000X) (Supplementary Fig. 12c) and the chromosomal region encoding P22 had twice the coverage of the chromosome (Supplementary Fig. 6). This suggests that IncI1 and P22 bacteriophages are capable of mobilizing ColpVC plasmids.

It is plausible that carriage of ColpVC provides conjugative plasmids with extra *oriT* that is required for conjugation, while ensuring their own mobilization. Consequently, encapsulation of ColpVC by bacteriophages not only enhances the stability of ColpVC but also helps maintain IncI1 plasmids in the bacterial host. Indeed, early studies on P22 transduction suggested that P22 infection aids productive rolling circle replication of ColE1 plasmids by providing a substrate for headful packaging (43). The 2.2kb size of ColpVC puts it in the upper end of the predicted genome size P22 can transduce. There is no reported benefit ColpVC gives the bacterial host, but it is prevalent in SH strains harboring IncX1 and/or pST12 (9). Rodriguez-Rubio et al (44) showed that encapsulation of multicopy Col plasmids by bacteriophages increased AR transfer 10,000-fold compared to encapsulation of low copy plasmids.

Discussion

Establishing a “balanced” gut microbiome through the administration of probiotics has gained a lot of attention and use in human and animal medicine, but its effect on AR transfer is not fully understood. Casaburi et al (46) showed that infants fed a probiotic strain of *Bifidobacterium longum* exhibited a change in their gut microbiome, resulting in a 90 % lower level of ARG compared to non-fed controls. In this study, we show that commensal bacterial populations from reused broiler chicken litter provided colonization resistance against the conjugal transfer of multidrug resistant plasmid to *S*. Heidelberg present in the gut of neonatal chicks. We identified several bacterial species from the genera *Bifidobacterium*, *Faecalibacterium, Corynebacterium* and *Escherichia* to be involved in the competitive exclusion of antibiotic resistant *Salmonella* and determined the metabolic pathways used. Though we measured only two cross-sectional timepoints for reused and fresh litter, the relative abundance of plant-metabolic related pathways in fresh litter compared to the enrichment of competitive molecules in reused litter suggests that a selective pressure gradient exists over time for the colonizing microbial populations in reused litter. Together, these characteristics suggests that reused litter is a highly competitive environment for *Salmonella* to successfully colonize.

Although neonatal chicks raised on reused litter did not acquire MDR S. Heidelberg, our analysis of the cecal metagenome and whole genome sequencing of *E. coli* strains originating from reused litter confirmed that transferable plasmids were indeed present in reused litter. Additionally, a competition experiment involving *E. coli* strains from fresh and reused litter suggested that selection for fumarate encoding genes led to the development of β-lactam resistant *S*. Heidelberg populations and not antibiotic selection. The ampC/frd region is part of a large mobile genomic island-like (~140kb)

chromosome region encoding P4 bacteriophage in some *Enterobacteriaceae* species (47). We confirmed the location of the island in one of the *E. coli* donor strain from fresh litter (Supplementary Fig. 13). The mobility of this region following *E. coli* lysis was key to the emergence and transfer of the most reported (48, 49) plasmid-mediated AmpC β-lactamase gene (bla_{CMY-2}) gene to *S*. Heidelberg in our study.

The vertebrate host uses multiple defense mechanisms to protect the gut against an invading pathogen including its microbiome. The microbiome competes with pathogens for space and nutrients, and through this process confers colonization resistance against an invader. Consequently, the microbiome regulates a variety of host immune responses and has been demonstrated to shape the gut-brain communication (50, 51). This communication between the host and its microbiome ensures that gut conditions are unfavorable to a pathogen such as *Salmonella*. This can be achieved by reducing the stomach pH and oxygen levels to levels that only favor short chain fatty acid producers (52) and oxygen-sensitive bacterial species (53) including members of the phylum Firmicutes. However, some strains/serotypes of non-typhoidal *Salmonella enterica* have developed mechanisms to evade the host microbiome-imposed colonization resistance. Through evolution, these strains have acquired repertoire of genes for acid resistance and anaerobic metabolism. Furthermore, *Salmonella* has been shown to modulate the microbiome present in the gut of broiler and layer chickens after challenge with *Salmonella* Enteritidis or Typhimurium (52, 54).

Pathogen induced gut 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 antibiotic

transfer (55, 56). In this study, we observed that competition from commensal bacteria in reused litter significantly reduced *S*. Heidelberg population that acquired AR from *E. coli* strains harbored by neonatal chicks. However, when we performed competition experiments with a mixture of divergent *E. coli* strains originating from fresh and reused litter, *S*. Heidelberg strains emerged with newly acquired antibiotic resistance genes. This result suggests that competition for space and nutrients between closely related species that differ in virulence and resistome can lead to a negative outcome i.e., the development of new multidrug resistant pathogenic strains. Thus, identifying gut conditions that maintains an abundance of beneficial microbes while limiting such "adverse" competition warrants further study.

Materials and Methods

The details of the methods used for preparing S. Heidelberg (SH) inocula, challenging neonatal chicks, bacterial and DNA analyses, whole genome, and metagenome sequencing and bioinformatics have been described before for neonatal chicks raised on fresh pine shavings (7).

SH inoculum preparation. The SH strain was made resistant to 200 ppm of nalidixic acid (nal^R) for selective enumeration. The inoculum was grown overnight in poultry litter extract, centrifuged, and resuspended in 1X phosphate buffered saline (PBS). The resuspended cells were used as inocula. The complete genome of the ancestor to SH-2813nal^R is available under GenBank accession number : CP066851.

Challenging broiler chicks with SH Two hundred one-day-old Cobb 500 broiler chicks were either uninoculated, gavaged or cloacally inoculated with ~10^6 colony forming units (CFU) of SH-2813nal^R. We included a seeder-bird colonization method, whereby five

chicks were gavaged and mingled with twenty uninoculated chicks. Afterwards, chicks were placed in floor pens at a stocking density of 0.65 m^2/chick on fresh pine shavings (n =100) or reused litter (n =100). The reused litter was previously used to raise three flocks of broiler chicken's antibiotic-free under simulated commercial poultry production conditions and was top dressed with 0.5 cm of fresh pine shavings before the placement of each flock. Broiler chicks on fresh (FL) and reused litter (RL) were housed separately.

Broiler chicks were raised for 14 days on antibiotic-free starter diet and water. At 14 days, forty-five chicks from FL and RL (ten chicks from gavaved, cloacal and uninoculated groups and fifteen chickens from the seeder method) were euthanized to determine the extent of SH colonization in ceca. Additionally, litter samples were collected as grab samples from each pen after chicks were euthanized. The experiments were performed in April 2018. The study was approved by the University of Georgia Office of Animal Care and Use under Animal Use Protocol: A2017 04-028-A2.

Determination of cecal and litter *Salmonella* counts Ceca were removed from the eviscera of broiler chicks and stomached for 60s after the addition 3X volume to the weight (v/w) of buffered peptone water. Litter was collected as grab samples from seven locations (4 corners of the pen and under the waterer) in each pen after chicks were removed. The litter samples were pooled and 30g was processed in duplicates from each pen as previously described (45). Serial dilutions of the cecal and litter slurry were plated onto Brilliant Green Sulfa agar (BGS) (BD Difco, MD, USA) containing 200-ppm nal and plates were incubated for 24 h at 37°C.

After incubation, colonies were counted and calculated per gram of ceca or litter dry weight. When no colonies appeared, pre-enriched cecal and litter slurry was streaked onto BGS agar supplemented with nal and incubated overnight. BGS plates were examined for the presence/absence of *Salmonella* colonies after overnight incubation. Broiler chicken litter was collected as grab samples from seven locations (4 corners of the pen and under the waterer) in each pen after chicks were removed. The litter samples were pooled and 30g was processed in duplicates from each pen as previously described[37]. Serial dilutions of the litter slurry were made and plated onto BGS agar.

Litter pH and moisture was determined as described previously (45). We selected randomly 2 – 6 single colonies from BGS plates supplemented with nal from each cecal and litter sample and archived them in 30% LB glycerol at -80 ^{o}C. In addition, cecal slurry was saved at a 4:1 ratio in Luria Bertani (LB) broth (BD Difco, MD, USA) containing 30% glycerol at –80°C, while litter samples were stored in vacuum sealed whirl pak bags at -20°C.

DNA extraction. DNA was extracted and purified from bacterial colonies using FastDNA spin Kit (MP Biomedicals, LLC, CA, USA), while 250 mg of cecal slurry (previously saved in LB broth containing glycerol) and litter were extracted with Qiagen DNeasy Power-Soil DNA kit (Hilden, Germany). Extracted DNA was used for qPCR, 16S rRNA gene sequencing, whole genome and metagenome sequencing.

Real-time qPCR. Real-time qPCR amplification was performed on DNA extracted from the ceca and litter of broiler chicks as described previously (45) using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Inc., Hercules, CA). Reaction mixtures (20 µl) for all assays contained 1X SsoAdvanced Universal SYBR Green Supermix (Bio-

Rad Inc., Hercules, CA), 600 nM (each) primers (Supplementary Table 6), and 2 µl of cecal/litter DNA. Calibration curves used for converting qPCR cycle threshold values to gene copies per gram were determined using genomic DNA of *E. coli* or *Salmonella* strains harboring the region or gene of interest

16S rRNA gene sequencing. The V4 hypervariable region of the 16S rRNA gene was sequenced using the paired-end (250 × 2) method on the Illumina MiSeq platform. The samples were part of a larger sequencing run and processed together with other samples. Thus, detailed sequence processing parameters are described in (7). To avoid bias introduced by spurious amplicon sequence variants (ASVs) or from samples with low sequencing depth, any ASVs with less than 5 reads and samples with less than 5000 reads were removed from the dataset before further analysis.

Statistical analysis of microbial communities was performed in the R environment using the packages “phyloseq”, “Ampvis2”, and “vegan”. Alpha diversity indices were calculated with a dataset rarefied to the minimum sample size (8740 sequences). After assessing normal distribution by qqplots, histograms, and the Shapiro-Wilk normality test, the not normal distributed groups were compared using the Wilcoxon Signed Rank test. The principal coordinates analysis based on Bray-Curtis distances was calculated with no initial data transformation and ASVs that were not present in more than 0.1% relative abundance in any sample have been removed. All raw FASTQ files for 16S rRNA gene sequences have been deposited under NCBI accession number: PRJNA669215.

Cecal metagenome sequencing Two hundred and fifty milligrams of cecal slurry from chicks cloacally challenged with SH and raised on fresh (n = 2) or reused litter (n = 2)

were used for generating shotgun and Hi-C DNA library. For shotgun library, cecal DNA was extracted with Qiagen DNeasy Power-Soil DNA kit (Hilden, Germany) and Nextera™ XT library preparation kit was used to make the library. Hi-C library was made using Phase Genomics (Seattle, WA, USA) ProxiMeta Hi-C Microbiome Kit following the manufacturer's instructions. Libraries were sequenced by Novogene corporation (Sacramento, CA, USA) on the Illumina HiSeq platform using 150bp paired end reads. Two libraries were sequenced per HiSeq flowcell lane resulting in a total of ~111 million shotgun reads, and ~213 million Hi-C reads per sample.

Shotgun reads were filtered and trimmed for quality and normalized before assembly was performed with metaSPAdes (57) using default options. Hi-C reads were then aligned to the assembly following the Hi-C kit manufacturer's recommendations (https://phasegenomics.github.io/2019/09/19/hic-alignment-and-qc.html). Metagenome deconvolution was performed with ProxiMeta (58, 59), resulting in the creation of putative genome and genome fragment clusters. Hi-C clusters were assessed for quality using CheckM (59) and assigned taxonomic classifications with Mash v2.1. Quality controlled shotgun reads were additionally classified using Kraken2 (v2.0.8 beta) (30) to create a count profile of the metagenome. To identify the plasmids and ARGs present in metagenome assembled genomes, we used Minimap2 (61) to align assembled metagenome contigs to PlasmidFinder (29) and ResFinder (62) database. For contigs aligned using Minimap, a 75% identity threshold was used for the alignment match, whereas contigs with significant homology (E value < 0.05) were considered contigs of the respective plasmid or ARG using Geneious Prime®.

Functional Pathway Analysis To assess enrichment of functional pathways, both the

shotgun sequences and the Hi-C deconvoluted genomes were utilized for each sample. The shotgun metagenomic sequences were aligned to the Hi-C genomic contigs using the BWA-MEM algorithm as implemented in the Burrows-Wheeler Aligner software (v0.7.17) (63). The Hi-C genomic contigs were then annotated using Prokka (v1.13) (64). For each annotated region on the Hi-C genomic contigs, sequences overlapping the annotated region in the sequence alignments were counted using a custom Python script to produce a count matrix of genes for all samples. Gene counts were normalized by gene length and using total sum scaling normalization to control for differences in sequencing depth (65).

Enzyme Commission identifiers (EC ID) were extracted from the Prokka annotations for each gene and used to translate counts for genes into counts for each EC ID. Genes that did not have an annotated EC ID were not included in the analysis. For ambiguous EC IDs (e.g., 3.6.3.-), counts were divided evenly into the lowest-level, non-ambiguous EC IDs that were children of that EC ID. For example, if 3.6.3.- included 3.6.3.1, 3.6.3.2, and 3.6.3.3, then a count of 9 for the ambiguous EC ID 3.6.3.- would result in each of the three children EC IDs having a count of 3. EC IDs were then mapped to KEGG functional pathways according to the KEGG ontology. KEGG pathway XML files were retrieved from the KEGG database via HTTP REST query using a custom R script. For each pathway, the adjacency graph was reconstructed from the KEGG XML files, and connected components were determined using depth-first search (66). Scores were calculated for pathways and connected components for each sample using the following equation:

$$\text{Score} = \frac{1}{K}\sum_{k=1}^{K}\frac{x_k - \bar{x}}{\sigma}$$

where *K* is the number of nodes in each pathway or connected component, x_k is the integer count for node *k* in that pathway, $\bar{x}$ is the mean of the node counts in that pathway, and σ is the standard deviation of the node counts in that pathway. Positive scores indicate average increased "flux" through the pathway or connected component, while negative scores indicate average decreased "flux." This approach is equivalent to averaging Z-scores for each node in each pathway or connected component. Statistical testing was then performed by comparing scores to the Z-distribution. All codes, database files, and analytic data used for functional pathway analysis are available on GitHub : https://github.com/lakinsm/oladeinde-competitive-exclusion.

Isolation of *E. coli* from the ceca of broiler chicks. The method used for screening frozen cecal contents has been reported (7). Briefly, two cecal slurry from challenged chicks raised on fresh and reused litter were spread plated onto CHROMagar™ plates supplemented with or without gentamicin (8 ppm) and tetracycline (8 ppm), ampicillin (16 ppm) and cefoxitin (16 ppm) (Thermo Fisher, Waltham, MA). After 24 h incubation, blue green and blue-cream colonies were counted as presumptive *E. coli* and 4 - 5 colonies (n = 27) from each spread plate were picked and restruck for colony purification.

After incubation, a colony representing the different morphologies observed was struck on Eosin Methylene Blue (EMB) (Remel Inc., KS, USA) and incubated overnight at 37 ^{o}C. A characteristic green metallic sheen growth on EMB was used to confirm an

isolate as *E. coli*. Colonies were preserved in LB broth containing glycerol and frozen before they were used further including solid agar competition with SH.

Solid agar competition between SH and *E. coli*. Four *E. coli* strains (GenBank/Sample accession numbers: CP066836, JAFCXR000000000, CP066839, SAMN17168891) were selected as AR donors and SH-2813nal^R (Sample accession number: SAMN17171078) was used as the recipient (see Fig. 4a for experimental design). Four colonies from SH-2813nal^R and one colony from each *E. coli* donor strain (~10^8 CFU in a colony) were selected from overnight cultures grown on sheep blood agar (Remel Inc, San Diego, CA) and resuspended in 1.7 ml microcentrifuge tubes containing 900 µl of 1× PBS. The four SH and *E. coli* colonies were resuspended in separate microcentrifuge tubes i.e., one tube for SH and one tube for all four *E. coli*. Afterwards, recipient and donor were diluted 10,000- and 100,000-fold, respectively, using 1× PBS. Thereafter, 100 µl from the 1:10,000 recipient dilution was spread onto CHROMagar™ ECC (Paris, France) supplemented with or without 10mM $CaCl_2$ (Sigma-Aldrich, St. Louis, MO), 20mM sodium chloride citrate buffer ($Na_3C_6H_5O$) (Sigma-Aldrich, St. Louis, MO) or 1mM 2-hexadecynoic acid (2-HDA) (MJM chemical LLC, Athens, GA; see supplementary methods for 2-HDA preparation) and incubated for 6 h. Following incubation, 100 µl from the 1:100,000 donor dilution was spread onto the recipient bacterial lawn and incubation continued overnight (n = 3 CHROMagar™ plates per treatment).

After incubation, two areas of equal sizes (~0.3 cm^2) were excised from each CHROMagar™ plate and transferred to 900 µl of 1× PBS. Vigorous shaking was done at 1,800 rpm with a Fastprep-96 bead beater (MP Biomedicals, Solon, Ohio) for 1 min

to suspend the cells from the agar plug. Serial dilutions were then spread onto BGS agar and CHROMagar™ supplemented with or without gentamicin (gen) (16 ppm) or amp (8 ppm). Experiments were performed twice, and colonies were counted 18 - 48 h after incubation using a calibrated automated ProtoCOL 3 HD colony counter (Synoptics Inc, MD, USA). Additionally, three SH colonies per replicate were selected from BGS agar plates supplemented with either gen or amp for antibiotic susceptibility testing.

DNA extraction from agar plug lysate We modified a rapid method developed for extracting DNA from bacteriophages (66). First, we combined triplicates of the agar plug resuspension from the sodium citrate treated agar i.e., excised agar and 1X PBS (~ 1.2 ml) and added 120 µl of 10X DNAse buffer before incubating in a dry water bath at 40°C for 30 min. Afterwards, we passed the resuspended agar through a 0.22 µm syringe filter and added 2U of DNase (TURBO DNA-free™ Kit, Thermo Fisher) to the lysate recovered. The DNAse-treated lysate was incubated at 37 ^{o}C for 30 min before the DNase was inactivated with 10 µl of 50 mM EDTA (Sigma-Aldrich, St. Louis, MO).

Next, we used a 30kDa Amicon® Ultra-15 centrifugal filter unit (Thermo Fisher) to concentrate the lysate for 5 min at 4,000 x *g* in a swing bucket centrifuge (Eppendorf ; model 5804 R, Hamburg, Germany) . We added 10 µl of 10% sodium dodecyl sulfate solution (Media Tech Inc., VA, USA) and 5 µl of proteinase K (Thermo Fisher) to the concentrated lysate, followed by incubation at 55 ^{o}C for 30 min. Afterwards, lysate DNA was incubated at 70 ^{o}C for 10 min to inactivate proteinase K and reconcentrated in an Eppendorf vacufuge plus concentrator (Thermo Fisher) at 30 °C. Pelleted DNA was reconstituted in 10 µl 1× Tris-EDTA buffer (10 mM Tris-HCL, 1 mM EDTA, pH 7.5), and purified with Zymogen PCR inhibitor removal kit (Zymo Research Corp., CA, USA) as

per the manufacturer's instructions. DNA was used for making Nextera XT (Illumina, Inc., CA, USA) libraries in duplicates and sequencing was performed on an Illumina MiSeq platform with 150 paired end reads.).

Antibiotic resistance phenotype determination. Antimicrobial susceptibility testing (AST) was done on SH and *E. coli* isolates by following the National Antimicrobial Resistance Monitoring System (NARMS) protocol for Gram-Negative bacteria. Results were interpreted according to clinical and laboratory standards institute guidelines and breakpoints established by NARMS. For presumptive SH recombinants selected from mating experiments, we screened sixty colonies on BGS agar supplemented with 8 ppm amp or 16 ppm gen before forty-eight isolates were selected for AST using the NARMS protocol.

Whole genome sequencing and analyses. DNA was extracted and purified from bacterial colonies using FastDNA spin Kit (MpBio) and Illumina short read sequencing was performed on DNA extracted. Libraries were prepared using either Nextera™ XT or Nextera™ DNA Flex library preparation kits (Illumina, Inc., CA, USA) following the manufacturers protocol. Libraries were sequenced on the Illumina MiSeq platform with 150 or 250-bp paired end reads. Additionally, two SH and two *E. coli* isolates were selected for long read sequencing using Sequel II System (PacBio Biosciences Inc., CA, USA). Preparation and sequencing of long read libraries were done by sequencing core centers of University of Georgia and Colorado State University. The method used for read quality control and demultiplexing has been reported (7).

Genome assembly, resistome characterization, and quality assessment of assemblies was done using Reads2Resistome pipeline v.1.1.1 (68). We assembled

bacterial genomes using either Illumina reads only or Illumina and PacBio reads (Supplementary File 7). Genome annotation was done using Prokka (64), Rapid Annotation using Subsystem Technology (RAST) (69) and BlastKOALA (70). We subtyped the *E. coli* strains using ClermonTyping (71) and Multi-Locus Sequence Typing (MLST) (72), whereas S. Heidelberg was subtyped using cgMLSTFinder v. 1.1 (73). For plasmid typing, we used plasmid MLST (29). To identify prophages, we used PHAST(74). ProgressiveMAUVE v. 1.1.1 (75) and MAFFT v. 1.4.0 (76) implemented in Geneious Prime® v 2020.0.1 were used to align and compare sequences. Roary (77) was used for pan genome analysis and phylogenetic trees were reconstructed using the maximum likelihood (ML) method implemented in RAxML-NG v 1.0.0 (78). IslandViewer (79) was used to predict genomic islands. BLAST database of the bacterial genomes sequenced in this study was made using Geneious Prime®.

SNP and Indel identification Illumina reads were used to determine single nucleotide polymorphisms (SNPs) and indels present in SH isolates. Alignment of raw FASTQ reads to the genome of SH-ancestor was done using Burrows-Wheeler Aligner (BWA) (63) and SNPs/indels were called using Genome Analysis Toolkit (80). Variant call format (VCF) files of SNPs/Indels are available in Dryad Digital Repository: https://doi.org/10.5061/dryad.4tmpg4f8d. BlastKOALA was used to identify KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways that sequenced genomes or proteins are capable of. For other bioinformatic analyses performed including read coverage determination through FASTQ alignment and CRISPR array identification, see supplementary methods. All raw FASTQ reads for sequenced

bacterial genomes and agar lysate are publicly available under NCBI accession numbers: PRJNA683658, PRJNA684578 and PRJNA684580.

Statistical analyses. Continuous variables did not meet the assumption of a normal distribution; therefore, non-parametric testing for direct comparisons was performed using Wilcoxon rank sum and signed rank tests, and the Kruskal-Wallis rank sum test was used for one-way analysis of variance tests. Furthermore, continuous variables were log-transformed before any statistical tests were performed. Statistical analyses were performed using R (v 3.4.1).

Data availability. All raw short and long FASTQ reads are publicly available under NCBI accession numbers: PRJNA683658, PRJNA684578 and PRJNA684580. Shotgun and Hi-C reads are publicly available under NCBI accession number: PRJNA688069 and 16S rRNA gene sequences under NCBI accession number: PRJNA669215. Complete genomes for *E. coli* strain Ec-RL-1X is available under GenBank accession number: 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.4tmpg4f8d.

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.

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Author's contributions. A.O., Z.A., and K.C. 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 sequencing. A.O. performed cecal shotgun and Hi-C library preparation. T.L. made 16S rRNA gene libraries and sequencing. B.Z. performed 16S rRNA bacterial community analysis and interpretation. A.O., Z.A., M.O.P., J.C.T.,

S.M.L., R.W., J.L., and D.C. performed bioinformatic analyses and data curation. A.O., J.L. and D.C. performed solid agar mating experiment. A.O., and B.Z. performed statistical analyses. A.O., Z.A., K.C., N.A.C., B.Z., R.W., M.P., S.M.L., G.Z., and J.L. drafted the manuscript, which was reviewed and edited by all authors. A.O., Z.A., and S.A.A. supervised the study.

Reference:

1. C. J. Von Wintersdorff et al., Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. Frontiers in microbiology 7, 173 (2016).
2. C. Ventola (2015) The antibiotic resistance crisis: part 1: causes and threats. PT 40 (4): 277–283.
3. F. J. Angulo, N. L. Baker, S. J. Olsen, A. Anderson, T. J. Barrett (2004) Antimicrobial use in agriculture: controlling the transfer of antimicrobial resistance to humans. in Seminars in pediatric infectious diseases (Elsevier), pp 78-85.
4. K. L. Tang et al., Restricting the use of antibiotics in food-producing animals and its associations with antibiotic resistance in food-producing animals and human beings: a systematic review and meta-analysis. The Lancet Planetary Health 1, e316-e327 (2017).
5. R. Singer, L. Porter (2019) Estimates of On-Farm Antimicrobial Usage in Broiler Chicken and Turkey Production in the United States, 2013-2017. . (Mindwalk Consulting Group, LLC. Falcon Heights, MN, 55113, USA.).
6. C. Kirchhelle, Swann Song: Antibiotic Regulation in British Livestock Production (1953-2006). B Hist Med 92, 317-351 (2018).

7. A. Oladeinde et al., Gene flux and acid-imposed selection are the main drivers of antimicrobial resistance in broiler chicks infected with Salmonella enterica serovar Heidelberg. bioRxiv 10.1101/2021.02.25.432983, 2021.2002.2025.432983 (2021).

8. L. Gieraltowski et al., National Outbreak of Multidrug Resistant Salmonella Heidelberg Infections Linked to a Single Poultry Company. PLoS One 11, e0162369 (2016).

9. R. Edirmanasinghe et al., A Whole-Genome Sequencing Approach To Study Cefoxitin-Resistant Salmonella enterica Serovar Heidelberg Isolates from Various Sources. Antimicrob Agents Chemother 61 (2017).

10. D. Ceccarelli et al., Competitive Exclusion Reduces Transmission and Excretion of Extended-Spectrum-beta-Lactamase-Producing Escherichia coli in Broilers. Appl Environ Microbiol 83 (2017).

11. I. Chantziaras et al., The effect of a commercial competitive exclusion product on the selection of enrofloxacin resistance in commensal E. coli in broilers. Avian Pathology 47, 443-454 (2018).

12. M. Raffatellu, Learning from bacterial competition in the host to develop antimicrobials. Nature medicine 24, 1097-1103 (2018).

13. C. S. Achard et al., Variability of the ability of complex microbial communities to exclude microbes carrying antibiotic resistance genes in rabbits. Frontiers in microbiology 10, 1503 (2019).

14. M. Baumgartner, F. Bayer, K. R. Pfrunder-Cardozo, A. Buckling, A. R. Hall, Resident microbial communities inhibit growth and antibiotic-resistance evolution

of Escherichia coli in human gut microbiome samples. PLoS Biol 18, e3000465 (2020).

15. A. Dame-Korevaar et al., Early life supply of competitive exclusion products reduces colonization of extended spectrum beta-lactamase-producing Escherichia coli in broilers. Poultry science 99, 4052-4064 (2020).

16. U. Methner, U. Rösler, Efficacy of a competitive exclusion culture against extended-spectrum β-lactamase-producing Escherichia coli strains in broilers using a seeder bird model. BMC veterinary research 16, 1-7 (2020).

17. L. Wang, M. Lilburn, Z. Yu, Intestinal Microbiota of Broiler Chickens As Affected by Litter Management Regimens. Front Microbiol 7, 593 (2016).

18. A. Figueroa et al., Persistence of low and highly pathogenic avian influenza virus in reused poultry litter, effects of litter amendment use, and composting temperatures. Journal of Applied Poultry Research, 100096 (2020).

19. S. Wadud et al., Bacterial and fungal community composition over time in chicken litter with high or low moisture content. British poultry science 53, 561-569 (2012).

20. National Farm Animal Care Council. Code of Practice: For the care and handling of hatching eggs, breeders, chickens, and turkeys. Retrieved from https://www.nfacc.ca/codes-of-practice/chickens-turkeys-and-breeders. (2016).

21. ADAS UK Ltd. Comparison of the Regulatory Framework and Key Practices in the Poultry Meat Supply Chain in the EU and USA : a study for the association of poultry processors and poultry trade in the EU Countries. (2016) Retrieved

from https://www.britishpoultry.org.uk/identity-cms/wp-content/uploads/2018/05/2016-ADAS-EU-US-comparison.pdf.22.

22 K. C. Dornelas et al., Chicken bed: a review on reuse, treatment and influence on ambience. Poultry Science (2020).

23. E. Muniz, D. Mesa, R. Cuaspa, A. M. Souza, E. Santin, Presence of Salmonella spp. in reused broiler litter. Revista Colombiana de Ciencias Pecuarias 27, 12-17 (2014).

24. U. S. Yamak, M. Sarica, M. A. Boz, A. Ucar, Effect of reusing litter on broiler performance, foot-pad dermatitis and litter quality in chickens with different growth rates. Kafkas Univ. Vet. Fak. J 22, 85-91 (2016).

25. R. Casey, B. Fairchild, M. Lacy (2005) Litter quality and broiler performance. ed C. e. S. Bulletin.

26. V. F. Roll, M. A. Dai Pra, A. P. Roll, Research on Salmonella in broiler litter reused for up to 14 consecutive flocks. Poult Sci 90, 2257-2262 (2011).

27. J. Zucko et al., Probiotic–friend or foe? Current Opinion in Food Science 32, 45-49 (2020).

28. S. Gupta, E. Allen-Vercoe, E. O. Petrof, Fecal microbiota transplantation: in perspective. Therapeutic advances in gastroenterology 9, 229-239 (2016).29.

29 A. Carattoli *et al.*, In Silico Detection and Typing of Plasmids using PlasmidFinder and Plasmid Multilocus Sequence Typing. *Antimicrob Agents Ch* **58**, 3895-3903 (2014).

30. D. E. Wood, J. Lu, B. Langmead, Improved metagenomic analysis with Kraken 2. *Genome biology* **20**, 257 (2019).

31. J. Haaber *et al.*, Bacterial viruses enable their host to acquire antibiotic resistance genes from neighbouring cells. *Nature Communications* **7** (2016).

32. M. Getino *et al.*, Synthetic Fatty Acids Prevent Plasmid-Mediated Horizontal Gene Transfer. *mBio* **6**, e01032-01015 (2015).

33. K. Bohm *et al.*, Genes affecting progression of bacteriophage P22 infection in Salmonella identified by transposon and single gene deletion screens. *Mol Microbiol* **108**, 288-305 (2018).

34. N.-F. Alikhan, Z. Zhou, M. J. Sergeant, M. Achtman, A genomic overview of the population structure of Salmonella. *PLoS genetics* **14**, e1007261 (2018).

35. N. Shariat, C. H. Sandt, M. J. DiMarzio, R. Barrangou, E. G. Dudley, CRISPR-MVLST subtyping of Salmonella enterica subsp. enterica serovars Typhimurium and Heidelberg and application in identifying outbreak isolates. *BMC Microbiol* **13**, 254 (2013).

36. A. Biswas, J. N. Gagnon, S. J. Brouns, P. C. Fineran, C. M. Brown, CRISPRTarget: bioinformatic prediction and analysis of crRNA targets. *RNA Biol* **10**, 817-827 (2013).

37. S. Bergstrom, F. P. Lindberg, O. Olsson, S. Normark, Comparison of the overlapping frd and ampC operons of Escherichia coli with the corresponding DNA sequences in other gram-negative bacteria. *J Bacteriol* **155**, 1297-1305 (1983).

38. H. M. Jones, R. P. Gunsalus, Regulation of Escherichia coli fumarate reductase (frdABCD) operon expression by respiratory electron acceptors and the fnr gene product. *J Bacteriol* **169**, 3340-3349 (1987).

39. J.-S. Kim *et al.*, Fumarate-mediated persistence of Escherichia coli against antibiotics. *Antimicrob Agents Ch* **60**, 2232-2240 (2016).

40. R. Nakano *et al.*, CFE-1, a novel plasmid-encoded AmpC beta-lactamase with an ampR gene originating from Citrobacter freundii. *Antimicrob Agents Chemother* **48**, 1151-1158 (2004).

41. C. M. Chen, M. Huang, H. J. Wu, M. K. Guo, L. T. Wu, Identification of CFE-2, a new plasmid-encoded AmpC beta-lactamase from a clinical isolate of Citrobacter freundii. *Int J Antimicrob Agents* **52**, 421-424 (2018).

42. S. Casjens, P. Weigele, "DNA packaging by bacteriophage P22" in Viral Genome Packaging Machines: Genetics, Structure, and Mechanism. (Springer, 2005), pp. 80-88.

43. B. A. Mann, J. M. Slauch, Transduction of low-copy number plasmids by bacteriophage P22. *Genetics* **146**, 447-456 (1997).

44. L. Rodriguez-Rubio *et al.*, Extensive antimicrobial resistance mobilization via multicopy plasmid encapsidation mediated by temperate phages. *J Antimicrob Chemother* **75**, 3173-3180 (2020).

45. A. Oladeinde *et al.*, Hotspot mutations and ColE1 plasmids contribute to the fitness of Salmonella Heidelberg in poultry litter. *PLoS One* **13**, e0202286 (2018).

46. S. Rao *et al.*, Pathogen-mediated inhibition of anorexia promotes host survival and transmission. *Cell* **168**, 503-516. e512 (2017).

47. K. V.-A. Johnson, K. R. Foster, Why does the microbiome affect behaviour? *Nature reviews microbiology* **16**, 647-655 (2018).

48. S. Khan, K. K. Chousalkar, Salmonella Typhimurium infection disrupts but continuous feeding of Bacillus based probiotic restores gut microbiota in infected hens. *Journal of animal science and biotechnology* **11**, 1-16 (2020).

49. Y. Litvak *et al.*, Commensal Enterobacteriaceae Protect against Salmonella Colonization through Oxygen Competition. *Cell Host Microbe* **25**, 128-139 e125 (2019).

50. K. K. Mon *et al.*, Salmonella enterica serovars Enteritidis infection alters the indigenous microbiota diversity in young layer chicks. *Frontiers in veterinary science* **2**, 61 (2015).

51. B. Stecher *et al.*, Gut inflammation can boost horizontal gene transfer between pathogenic and commensal Enterobacteriaceae. *Proceedings of the National Academy of Sciences* **109**, 1269-1274 (2012).

52. A. W. Rogers, R. M. Tsolis, A. J. Bäumler, Salmonella versus the Microbiome. *Microbiol Mol Biol R* **85** (2021).

53. G. Casaburi *et al.*, Early-life gut microbiome modulation reduces the abundance of antibiotic-resistant bacteria. *Antimicrobial Resistance & Infection Control* **8**, 1-18 (2019).

54. Nepal, Suruchi, et al. "An ancient family of mobile genomic islands introducing cephalosporinase and carbapenemase genes in Enterobacteriaceae." Virulence 9.1 (2018): 1377-1389.

55. S. Meini, C. Tascini, M. Cei, E. Sozio, G. M. Rossolini, AmpC β-lactamase-producing Enterobacterales: what a clinician should know. *Infection* **47**, 363-375 (2019).

56. F. Maguire, M. A. Rehman, C. Carrillo, M. S. Diarra, R. G. Beiko, Identification of primary antimicrobial resistance drivers in agricultural nontyphoidal Salmonella enterica serovars by using machine learning. *Msystems* **4** (2019).

57. S. Nurk, D. Meleshko, A. Korobeynikov, P. A. Pevzner, metaSPAdes: a new versatile metagenomic assembler. *Genome Res* **27**, 824-834 (2017).

58. M. O. Press *et al.*, Hi-C deconvolution of a human gut microbiome yields high-quality draft genomes and reveals plasmid-genome interactions. *biorxiv*, 198713 (2017).

59. R. D. Stewart *et al.*, Assembly of 913 microbial genomes from metagenomic sequencing of the cow rumen. *Nat Commun* **9**, 870 (2018).

60. D. H. Parks, M. Imelfort, C. T. Skennerton, P. Hugenholtz, G. W. Tyson, CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res* **25**, 1043-1055 (2015).

61. H. Li, Minimap2: pairwise alignment for nucleotide sequences. *Bioinformatics* **34**, 3094-3100 (2018).

62. E. Zankari *et al.*, Identification of acquired antimicrobial resistance genes. *J Antimicrob Chemother* **67**, 2640-2644 (2012).

63. H. Li, R. Durbin, Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* **25**, 1754-1760 (2009).

64. T. Seemann, Prokka: rapid prokaryotic genome annotation. *Bioinformatics* **30**, 2068-2069 (2014).

65. J. N. Paulson, O. C. Stine, H. C. Bravo, M. Pop, Differential abundance analysis for microbial marker-gene surveys. *Nature methods* **10**, 1200-1202 (2013).

66. G. Csardi, T. Nepusz, The igraph software package for complex network research. *InterJournal, complex systems* **1695**, 1-9 (2006).

67. W. Kot, F. K. Vogensen, S. J. Sorensen, L. H. Hansen, DPS - a rapid method for genome sequencing of DNA-containing bacteriophages directly from a single plaque. *J Virol Methods* **196**, 152-156 (2014).

68. R. R. Woyda, A. Oladeinde, Z. Abdo, Reads2Resistome: An adaptable and high-throughput whole-genome sequencing pipeline for bacterial resistome characterization. *bioRxiv* 10.1101/2020.05.18.102715, 2020.2005.2018.102715 (2020).

69. T. Brettin *et al.*, RASTtk: a modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. *Sci Rep* **5**, 8365 (2015).

70. M. Kanehisa, Y. Sato, K. Morishima, BlastKOALA and GhostKOALA: KEGG Tools for Functional Characterization of Genome and Metagenome Sequences. *J Mol Biol* **428**, 726-731 (2016).

71. J. Beghain, A. Bridier-Nahmias, H. Le Nagard, E. Denamur, O. Clermont, ClermonTyping: an easy-to-use and accurate in silico method for Escherichia genus strain phylotyping. *Microb Genom* **4** (2018).

72. M. V. Larsen *et al.*, Multilocus sequence typing of total-genome-sequenced bacteria. *J Clin Microbiol* **50**, 1355-1361 (2012).

73. P. T. Clausen, F. M. Aarestrup, O. Lund, Rapid and precise alignment of raw reads against redundant databases with KMA. *BMC bioinformatics* **19**, 1-8 (2018).

74. Y. Zhou, Y. Liang, K. H. Lynch, J. J. Dennis, D. S. Wishart, PHAST: a fast phage search tool. *Nucleic Acids Res* **39**, W347-352 (2011).

75. A. E. Darling, B. Mau, N. T. Perna, progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. *PLoS One* **5**, e11147 (2010).

76. K. Katoh, D. M. Standley, MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* **30**, 772-780 (2013).

77. A. J. Page *et al.*, Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* **31**, 3691-3693 (2015).

78. A. M. Kozlov, D. Darriba, T. Flouri, B. Morel, A. Stamatakis, RAxML-NG: a fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference. *Bioinformatics* **35**, 4453-4455 (2019).

79. Bertelli, Claire, et al. "IslandViewer 4: expanded prediction of genomic islands for larger-scale datasets." Nucleic acids research 45.W1 (2017): W30-W35.

80. A. McKenna *et al.*, The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res* **20**, 1297-1303 (2010).

Supplementary files

Supplementary file 1: Metagenome read abundance for members of bacterial genera with higher abundance in neonatal chicks raised on reused litter compared to fresh litter.

Supplementary file 2: Virulence genes present in the four *E. coli* donor strains used for solid agar competition with SH. The number of virulence genes found in each genome is written in the column headers.

Supplementary file 3: Mutations found in all *S*. Heidelberg amp^R recombinants and agar lysate from this study.

Supplementary file 4: Fifty bacterial genomes encoding ISEc9 on their chromosome.

Supplementary file 5: Cecal metagenome contigs that have identical protein sequences matching IncI1-pST12.

Supplementary file 6: Hotspot mutations found in every *S*. Heidelberg carrying ColpVC.

Supplementary file 7: Assembly statistics of bacterial genomes sequenced using short and long read technology.

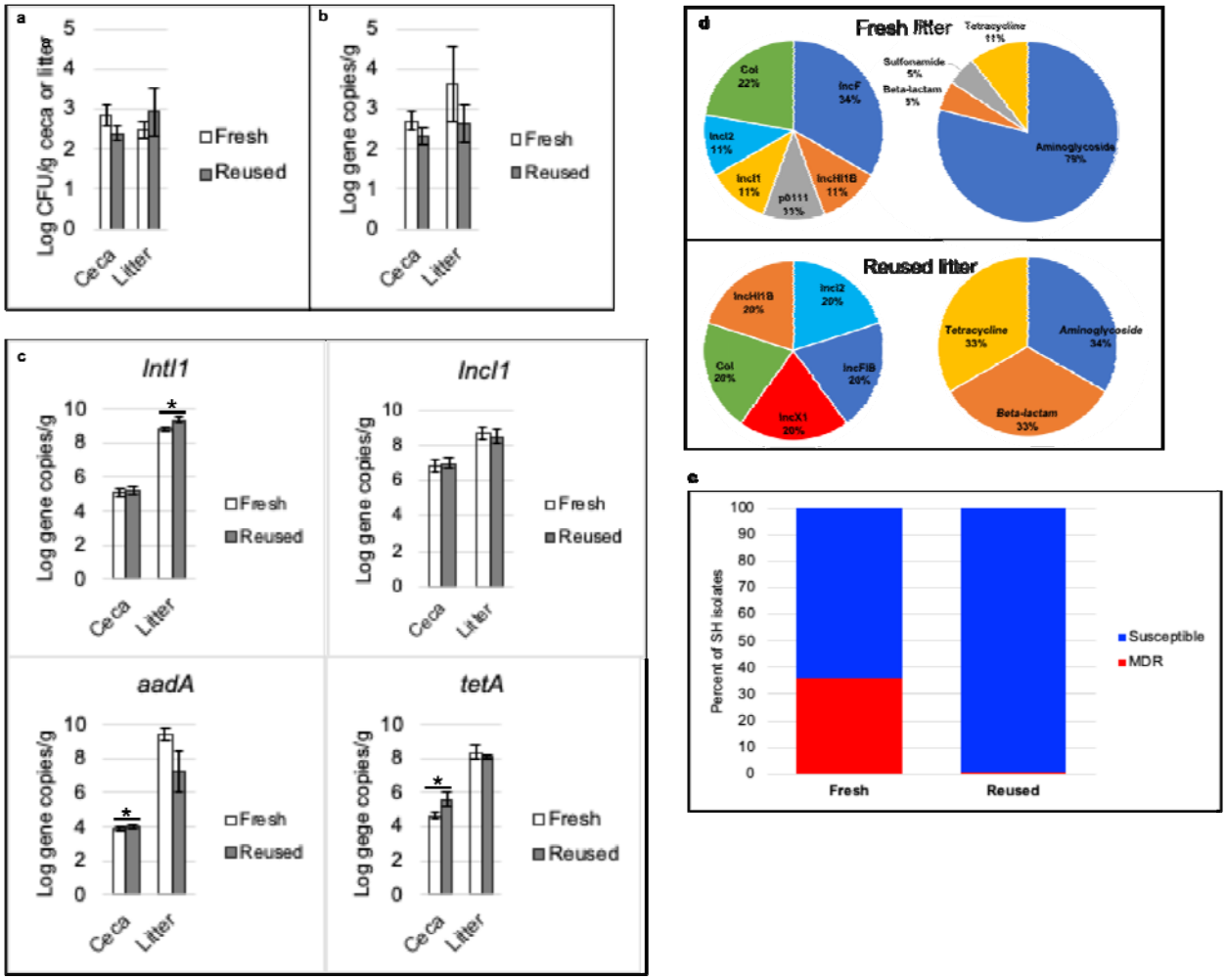

Fig 1. Neonatal chicks raised on reused litter carried lower levels of multidrug resistant *S.* Heidelberg. SH load in the ceca and litter (dry weight of litter) of broiler chicks (n = 70) raised on fresh or reused litter was determined using direct culture method (**a**) and real-time quantitative PCR (reported as gene copies) **(b**). **(c)** Gene copies of IncI1 encoded genes in the ceca (n =70) and litter (n = 12) of neonatal chicks raised on fresh or reused litter. (*P < 0.05 (Kruskal-Wallis test, Error bar = Standard error)). (**d**) Relative number of transferrable Enterobacteriaceae plasmids and ARG's found in the metagenome assembled genomes of chicks raised on fresh (n=2) and reused litter (n =2). **(e**) Percentage of SH isolates in the ceca and litter that acquired multidrug resistance in fresh (n = 158) and reused litter (n = 141).

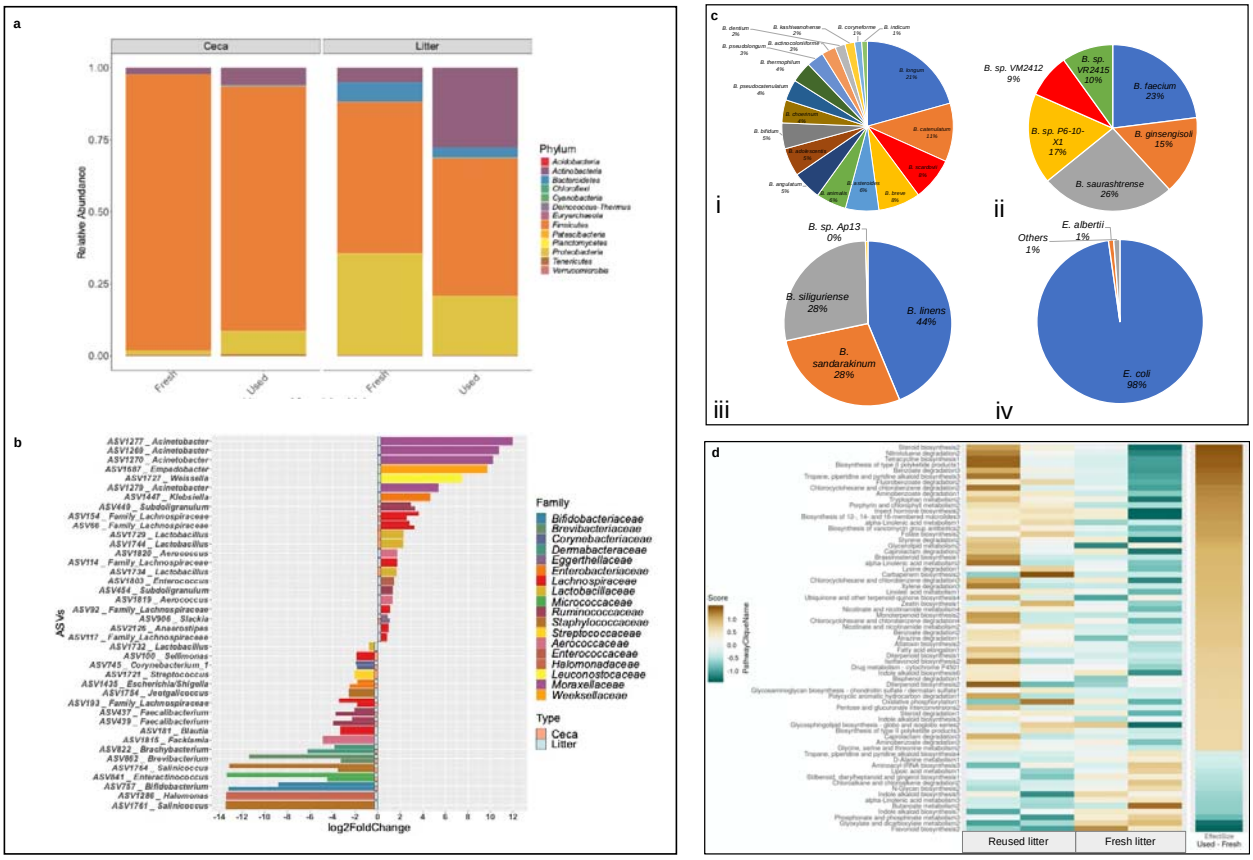

Fig 2. Reused litter is diverse in beneficial bacteria and antimicrobial metabolic pathways. (**a)** Phylum-level classification of 16S rRNA gene sequence reads in ceca and litter grouped by litter type. Data represent average of amplicon sequence variant (ASV) counts from replicate libraries for each category. (**b)** Plots of amplicon sequence variants (ASVs) that were significantly differentially abundant in the ceca and litter of chicks raised on fresh compared to reused litter ($p_{adj} < 0.05$). Significant ASVs are plotted individually and colored according to their family-level classification. **(c)** Relative abundance of bacterial species found using cecal metagenome shot gun reads to be associated with 16S rRNA amplicon sequence variants of the genera (i) *Bifidobacterium* (ii) *Brachybacterium* (iii) *Brevibacterium* and (iv) *Escherichia*. **(d)** Functional metabolic pathways that were enriched in the ceca of chicks from fresh compared to reused litter using metagenome assembled genomes. For a pathway or connected component within a pathway (clique), the flux is the Z-score deviation from the mean taken across all samples (n = 4). Pathway scores for each connected component are shown for each sample, and the difference of used minus fresh litter scores is shown on the right column labelled “effect size. Higher effect size indicates upregulation of the pathway in the reused litter, while lower effect size indicates upregulation in fresh litter.

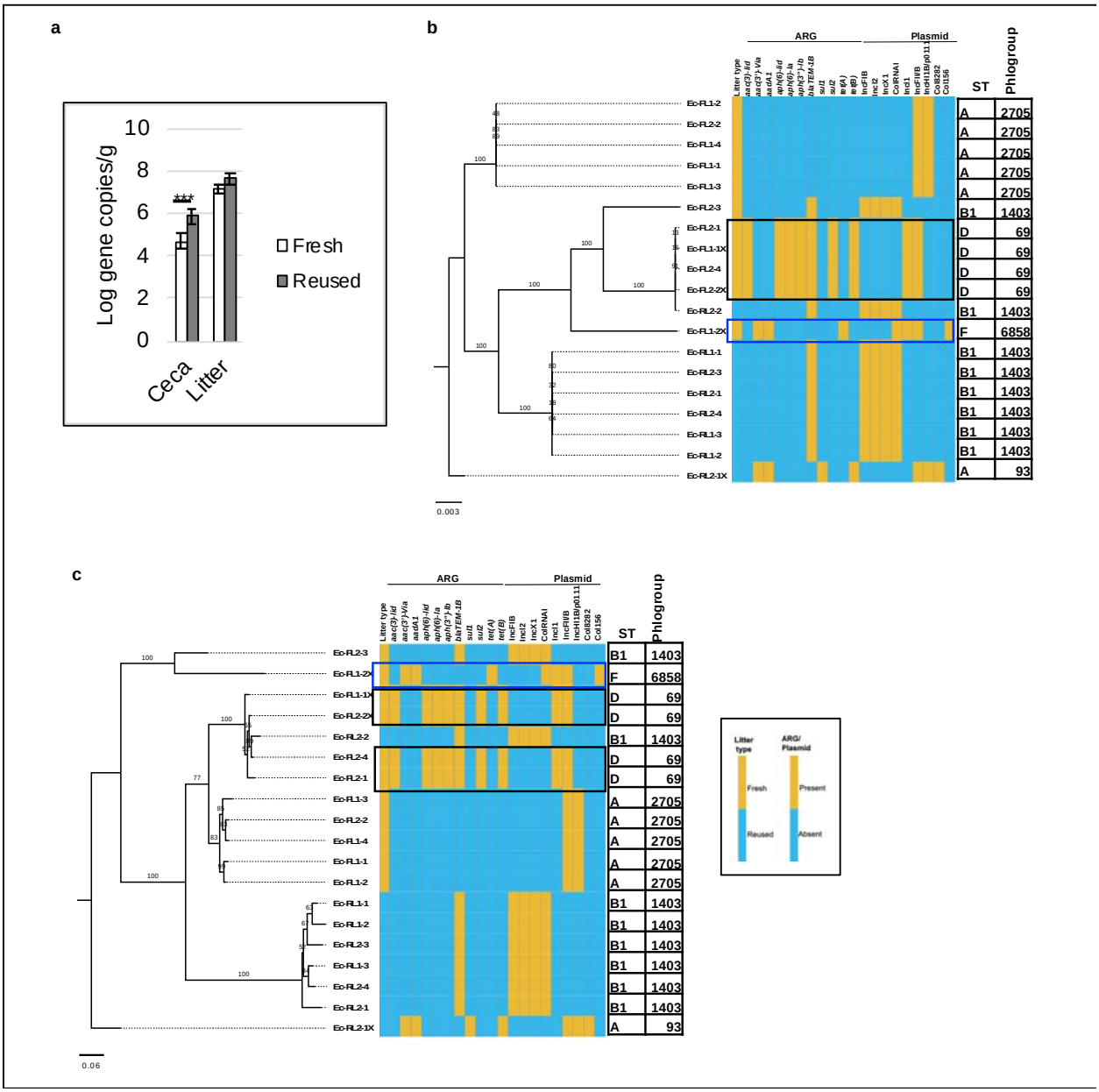

Fig 3. *Escherichia coli* from fresh litter was the main reservoir of transferable ARG. a) *E. coli* load in the ceca and litter (dry weight of litter) of broiler chicks of raised on fresh or reused litter was determined using direct culture method (**a**) and real-time quantitative PCR (reported as gene copies). Primers targeting the housekeeping gene (*gapA*) was used for *E. coli* quantification, (**b and c)** Whole genome sequencing were performed on *E. coli* strains (n =19) recovered from the ceca of four broiler chicks challenged with SH and raised on fresh (FL) or reused litter (RL). Maximum likelihood tree reconstructed using the core (**b)** and accessory **(c**) genes of the *E. coli* strains. 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. Tree was rooted with Ec-RL2-1X. (Note: ST – Multi locus Sequence Type). Blue rectangular box shows the *E. coli* donor strain carrying IncI1-pMLST26 that was acquired by SH. Black box denotes strains of *E. coli* where the ARG's were encoded on the chromosome.

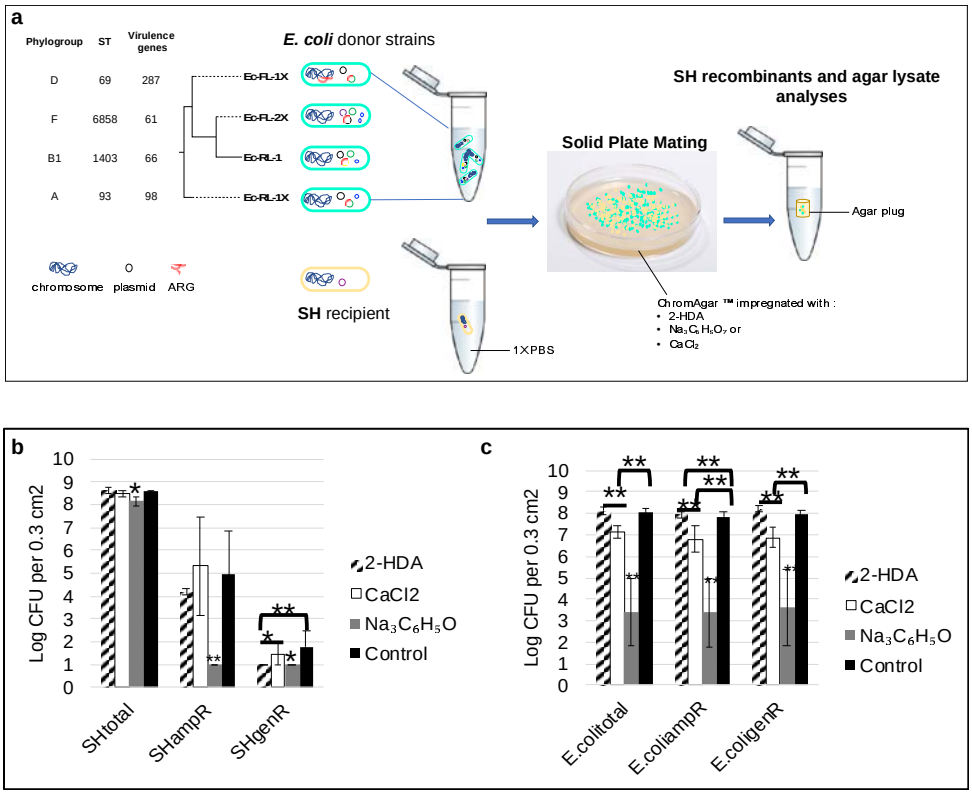

Fig 4. **Competition gave rise to ampicillin and gentamicin resistance *S*. Heidelberg population**. **(a)** Experimental design used for solid agar competition experiment between SH and *E. coli*. SH-2813nal^R recipient was spread plated onto CHROMagar™ supplemented with or without 2-Hexadecynoic acid (2-HDA), sodium citrate or calcium chloride and incubated for 6 h. Afterwards, four *E. coli* strains were spread plated onto SH -2813nal^R bacterial lawn and incubation continued for another 20 – 24 h. Thereafter, agar plugs were used for bacterial enumeration and agar lysate extraction. **(b and c)** SH recombinants (**b**) and *E. coli* donor population (**c**) in agar plugs was determined on Brilliant Green Sulfur agar and CHROMagar™ supplemented with or without gentamicin (16 ppm) or ampicillin (8 ppm) (total = bacterial population on agar with no antibiotic, amp^R/gen^R – bacterial population on agar supplemented with ampicillin or gentamicin). Colonies were counted using a calibrated automatic colony counter (ProtoCOL 3- Synoptics Ltd) (*P < 0.05, **P < 0.01 (Kruskal-Wallis test, multiple comparison correction; Error bar = Standard deviation)).

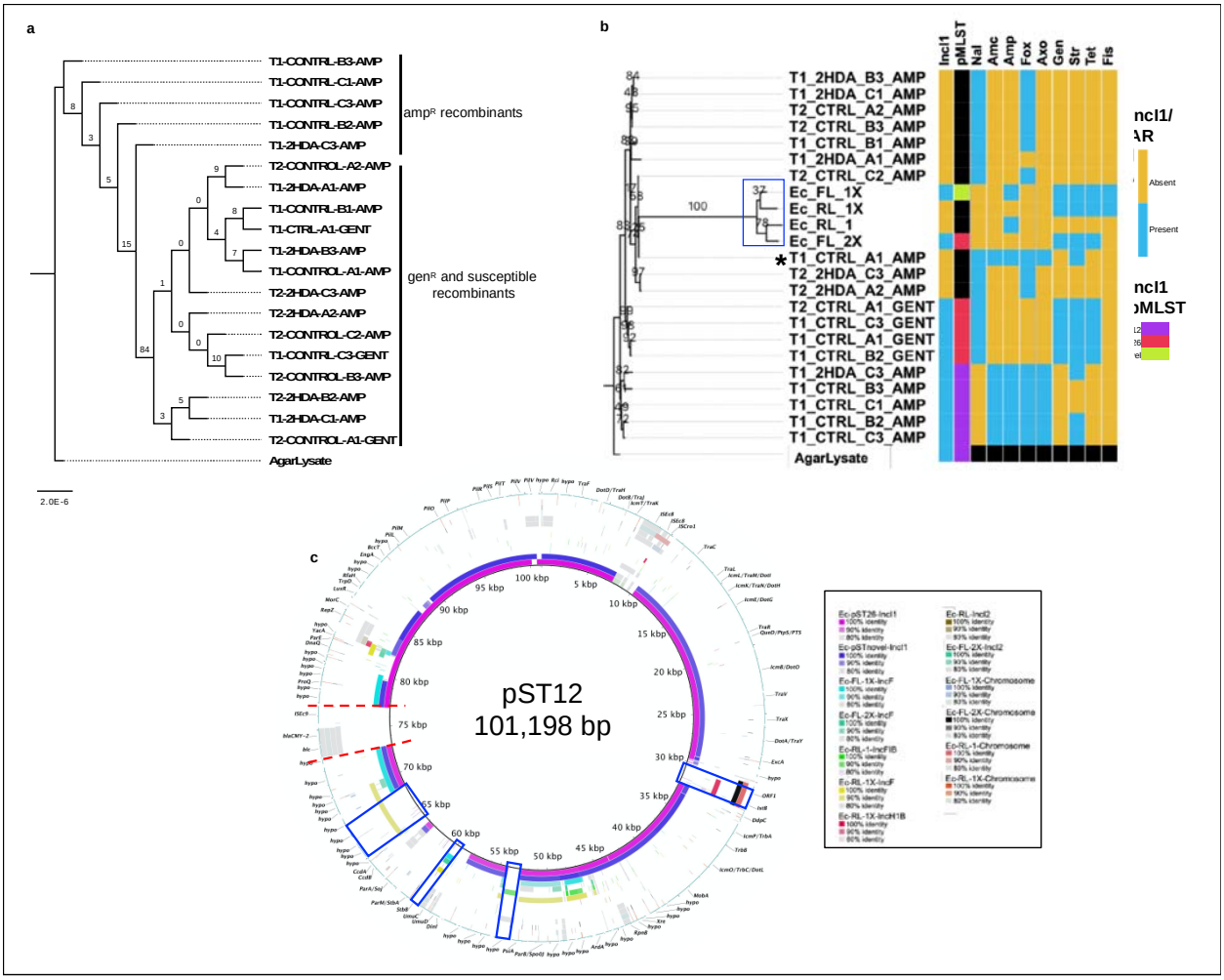

Fig 5. Multidrug resistance strains of *S.* Heidelberg evolved through genetic recombination with *E. coli*. (**a and b**) Maximum likelihood tree reconstructed using SNPs/Indel (**a**) found on the chromosome of SH recombinants and agar lysate, and the accessory genes they carried (**b**) GTR and JC model of nucleotide substitution was used for SNP and accessory gene tree, respectively and the GAMMA model of rate heterogeneity was used for sequence evolution prediction. Rectangular blue box is to highlight the four *E. coli* donor strains used for mating and asterisk denotes one β-lactam resistant SH recombinant that lost the bla_{CMY-2}. genetic determinants prior to sequencing. (Legend: Nal – nalidixic acid, Gen – gentamicin, Str – streptomycin, Tet – tetracycline, Fis – sulfisoxazole, Amc - amoxicillin-clavulanic acid, Amp – ampicillin, Fox – cefoxitin, Axo – Ceftriaxone, pMLST – plasmid multilocus sequence type). Numbers shown next to the branches represent the percentage of replicate trees where associated isolates cluster together based on ~100 bootstrap replicate. Tree was rooted with agar lysate. (**c**) BLASTn alignment of chromosome and all plasmids harbored by *E. coli* donors except IncX1 and Col-like plasmids. The IncI1 plasmid encoding bla_{CMY-2} (pST12 -102 kb) found in amp^R recombinants was used as the reference genome. Red dashed vertical lines highlights pST12 region encoding the bla_{CMY-2} operon whereas blue rectangular box highlights regions encoded by IncFII/FIB and IncH1B plasmids.