Litter Commensal Bacteria Can Limit the Horizontal Gene Transfer of Antimicrobial Resistance to *Salmonella* in Chickens

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

**IMPORTANCE** 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 organizations have adopted a One Health approach to curtail the proliferation of antimicrobial-resistant bacteria. This includes the removal and reduction of antibiotics used in food animal production and the development of alternatives to antibiotics. However, there is still a significant knowledge gap in our understanding of how resistance spreads in the absence of antibiotic selection and the role commensal bacteria play in reducing antibiotic resistance transfer. In this study, we show that commensal bacteria play a key role in reducing the horizontal gene transfer of antibiotic resistance to *Salmonella*, provide the identity of the bacterial species that potentially perform this function in broiler chickens, and also postulate the mechanism involved.

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

There is limited research on if and how commensal bacteria reduce AMR in foodborne pathogens (10–16), such as Salmonella enterica, and the role it plays in AMR reduction and transfer. Therefore, the goal of this study was to determine the role commensal bacteria play in limiting Salmonella enterica from acquiring AMR and to provide information on the bacterial species and mechanism involved. To do this, we compared the dynamics of AMR transfer in neonatal broiler chicks raised on fresh litter to that of chicks raised on reused litter. We chose reused litter because it is a complex environment made up of decomposing plant-based bedding (e.g., wood shavings, sawdust, and rice or peanut hulls) mixed with chicken feces, uric acid, feathers, feed, insects, and other broiler-sourced materials. Therefore, reused litter carries a unique and complex population of bacteria, fungi, and viruses (17–19) interacting with various forms of eukaryotes, making it a suitable environment to study competitive exclusion. Broiler chickens are commonly raised on litter, but how litter is managed differs between countries, producers, and farmers. For instance, the practice of reusing litter over multiple flocks of broiler chickens is a widespread practice in the United States and Brazil, while Canada (20) and Europe (21) recommend fresh litter bedding for every flock. One argument against litter reuse is that it harbors pathogenic bacteria that can be transferred to the next flock (22). Contrastingly, proponents of litter reuse argue that it confers competitive exclusion against pathogens when effectively managed and it is cost-effective (23–26). Beyond broiler chicken production, competitive exclusion by commensal bacteria has received enormous attention in the 20th century, resulting in the bloom of commercially marketed probiotics (27) and the underlying theory behind the application of fecal microbiota transplantation in human medicine (28).

RESULTS

S. Heidelberg abundance and prevalence in ceca and litter. We confirmed that neonatal chicks were Salmonella free by testing the chick pads used for transportation from the hatchery for Salmonella. None of the chick pads was positive for Salmonella. The reused litter for this study was confirmed to be Salmonella free in earlier studies (29, 30). To determine if the microbiome offers protection against Salmonella colonization, we challenged neonatal broiler chicks with a nalidixic acid-resistant (nalR) strain of S. Heidelberg and raised the chicks on either new bedding made up of fresh pine shavings (here referred to as fresh litter) \((n = 75)\) or reused litter (litter previously used to raise three flocks of broiler chickens) \((n = 75)\). Chicks were challenged either through oral gavage \((n = 25)\), cloacal inoculation \((n = 25)\), or by the seeder method (i.e., a few chicks \([n = 5]\) were challenged orally and comingled with unchallenged chicks \([n = 20]\) \((7)\). Unchallenged chicks on fresh \((n = 25)\) and reused litter \((n = 25)\) were used as controls. Broiler chicks on fresh and reused litter were housed separately for 14 days (four separate pens for each treatment in a house), and chicks were not administered any medication or antibiotics for the duration of the study.

Afterwards, we determined the concentration of nalR S. Heidelberg in the ceca and litter
of chicks 14 days after they were challenged. The average nal\(^R\) S. Heidelberg concentrations in the ceca (\(n = 10\) for oral and cloacal, \(n = 15\) for seeder) were not significantly different between chicks raised on fresh litter compared to those raised on reused litter (shown here as \(W\) statistic, \(P\) value: oral = 41, 0.15; cloacal = 41, 0.96; seeder = 5, 1.00) (Fig. 1A). However, the ceca of chicks raised on fresh litter were more likely to be positive for nal\(^R\) S. Heidelberg (\(x^2 = 16.07\), degree of freedom [df] = 2, \(P = 0.0003\)), than chicks on reused litter. The percentages of cecal samples positive for nal\(^R\) S. Heidelberg were 100%, 100%, and 50%, respectively, for oral, cloacal, and seeder chicks raised on fresh litter, compared to 80%, 100%, and 33.3%, respectively, for chicks raised on reused litter. For the seeder treatment on fresh litter, one seeder was lost due to premature mortality and three of the four seeders were positive for nal\(^R\) S. Heidelberg, while the ceca of four of the 10 uninoculated contact chicks were positive. In contrast, the ceca of the five orally challenged seeders raised on reused litter were positive for nal\(^R\) S. Heidelberg, but all contact chicks were negative (\(n = 10\)).

For the litter, there was no significant difference in nal\(^R\) S. Heidelberg concentration in fresh litter compared to that in reused litter (\(P > 0.05\)); however, oral and seeder treatments carried higher levels of nal\(^R\) S. Heidelberg in fresh litter compared to reused litter, while cloacally inoculated chicks had higher levels in reused litter compared to fresh litter (Fig. 1B). All litter samples from challenged chicks were positive for nal\(^R\) S. Heidelberg by direct culture or after enrichment in buffered peptone water (BPW), except for one reused litter sample from the seeder treatment that was negative. For unchallenged chicks used as controls, one litter sample each from fresh and reused litter tested positive after enrichment in BPW. These data suggest that litter age/type did not have a significant effect on the abundance of nal\(^R\) S. Heidelberg in the ceca, but chicks raised on reused litter (66%) had a lower Salmonella positivity rate compared to chicks on fresh litter (79%) (\(P = 0.0003\)).

Broiler litter age/type affected the horizontal transfer of AMR. Next, we questioned if the litter plays a role in the HGT of AMR. First, we performed antibiotic susceptibility testing (AST) on S. Heidelberg isolates recovered from the ceca and litter of broiler chicks raised on fresh litter (\(n = 158\)) and reused litter (\(n = 141\)). On average, five nal\(^R\) S. Heidelberg isolates (range, 1 to 7) were randomly selected from the ceca of each challenged chick that was Salmonella positive (fresh litter, \(n = 25\) chicks; reused litter, \(n = 23\) chicks) and used for AST. For AST of litter isolates, an average of 11 nal\(^R\) S. Heidelberg isolates (range, 3 to 17) were randomly selected per pen for fresh and reused litter (\(n = 3\) pens each for challenged chicks on fresh or reused litter).

As expected, all S. Heidelberg isolates were resistant to nalidixic acid (Fig. 2). S. Heidelberg isolates from the ceca and litter of chicks on fresh litter were more likely to be resistant to one or more antibiotics compared to isolates from chicks on reused litter (ceca, \(x^2 = 138\), df = 2, \(P < 2.2e-16\); litter, \(x^2 = 11.65\), df = 2, \(P = 0.003\)). Thirty-one percent of isolates from the ceca (37/118) and 37.5% of those from the litter (15/40) of
chicks on fresh litter acquired resistance to gentamicin, tetracycline, and streptomycin (7) (Fig. 2A). Contrastingly, no S. Heidelberg isolate from the ceca or litter of chicks on reused litter acquired resistance to gentamicin, tetracycline, and streptomycin. Three percent of S. Heidelberg isolates from the ceca of chicks on reused litter acquired resistance to nalidixic acid. Nal, nalidixic acid; Gen, gentamicin; Str, streptomycin; Tet, tetracycline; Fox, cefoxitin; Cip, ciprofloxacin.
resistance to either cefoxitin (1/114) or tetracycline (2/114), while \(~18\%\) of isolates from reused litter acquired resistance to either ciprofloxacin (1/27), tetracycline (2/27), or streptomycin (2/27) (Fig. 2A). The percentage of S. Heidelberg isolates that acquired AMR in fresh litter differed by the route of inoculation (7). For instance, 40\% of isolates from orally challenged chicks acquired AMR compared to 24\% for cloacal isolates (7).

We previously demonstrated that S. Heidelberg isolates from fresh litter that acquired resistance to gentamicin, tetracycline, and streptomycin harbored Inc1 plasmids (plasmid MLST 26; here referred to as Inc1-pST26) (7). The Inc1-pST26 plasmid carries ARGs for amnoglycoside (aadA1), tetracycline (tetA), and mercury (mer operon) resistance (see Fig. S1 in the supplemental material). Here, we investigated if S. Heidelberg isolates recovered from chicks raised on reused litter differed in their core genome or in plasmids harbored, compared to isolates from fresh litter. To answer this question, we performed whole-genome sequence (WGS) analysis on S. Heidelberg isolates (n = 29) recovered from the ceca of two cloacally challenged chicks from fresh litter (SH4 and SH6) and reused litter (SH16 and SH20), as well as the litter samples collected from the floor pen. We chose cloacal samples because only this route of challenge resulted in 100\% colonization of the ceca of chicks raised on fresh litter and reused litter.

There was no difference in the core genome multilocus sequence types (cgMLSTs) of evolved S. Heidelberg isolates from fresh versus reused litter; however, 28 of the 29 evolved isolates had a different cgMLST from the ancestral nalR S. Heidelberg (Fig. 2B). We used the genomes sequenced to construct a maximum likelihood (ML) tree based on the pangenome and mutations of S. Heidelberg strains recovered. The core genome (genes present in \(\geq 95\%\) of the strains) and accessory genome (genes present in \(< 95\%\) of the strains) were composed of 4,373 and 356 genes, respectively. After removing 10 isolates with identical mutations, a total of 118 informative sites (single nucleotide polymorphisms [SNPs] and indels) was used for ML SNP tree construction.

The three constructed ML trees did not group isolates by litter age/type (fresh versus reused) (Fig. 2B; see Fig. S2A and B in the supplemental material), but S. Heidelberg isolates that harbored Inc1-pST26 (n = 5) formed a separate clade on the accessory genome tree (Fig. 2B). Isolates from the ceca (n = 3) and litter (n = 1) of chicks on fresh litter that harbored Inc1-pST26 displayed the antibiotic resistance phenotype predicted by WGS (Fig. 2B). Contrastingly, one isolate from reused litter carried Inc1-pST26 but was found to be susceptible to all antibiotics tested, except for the ancestral nalidixic resistance. These results indicated that chicks grown on reused litter in this study were less likely to carry S. Heidelberg isolates harboring AMR compared to chicks on fresh litter.

The microbiomes of neonatal chicks differed for fresh and reused litter. We used 16S rRNA gene sequencing to examine the differences in the microbiome between neonatal chicks raised on fresh litter and those raised on reused litter. The beta diversities of the cecal (n = 59) and litter (n = 22) microbiome of chicks were significantly different between fresh litter and reused litter (ceca, \(P = 0.0002\); litter, \(P = 0.0002\)) (Fig. 3A). Furthermore, the route of inoculation used for S. Heidelberg affected the beta diversities of the ceca and litter for fresh litter and reused litter (\(P < 0.05\)). For example, chicks challenged orally (n = 10) or through the cloaca (n = 10) and raised on fresh litter harbored a significantly different beta diversity than uninoculated chicks (n = 10) in the ceca (\(P < 0.002\)) (Fig. 3A). Likewise, the beta diversity of the ceca of chicks challenged orally (n = 10) differed from those of cloacal (n = 9) and uninoculated controls (n = 10) for reused litter (\(P < 0.05\)). In the litter, pairwise comparisons between routes of inoculation were not significant (\(P > 0.05\)) for beta diversity. Nevertheless, fresh litter samples (n = 3) from orally challenged chicks clustered next to litter samples (n = 3) from seeder treatments, while litter samples (n = 2) from cloacally inoculated chicks were different from those of oral and seeder treatments (Fig. 3A). For reused litter samples, litter from orally (n = 3) and cloacally (n = 3) challenged chicks clustered together, while litter (n = 2) from seeder treatments was similar to litter samples (n = 3) from control chicks. Finally, the bacterial community structure (assessed using beta dispersion) of reused litter was less variable than that of fresh litter (\(P = 0.001\)); however, there was no difference in variability for the ceca...
Moreover, the route of challenge affected the bacterial community beta dispersion in the ceca and litter of chicks on fresh litter and reused litter ($P = 0.001$). The alpha diversity (i.e., the number of amplicon sequence variants [ASVs]) of the microbiome present in the ceca and litter of chicks on reused litter was higher for the observed species measure of diversity (ceca, $P < 0.001$; litter, $P = 0.001$), but not for Shannon (ceca, $P = 0.25$; litter, $P = 0.05$) and Simpson (ceca, $P = 0.97$; litter, $P = 0.28$) indices. The route of $S$. Heidelberg challenge affected the alpha diversity of the cecal and litter microbiomes ($P < 0.05$) (Fig. 3B). The alpha diversity of the ceca of challenged and control chicks raised on reused litter was higher than that of chicks grown on fresh litter for the observed species measure of diversity ($P < 0.01$). Cloacally challenged chicks raised on reused litter had higher alpha diversity in their ceca compared to chicks on fresh litter for the Shannon and Simpson indices ($P < 0.05$). Uninoculated control chicks on fresh litter had higher alpha diversity in their ceca than control chicks on reused litter for the Simpson index (Fig. 3B). The litter of cloacal, seeder, and control chicks on reused litter had higher alpha diversity than the litter of chicks on fresh litter for the Shannon measure of diversity ($P < 0.05$) (Fig. 3B). In contrast, the litter of orally challenged chicks on fresh litter had higher alpha diversity than the litter of gavaged chicks on reused litter for the Shannon index ($P = 0.0001$). There was no significant

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difference in alpha diversity between fresh litter and reused litter for the observed and Simpson indices ($P > 0.05$).

On the taxonomic level, uninoculated control chicks on fresh litter had higher relative abundance of *Actinobacteria* and *Firmicutes* in the ceca compared to control chicks on reused litter ($P = 0.004$), while the abundance of *Proteobacteria* and *Bacteroides* was higher in the ceca of control chicks on reused litter compared to that of control chicks on fresh litter ($P < 0.05$) (Fig. 3C). Orally challenged chicks on reused litter carried a higher abundance of *Actinobacteria* in the ceca compared to chicks on fresh litter ($P = 0.003$), while the abundance of *Proteobacteria* was higher in the ceca of oral and cloacal chicks on reused litter compared to chicks on fresh litter ($P < 0.01$). *Firmicutes* had higher relative abundance in the ceca of chicks on fresh litter compared to reused litter for all challenged chicks ($P < 0.01$), while the abundance of *Bacteroides* was higher in the ceca of cloacal chicks on reused litter compared to chicks on fresh litter ($P < 0.05$) (Fig. 3C).

For the litter, the abundance of *Actinobacteria* was higher in reused litter compared to fresh litter ($P < 0.05$), while the abundances of *Proteobacteria* and *Bacteroides* were higher in fresh litter compared to reused litter ($P < 0.05$). There was no significant difference in phylum abundance between routes of inoculation ($P > 0.05$) for the litter. These results shows that the microbiome of chicks raised on fresh litter differed from that of chicks grown on reused litter, and reused litter cecal and litter samples of challenged chicks were more uniform than fresh litter samples.

### S. Heidelberg Challenge Modulated the Gut Microbiome

We determined that the core microbiome (i.e., ASVs of $>0.1\%$ relative abundance and present in at least $80\%$ of samples) of fresh litter differs from that of reused litter (see Table S1 in the supplemental material) and found that *S. Heidelberg* challenge perturbed the cecal and litter microbiomes of broiler chicks (Fig. 4). For example, *Enterobacteriaceae* (*Klebsiella pneumoniae* and *Proteus mirabilis*) were part of the core microbiome in fresh litter, while they were not part of the core microbiome of reused litter (Table S1). Contrastingly, *Actinobacteria* (23 ASVs) were part of the core microbiome of reused litter, while only one ASV classified as *Actinobacteria* was determined to be a core member of fresh litter (Table S1). Unsurprisingly, the bacterial ASVs that increased or decreased in abundance after *S. Heidelberg* challenge differed between chicks on fresh litter and reused litter (Fig. 4A and B).

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

For the ceca of chicks on reused litter, the ASVs modulated were assigned to 12 bacterial families. Amplicon sequence variants matching *Bifidobacteriaceae*, order *Clostridiales*, *Moraxellaceae*, *Rikenellaceae*, *Eggerthellaceae*, *Enterococcaceae*, family *XIII*, *Lactobacillaceae* ($n = 2$), *Ruminococcaceae* ($n = 2$), and *Lachnospiraceae* ($n = 2$) increased in abundance in challenged chicks compared to control chicks. In contrast, ASVs classified as *Enterobacteriaceae*, *Enterococcaceae*, *Clostridiales* vadinBB6 group ($n = 2$), *Ruminococcaceae* ($n = 3$), and *Lachnospiraceae* ($n = 7$) decreased in abundance in challenged chicks compared to control chicks (Fig. 4A).

For the litter, the ASVs that were significantly modulated in fresh litter were assigned to 21 bacterial families (Fig. 4B). The ASVs that increased in abundance in fresh litter of challenged chicks belonged to *Caulobacteriaceae*, *Dysgonomonadaceae*, *Burkholderiaceae*, *Flavobacteriaceae*, *Cellvibrionaceae*, *Enterobacteriaceae*, *Enterococcaceae* ($n = 2$), *Lactobacillaceae* ($n = 2$), *Sphingobacteriaceae* ($n = 2$), *Pseudomonadaceae* ($n = 3$), *Ruminococcaceae* ($n = 3$), *Moraxellaceae* ($n = 4$), *Proteobacteriaceae* ($n = 3$), *Enterococcaceae* ($n = 7$), *Ruminococcaceae* ($n = 10$), *Lactobacillaceae* ($n = 2$), *Sphingobacteriaceae* ($n = 2$), *Pseudomonadaceae* ($n = 3$), *Ruminococcaceae* ($n = 3$), *Moraxellaceae* ($n = 4$), *Proteobacteriaceae* ($n = 3$), *Enterococcaceae* ($n = 7$), *Ruminococcaceae* ($n = 10$), *Lactobacillaceae* ($n = 2$), *Sphingobacteriaceae* ($n = 2$), *Pseudomonadaceae* ($n = 3$), *Ruminococcaceae* ($n = 3$), *Moraxellaceae* ($n = 4$),
Clostridiales vadinBB6 group (n = 4), and Lachnospiraceae (n = 4). Conversely, ASVs assigned to Staphylococcaceae, Brevibacteriaceae, order Micrococcales, Corynebacteriaceae, Xanthomonadaceae, Weeksellaceae, Bacillaceae (n = 2), Ruminococcaceae (n = 6), and Lachnospiraceae (n = 6) decreased in abundance in the litter of challenged chicks compared to control chicks (Fig. 4B).

Amplicon sequence variants modulated in reused litter after challenge were grouped into 24 bacterial families. The ASVs that increased in abundance in reused litter of challenged chicks were assigned to Lactobacillaceae, Saprospiraceae, Bifidobacteriaceae, Rikenellaceae, Pseudomonadaceae, Sphingobacteriaceae, Leuconostocaceae, Glycmyctecaceae, Streptococcaceae, Enterobacteriaceae, Wohlfahrtimonadaceae, Bacteroides (n = 2), Ruminococcaceae (n = 3), Planococcaceae (n = 3), Lachnospiraceae (n = 3), and Clostridiaceae 1 (n = 5). The ASVs that decreased in the reused litter of challenged chicks were classified as Burkholderiaceae, Microbacteriaceae, Micrococcaceae, Moraxellaceae (n = 2), Wohlfahrtimonadaceae (n = 2), Aeromonadaceae (n = 3), Clostridiales vadinBB6 group (n = 3), Ruminococcaceae (n = 4), Lachnospiraceae (n = 4), and Bacillaceae (n = 6).

Not all bacterial families were modulated by the cloacal route of S. Heidelberg challenge. For instance, Enterococcaceae was only modulated by oral challenge in the ceca of chicks grown on fresh and reused litter (Fig. 4A). Similarly, Pseudomonadaceae was only modulated in the litter of chicks that were orally challenged (Fig. 4B). Together,
these results show that the litter microbiomes modulated by S. Heidelberg challenge differed between chicks on fresh litter and those on reused litter, and the route of challenge affected the ASVs that increased or decreased in abundance.

The microbiomes of chicks raised on reused litter were enriched in functional pathways for the biosynthesis of antimicrobials. A functional protein pathway analysis was performed using Hi-C metagenome-assembled genomes (MAGs) of two cecal samples each from cloacally challenged chicks on fresh and reused litter. The average assembly size and number of contigs for the four cecal samples were 1,238,395,273 ± 167,820 bp and 1,797,423 ± 281,253, respectively. The number of MAGs found in cecal samples from fresh and reused litter was 456 ± 422. Pathways related to the biosynthesis of organic and antimicrobial molecules were enriched to a greater degree in the ceca of chicks on reused litter than those in chicks on fresh litter (see Fig. S3 in the supplemental material). Of note was the enrichment of biosynthetic pathways for antimicrobials and secondary metabolites in reused litter (e.g., macrolides, tetracyclines, carbapenems, vancomycin, and polyketides), suggesting that reused litter harbored a higher abundance of microbial species with antimicrobial properties than fresh litter. Contrastingly, pathways associated with the biosynthesis of secondary metabolites of plants, including flavonoids, indole-alkaloids, and stilbenoid, diaryleptanoid, and gingenol were enriched in samples from fresh litter (Fig. S3).

Enterobacteriaceae and Clostridiales were the major bacterial hosts for AMR. We also used Hi-C MAGs to explore the bacterial reservoir of AMR in the cecal microbiome. We previously used this approach to identify Escherichia coli as the main bacterial reservoir of transferable plasmids in fresh litter (7). Here, we extended our analysis to cecal samples from reused litter (n = 2). We searched the MAGs for sequences matching plasmid incompatibility (inc) groups and replicons available on the PlasmidFinder database. The inc groups found matched common Enterobacteriaceae plasmids, including IncF, IncI, IncX, IncY, IncB/O/K/Z, p0111, and CoI-like plasmids (Table 1). Three plasmid replicons were found for Gram-positive bacteria, including rep2, rep18b, and repA (Table 1). The low representation of Gram-positive plasmids is expected, since the PlasmidFinder database is much more comprehensive for Enterobacteriaceae than other bacterial species, and within the well-studied Proteobacteria, most plasmids cannot be typed (31).

Next, we used proximity ligation to find the bacterial host of ARGs and to determine if they are encoded on plasmids or the chromosome. One hundred ARGs were found in the MAGs, and the majority (~50%) were harbored by family Enterobacteriaceae (n = 3 MAGs) (Fig. 5). Escherichia coli was the only member of the Enterobacteriaceae found to be the putative host for these ARGs in fresh and reused litter. Members of the order Clostridiales were the putative hosts for 27% of ARGs, while unclassified bacterial species (n = 6) and members of the phyla Firmicutes (n = 9), Bacteroidetes (n = 3), and Actinobacteria (n = 1) were the hosts for the remaining ARGs. Fifty-four percent of the ARGs were found to be carried on the chromosome, while 46% were found on plasmids. One cecal sample from fresh litter (Fig. 5) harbored E. coli MAGs that contained silver and copper resistance genes (silABCFRS and pcoABCDRS) on the chromosome, while virulence genes (iroBCDEN) were found on plasmids. A cecal sample from a chick raised on reused litter harbored E. coli MAGs that carried ampicillin (blaTEM-1B),

| Bacterial group | Hi-C-FL1 | Hi-C-FL2 | Hi-C-RL1 | Hi-C-RL2 |
|----------------|---------|---------|---------|---------|
| Gram negative  | Col(MG828), ColI56, ColpVC, ColRNAI, IncFIB, IncFIC(FII), IncFIC(pRSB107), IncH11B(pNDM-CIT), IncI1-1(Alpha), IncI2(Delta), IncX1, IncX2, p0111 | Col(MG828), ColpVC, ColRNAI, IncB/O/K/Z, IncFIB, IncFII (pSFO), IncI2, IncX1, IncX2 | Col(MG828), Col(pHAD28), IncI1-I(Alpha), IncI2, IncX1, IncX2, IncY | Col(MG828), ColI56, IncFIB, IncFII(pCoo), IncH11B(pNDM-CIT), IncI1-1(Alpha) |
| Gram positive  | rep2, repA(pB82) | None | rep18b | repA(pB82) |

*Sample IDs: FL, fresh litter; RL, reused litter.

**The microbiomes of chicks raised on reused litter were enriched in functional pathways for the biosynthesis of antimicrobials.** A functional protein pathway analysis was performed using Hi-C metagenome-assembled genomes (MAGs) of two cecal samples each from cloacally challenged chicks on fresh and reused litter. The average assembly size and number of contigs for the four cecal samples were 1,238,395,273 ± 167,820 bp and 1,797,423 ± 281,253, respectively. The number of MAGs found in cecal samples from fresh and reused litter was 456 ± 422. Pathways related to the biosynthesis of organic and antimicrobial molecules were enriched to a greater degree in the ceca of chicks on reused litter than those in chicks on fresh litter (see Fig. S3 in the supplemental material). Of note was the enrichment of biosynthetic pathways for antimicrobials and secondary metabolites in reused litter (e.g., macrolides, tetracyclines, carbapenems, vancomycin, and polyketides), suggesting that reused litter harbored a higher abundance of microbial species with antimicrobial properties than fresh litter. Contrastingly, pathways associated with the biosynthesis of secondary metabolites of plants, including flavonoids, indole-alkaloids, and stilbenoid, diaryleptanoid, and gingenol were enriched in samples from fresh litter (Fig. S3).

**Enterobacteriaceae and Clostridiales were the major bacterial hosts for AMR.** We also used Hi-C MAGs to explore the bacterial reservoir of AMR in the cecal microbiome. We previously used this approach to identify Escherichia coli as the main bacterial reservoir of transferable plasmids in fresh litter (7). Here, we extended our analysis to cecal samples from reused litter (n = 2). We searched the MAGs for sequences matching plasmid incompatibility (inc) groups and replicons available on the PlasmidFinder database. The inc groups found matched common Enterobacteriaceae plasmids, including IncF, IncI, IncX, IncY, IncB/O/K/Z, p0111, and CoI-like plasmids (Table 1). Three plasmid replicons were found for Gram-positive bacteria, including rep2, rep18b, and repA (Table 1). The low representation of Gram-positive plasmids is expected, since the PlasmidFinder database is much more comprehensive for Enterobacteriaceae than other bacterial species, and within the well-studied Proteobacteria, most plasmids cannot be typed (31).

Next, we used proximity ligation to find the bacterial host of ARGs and to determine if they are encoded on plasmids or the chromosome. One hundred ARGs were found in the MAGs, and the majority (~50%) were harbored by family Enterobacteriaceae (n = 3 MAGs) (Fig. 5). Escherichia coli was the only member of the Enterobacteriaceae found to be the putative host for these ARGs in fresh and reused litter. Members of the order Clostridiales were the putative hosts for 27% of ARGs, while unclassified bacterial species (n = 6) and members of the phyla Firmicutes (n = 9), Bacteroidetes (n = 3), and Actinobacteria (n = 1) were the hosts for the remaining ARGs. Fifty-four percent of the ARGs were found to be carried on the chromosome, while 46% were found on plasmids. One cecal sample from fresh litter (Fig. 5) harbored E. coli MAGs that contained silver and copper resistance genes (silABCFRS and pcoABCDRS) on the chromosome, while virulence genes (iroBCDEN) were found on plasmids. A cecal sample from a chick raised on reused litter harbored E. coli MAGs that carried ampicillin (blaTEM-1B),

### Table 1: Plasmid incompatibility groups and replicons found in cecal metagenome-assembled genomes using PlasmidFinder

| Bacterial group | Hi-C-FL1 | Hi-C-FL2 | Hi-C-RL1 | Hi-C-RL2 |
|----------------|---------|---------|---------|---------|
| Gram negative  | Col(MG828), ColI56, ColpVC, ColRNAI, IncFIB, IncFIC(FII), IncFIC(pRSB107), IncH11B(pNDM-CIT), IncI1-1(Alpha), IncI2(Delta), IncX1, IncX2, p0111 | Col(MG828), ColpVC, ColRNAI, IncB/O/K/Z, IncFIB, IncFII (pSFO), IncI2, IncX1, IncX2 | Col(MG828), Col(pHAD28), IncI1-I(Alpha), IncI2, IncX1, IncX2, IncY | Col(MG828), ColI56, IncFIB, IncFII(pCoo), IncH11B(pNDM-CIT), IncI1-1(Alpha) |
| Gram positive  | rep2, repA(pB82) | None | rep18b | repA(pB82) |

*aSample IDs: FL, fresh litter; RL, reused litter.*
tetracycline (tetA), and mercury resistance (merCPRT) genes on plasmids (Fig. 5). This result suggests that the microbiomes of chicks on fresh litter and reused litter harbored AMR, and Enterobacteriaceae and Clostridiales were the putative bacterial hosts of the ARGs.

**E. coli isolates harbored AMR on plasmids and genomic islands.** In our earlier work, we found that E. coli MAGs were the primary hosts of IncI1 by retrospectively screening the cecal contents of chicks on fresh litter for E. coli isolates (7). Using this approach, we confirmed that the IncI1-pST26 plasmid acquired by S. Heidelberg was identical to the IncI1-pST26 plasmid present in E. coli strains (Fig. S1). Here, we performed AST and WGS on additional E. coli isolates recovered from the ceca of chicks used for Hi-C metagenomics (see Table S2 in the supplemental material), and a pan-genome analysis was done on 19 of them. The isolates were randomly selected from CHROMagar plates supplemented with or without gentamicin and tetracycline.

The inferred phylogeny of the E. coli strains by using their core (n = 3,385) and accessory (n = 6,024) genes grouped the isolates by the litter used for raising the chicken host, except for one isolate (Ec-RL2-2) from reused litter that clustered with E. coli strains from fresh litter (Fig. 6). The ML trees grouped the isolates into three main clades (clades I to III). One E. coli isolate from the ceca of chicks on reused litter made up clade I on the core and accessory gene tree, while the numbers of isolates in clade II and III strains differed between the core (Fig. 6A) and accessory (Fig. 6B) gene trees. Clade II isolates were clonal and recovered from chicks on fresh litter. Clade III was divided into two subclades represented by isolates from fresh litter (subclade IIIa) and reused litter (subclade IIIb), and subclade IIIb isolates were more clonal compared to subclade IIIa isolates. E. coli isolates classified as phylogroups A, D, and F were more likely to be found in chicks on fresh litter, while phylogroup B1 was found in the ceca of chicks from reused litter (Fig. 6; Table S2). Likewise, cgMLST classification showed that the sequence types ST69, ST2705, and ST6858 were associated with E. coli strains originating from chicks on fresh litter, while ST1403 was found in reused litter. These
results suggest that the litter type/age used for raising the chicks in this study affected the pangenome of \textit{E. coli} strains recovered from the ceca. Some \textit{E. coli} strains from fresh litter harbored ARGs on the chromosome and plasmids. \textit{E. coli} strains classified as the pandemic lineage of extraintestinal pathogenic ST69 from fresh litter harbored an $\sim128$-kb genomic island containing virulence genes (pyelonephritis-associated pilus [\textit{pap}] operon) and ARGs for aminoglycoside [\textit{strAB}] and \textit{aph(3'')-Ia}] tetracycline (\textit{tetB}), sulfonamide (\textit{sul2}), and metal resistance (silver [\textit{sil}] and copper [\textit{pco}] operons) (see Table S2 and Fig. S4 in the supplemental material). The presence of a genomic island encoding AMR suggests that the ARGs are mobile. We found DNA regions carrying identical metal resistance genes (10 to 40 kb), as seen in the genomic island in \textit{p0111/IncH1B} plasmids harbored by \textit{E. coli} strains from this study and in \textit{E. coli} genomes found in the NCBI database (Fig. S4). In addition, ST69 \textit{E. coli} strains harbored a multireplicon IncF plasmid carrying \textit{blaTEM-1B}, \textit{aac(3'')-Ild}, and virulence genes (colicin M and catecholate siderophore uptake system [\textit{iroBCDEN}]), as well as an unknown IncI1 plasmid (see Data Set S1). Data Sets S1 to S5 are available in the Dryad Repository at https://doi.org/10.5061/dryad.c866t1g6c.

As previously reported (7), \textit{E. coli} ST6858 from fresh litter carried an identical \textit{S. Heidelberg IncI1-pST26} plasmid and harbored IncI2, IncF, and cryptic Col-type plasmids (see Data Set S2 in the Dryad Repository). Antibiotic-susceptible ST2705 \textit{E. coli} isolates from fresh litter harbored no plasmid containing antibiotic resistance genes but carried a phage-like plasmid classified as \textit{p0111} and a multireplicon IncF plasmid containing metal resistance and virulence genes (see Data Set S3 in the Dryad Repository). The common ARG carried by ST1403 \textit{E. coli} strains from reused litter was \textit{blaTEM-1B}. The gene was carried on an IncX1 plasmid (see Data Set S4 in the Dryad Repository), and it conferred ampicillin resistance. Additionally, ST1403 \textit{E. coli} strains harbored IncFIB, IncI2, and ColRNAi plasmids. One \textit{E. coli} isolate (ST93) from reused litter harbored an IncH1B/\textit{p0111} plasmid encoding ARGs for aminoglycoside [\textit{aadA1}, \textit{aac(3)-Vla}], tetracycline (\textit{tetB}), sulfonamide (\textit{sul1}), and mercury resistance (\textit{mer} operon) and an IncF plasmid containing colicin M and \textit{iroBCDEN} genes (see Data Set S5 in the Dryad Repository).

**Bacterial hosts from reused litter maintained IncI1 plasmid at low copies.** The low rate of AMR acquisition by \textit{S. Heidelberg} isolates from reused litter made us hypothesize that IncI1-pST26 plasmids are present at lower copies in reused litter. To
test this hypothesis, we used quantitative PCR (qPCR) assays targeting the region upstream of the repA of IncI1 plasmid, including incRNAI (small antisense RNA essential for control of IncI plasmids replication), to determine their copy number in the ceca of chicks on fresh and reused litter. We normalized the abundance of IncI1 against glycer-aldehyde-3-phosphate dehydrogenase A (gapA) housekeeping gene carried on the chromosome of several enteric bacteria (32) including Salmonella and E. coli. We targeted Enterobacteriaceae with gapA because E. coli was determined to be the host for AMR by metagenomics and WGS in this study. The relative abundance of the Enterobacteriaceae population carrying IncI1 was higher in the ceca of orally challenged chicks on fresh litter compared to chicks on reused litter (W = 85, P = 0.00041) and was not significantly different for cloacally inoculated (W = 23, P = 0.24) and uninoculated control (W = 63, P = 0.16) chicks (Fig. 7).

To further explore the effect of low-copy IncI1 plasmids on AMR transfer, we selected an S. Heidelberg isolate from reused litter harboring IncI1-pST26 that was unexpectedly susceptible to antibiotics predicted by ARGs carried on the plasmid. The isolate (SH-16-0-5B) was confirmed twice by broth microdilution to be susceptible to gentamicin, streptomycin, and tetracycline and once on Mueller-Hinton agar supplemented with various concentrations (0, 2, 4, 8, and 16 mg/L) of gentamicin (see methods in the supplemental material). To ensure that the plasmid contig identified as IncI1-pST26 in this strain was not an artifact of short-read sequencing, we performed qPCR on plasmid DNA and targeted four coding DNA sequences on IncI1-pST26 including its incRNAI, class 1 integron (intI1), aminoglycoside (aadA1), and tetracycline resistance (tetA) genes (see Table S3 in the supplemental material). This confirmed that the strain carried IncI1-pST26. (The qPCR cycle threshold ranged from 20.2 to 27.9 for the four targets.) Afterwards, we used WGS (depth of coverage of IncI1-pST26 contig/depth of coverage of the largest chromosome contig) to compare the copy number of IncI1-pST26 in SH-16-0-5B to the copy number of IncI1-pST26 in four S. Heidelberg isolates (here referred to as SH-IncI1-FL) recovered from the ceca of cloacally inoculated chicks on fresh litter. This revealed that SH-16-0-5B maintained IncI1-pST26 at ~0.2 copy/cell (i.e., 1 of 5 cells harbored IncI1) compared to 2.8 ± 0.2 copies/cell in SH-IncI1-FL from fresh litter.

To investigate if the copy number difference determined using WGS exists under relevant environmental conditions, we first acclimatized the isolates in prereduced cecal extract (pH 6.5) for 2 h before exposing them to prereduced cecal extract at pH 2.5 under microaerophilic conditions. We reasoned that the low pH of the upper gastrointestinal tract (GIT) of the chicken host will interfere with the copy number of plasmids kept in a bacterial cell, which would have a direct effect on the rate of HGT. The copy number of IncI1-pST26 was significantly lower (0.00005 to 0.21 copy/per cell [i.e., 1 in 20,000 to 1 in 5 cells carried IncI1]) in SH-16-0-5B compared to SH-IncI1-FL (4.94 to

**FIG 7** Relative abundance of IncI in *Enterobacteriaceae* bacterial population of the ceca. Box plot of the ratio of IncI gene copies/gram of ceca to gene copies of gapA/gram of ceca for chicks raised on fresh litter (oral, n = 9; cloacal, n = 8; control, n = 9) and reused litter (oral, n = 10; cloacal, n = 9; control, n = 10). ***, P < 0.001 by Wilcoxon signed-rank test.
6.65 copies/per cell) at pH 6.5 ($W = 9, P = 0.1$) and pH 2.5 ($W = 81, P = 4.114e^{-05}$) (Table 2). These results suggest that \textit{Enterobacteriaceae} populations in reused litter harbored lower copies of IncI1-pST26 than the populations in fresh litter.

### DISCUSSION

Litter is commonly used as a bedding material for raising poultry, and it is ingested by chickens during pecking activities. Therefore, the microbiome of the litter is one of the first inocula that colonize the GIT of broiler chicks. Litter can be managed as single use (i.e., complete removal of litter from a broiler house after each flock), or it can be reused over multiple flocks. The benefits of litter reuse include lower cost for growing chickens and the sustainable management of litter waste (33). It has been shown that chickens raised on reused litter harbor a different microbiome than chicks grown on fresh litter (17, 34) and that reused litter stimulates higher humoral and cell-mediated immune responses than fresh litter in chickens (35). Muniz et al. (23) and Roll et al. (26) showed that the number of litter samples positive for \textit{Salmonella} significantly decreases as the number of litter reuses increased compared with the first use of the litter.

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

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

This was the case for the ASV assigned to \textit{Bifidobacterium} that increased in the ceca and litter of orally challenged chicks raised on reused litter but was not modulated in cloacally inoculated chicks or in chicks on fresh litter. Consequently, \textit{Bifidobacterium} was not part of the core microbiome of the ceca or litter in this study (Table S1) and most likely originated with neonatal chicks. \textit{Bifidobacterium} species are strict anaerobic \textit{Actinobacteria} and are among the first microbes to colonize the GIT of vertebrates (39). Additionally, \textit{Bifidobacterium} has been shown to affect the abundance of AMR and virulence genes in the GIT of infants (40–42). Casaburi et al. (42) reported that infants fed a probiotic strain of

### TABLE 2

| \textit{S. Heidelberg} strain ID | Time (h) | pH of cecal extract | IncI1 copy no./cell (mean ± SD)$^a$ | Coefficient of variation (%) |
|-------------------------------|----------|----------------------|-------------------------------------|-----------------------------|
| SH-16-0-SB-RL                 | 2        | 6.52                 | 0.001 ± 0.001                       | 155.8                      |
| SH-IncI1-FL                   | 2        | 6.52                 | 5.594 ± 0.582                       | 10.4                       |
| SH-16-0-SB-RL                 | 0.5      | 2.52                 | 0.001 ± 0.0003                      | 47.9                       |
| SH-IncI1-FL                   | 4        | 2.52                 | 0.141 ± 0.064                       | 46.0                       |
| SH-16-0-SB-RL                 | 24       | 2.52                 | 0.084 ± 0.038                       | 46.4                       |
| SH-IncI1-FL                   | 0.5      | 2.52                 | 6.304 ± 0.165                       | 2.6                        |
| SH-IncI1-FL                   | 4        | 2.52                 | 6.301 ± 0.343                       | 5.4                        |
| SH-IncI1-FL                   | 24       | 2.52                 | 5.892 ± 0.378                       | 6.4                        |

$^a$Three individual \textit{S. Heidelberg} isolates from fresh litter (FL) were used to determine the copy number of IncI1 for SH-IncI1-FL, while the IncI1 copy number in \textit{S. Heidelberg} strain SH-16-0-SB from reused litter (RL) was determined using three replicates.
Bifidobacterium longum harbored lower levels of ARG compared to nonfed controls. Alignment of shotgun metagenomic reads generated from the ceca of broiler chicks ($n = 4$) to the Kraken database (43) revealed that 21% and 11% of the reads mapping to the genus Bifidobacterium, were aligned to B. longum and Bifidobacterium catenulatum, respectively (see Fig. S5 in the supplemental material). Their increase in abundance for only orally challenged chicks on reused litter suggests that the route S. Heidelberg used for gaining entry into the GIT and the litter microbiome affected how Bifidobacterium was perturbed. Therefore, it is conceivable that the reused litter microbiome created conditions that allowed Bifidobacterium to flourish and proliferate, while the microbiome in fresh litter led to a reduction or elimination of Bifidobacterium species.

We used metagenomics and WGS to show that E. coli was the main reservoir of AMR in the cecal microbiomes of fresh and reused litter. Importantly, we found the identical IncI1-pST26 plasmid, acquired by S. Heidelberg, to be present in E. coli, suggesting that the plasmid was transferred in vivo within chicks raised on fresh litter. The possible sources of the E. coli strains in this study include the chicken host and the broiler house environment. Although we did not establish if the meconium harbored E. coli, it is plausible that post-hatch chicks were colonized with E. coli from the hatchery (44). The evolution of these ancestral E. coli strains would be shaped by selective pressures, such as exposure to antibiotics and metals, and competition with resident microbiota in the environment. For chicks placed on fresh litter, the bacterial community of the first fecal droppings will compete with mostly a pine shaving/plant-associated microbiome. In contrast, chicks placed on reused litter will compete with microbes and metabolites deposited from earlier flocks, including resident E. coli and Salmonella strains that are well adapted to the broiler house environment.

Also, the physiochemical properties of the litter will affect the litter structure, the native microbiome, and the survival of the invading pathogen. The difference in litter physiochemical parameters in this study was inferred using pH and moisture. The pH of reused litter was higher ($7.12 \pm 0.26$) than that of fresh litter ($6.54 \pm 0.17$) ($P = 0.028$), while fresh litter had higher moisture ($20.83\% \pm 3.54\%$) than reused litter ($14.65\% \pm 3.51\%$) ($P = 0.11$). Litter moisture and pH are known factors that affect pathogen survival and microbial diversity (45, 46). We did not administer antibiotics to chicks in this study and have shown that metals did not significantly affect the metabolism of S. Heidelberg (7). Hence, the litter microbiome and physiochemical properties are the major selection pressure that could explain the evolutionary trajectory of E. coli in this study. This hypothesis was further supported after a functional protein pathway analysis was performed on the cecal metagenome of chicks raised on fresh versus reused litter. Although we measured only two cross-sectional time points for reused litter and fresh litter, the relative abundance of plant metabolic-related pathways in fresh litter compared to the enrichment of antimicrobial molecules in reused litter suggests that a selective pressure gradient exists over time for the colonizing microbiome in reused litter. Thus, the microbiome of reused litter has the antimicrobial capability to competitively exclude invading pathogens, including strains carrying AMR-encoding plasmids.

Plasmid carriage is expected to impose a fitness cost on the host, but plasmid-bearing microbes are pervasive in nature (47). We showed previously that carriage of IncI1-pST26 by S. Heidelberg isolates from fresh litter presented a variable fitness cost to the host (7). We found here that Enterobacteriaceae populations in the ceca of chicks on reused litter harbored lower copies of the IncI1 plasmid per cell compared to chicks on fresh litter. In addition, we did not obtain any E. coli isolate carrying IncI1 from the ceca of chicks raised on reused litter. The limited number of members of the Enterobacteriaceae IncI1 donor population would affect the rate of transfer to S. Heidelberg recipients as direct contact is a requirement for conjugation (48). Nevertheless, we found one S. Heidelberg isolate from the ceca of one chick on reused litter that harbored IncI1-pST26; however, only a fraction of its population harbored the IncI1 plasmid. This isolate did not exhibit the resistance phenotype predicted by the ARGs carried on the IncI1-pST26 plasmid. These results suggest
that IncI1 plasmid carriage in reused litter posed a fitness cost on the bacterial host, but this cost was ameliorated by maintaining IncI1 at low copies in our study.

It is important to mention that the litter used for this study is not representative of all broiler litter. Litter compositions may differ between farms, and there are multiple physiochemical factors that can affect the litter microbiome: e.g., moisture and pH, antibiotic usage, and feed additives. Furthermore, the age of the litter (i.e., numbers of flocks that have been grown on the litter) and the length of the downtime between flocks are examples of management practices that may influence microbiome succession. Nonetheless, our study showed that the litter microbiome significantly affects the bacterial diversity of broiler chicks and the HGT of AMR.

MATERIALS AND METHODS

Details of methods used for preparing the S. Heidelberg 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 litter (7). We briefly redescribe these methods and present others below.

**Determining if post-hatch chicks were Salmonella free.** Chicks pads that conveyed chicks from the hatchery were preenriched in 500 mL of buffered peptone water (BPW) for 18 to 24 h at 37°C. Two different enrichment broths were used to isolate Salmonella from the BPW preenrichment broth: tetraphionate (TT) broth (Becton, Dickinson, Sparks, MD) and Rappaport-Vassiliadis (RV) medium (Becton, Dickinson). After overnight incubation at 42°C in both enrichment broths, 10-μL aliquots from each enrichment broth were spread on two different differential media—Brilliant green sulfa (BGS) agar (Becton, Dickinson) and xylose lysine tergitol-4 agar (XLT-4) (Becton, Dickinson)—and incubated for 18 to 24 h at 37°C. Isolated colonies characteristic of *Salmonella* were stabbed and streaked onto triple sugar iron agar (TSI) (Becton, Dickinson) and lysine iron agar fermentation (LIA) (Becton, Dickinson) and incubated for 18 to 24 h at 37°C for biochemical confirmation.

**S. Heidelberg inoculum preparation.** The S. Heidelberg strain was made resistant to 200 ppm of nalidixic acid (nalfl S. Heidelberg) for selective enumeration and was grown overnight in poultry litter extract, centrifuged, and resuspended in 1× phosphate-buffered saline (PBS). The resuspended cells were used as inocula. The complete genome of the ancestor to nalfl S. Heidelberg is available under GenBank accession no. CP066851.

**Challenging broiler chicks with nalfl S. Heidelberg.** One-day-old Cobb 500 broiler chicks were either uninoculated (n = 25), gavaged (n = 25), or (n = 25) cloacally inoculated with ~10^6 CFU of nalfl S. Heidelberg. We also included a seeder bird colonization method, whereby five chicks were gavaged and mingled with 20 uninoculated chicks. Afterwards, chicks were placed in floor pens at a stocking density of 0.65 m²/chick on fresh pine shavings (fresh litter, n = 100) or reused litter (n = 100). The reused litter was previously used to raise three flocks of broiler chickens 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 and reused litter were housed separately and were raised for 14 days on antibiotic-free starter diet and water. At 14 days, 45 chicks from each fresh litter and reused litter (10 chicks from the gavaged, cloacal, and uninoculated groups and 15 chicks from the seeder method [5 seeder and 10 contact chicks]) were euthanized to determine the extent of nalfl S. Heidelberg 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’s Office of Animal Care and Use under Animal Use Protocol A2017 04-028-A2.

**Determination of nalfl S. Heidelberg concentration in the ceca and litter.** Cecae were removed from the eviscerae of broiler chicks and stomached for 60 s after the addition of 3× volume to the weight (vol/vt) of BPW. Litter was collected as grab samples from seven locations (4 corners of the pen and 3 locations under the waterer) in each pen after chicks were removed. The litter samples were pooled, and 30 g was subsampled in duplicates from each pen as previously described (29). Serial dilutions of the cecal and litter slurry were plated onto BGS containing 200 ppm nalidixic acid, 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, preenriched cecal and litter slurry was streaked onto BGS agar supplemented with nalidixic acid and incubated overnight. These plates were then examined for the presence/absence of *Salmonella* colonies. Two to six single colonies were randomly selected from each plate and archived in 30% LB glycerol at −80°C. In addition, cecal slurry was saved at a 4:1 ratio in 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. Litter pH and moisture were determined as described previously (29).

**Antibiotic resistance phenotype determination.** Antibiotic susceptibility testing was done on S. Heidelberg 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. In addition, we used agar dilution to
determine the MIC of gentamicin for one S. Heidelberg isolate (SH-16-0.5B) (see methods in the supplemental material).

**Genomic, environmental, and plasmid DNA extraction.** Unless otherwise noted, DNA was extracted and purified from bacterial cultures by using FastDNA spin kit for soil (MP Biomedicals, LLC, CA, USA), while 250 mg of cecal slurry (previously saved in LB broth containing glycerol) and litter were extracted with the Qiagen DNeasy PowerSoil DNA kit (Hilden, Germany). Extracted DNA were used for qPCR, 16S rRNA gene sequencing, shotgun metagenomics, and whole-genome sequencing. Plasmid DNA was extracted from two S. Heidelberg isolates that harbored IncI1. Isolates were cultured overnight on sheep blood agar, and plasmid DNA was extracted using the Qiagen Plasmid Midi kit (Qiagen, Inc., Germantown, MD) as per the manufacturer’s instructions. Plasmid DNA was used to construct a calibration curve for IncI1 plasmid copy number determination.

**Real-time quantitative PCR.** Real-time qPCR amplification was performed as described previously (45) using a CFX96 Touch real-time PCR detection system (Bio-Rad, Hercules, CA). Reaction mixtures contained 1× SensiFast Universal SYBR green Supermix (Bio-Rad, Hercules, CA), 600 nM (each) primers, and 2 μL of DNA. The primers used in this study are shown in Table S3. Unless otherwise stated, primers were designed with Beacon Designer (Premier Biosoft, Palo Alto, CA) and synthesized by Integrated DNA Technologies (Coralville, IA). Calibration curves used for converting qPCR cycle threshold values to gene copies per gram of ceca were determined using the genomic DNA from an E. coli strain that harbored IncI1-pST26 plasmid. To convert qPCR cycle threshold values to IncI1 copies per cell of S. Heidelberg, plasmid DNA of a relevant S. Heidelberg strain harboring IncI1-pST26 was used for calibration curve construction (Table S3). Two primer sets specific to the gapA gene and the region upstream of the repA gene of IncI1, including incRNAi, were used to determine the gene copies of IncI1 in the ceca. gapA is a housekeeping gene carried on the chromosome of enteric bacteria, including E. coli, Klebsiella, Citrobacter, and Salmonella (32). The number of gene copies of IncI1 per cell was determined as the copy ratio of incRNAi to gapA (49).

**Whole-genome sequencing and analysis.** Whole-genome sequencing libraries were prepared using either Nextera XT or Nextera DNA Flex library preparation kits (Illumina, Inc., CA, USA) following the manufacturer’s protocol. Libraries were sequenced on the Illumina MiSeq platform with 150- or 250-bp paired-end reads. Additionally, five E. coli isolates were selected for long-read sequencing using the Sequel II system (PacBio Biosciences, Inc.) or MinION device (Oxford Nanopore Technology) (Table S4). 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 previously (7).

**Genome assembly, resistome characterization, and quality assessment of assemblies were done using** Reads2Resistome pipeline v.1.1.1 (50). For tools used for genome annotation and bacterial and plasmid typing, see the methods in the supplemental material. MAFFT v.1.4.0 (51), implemented in Geneious Prime v.2020.0.1, was used to align and compare sequences. Roary (52) was used for pangenome analysis, and phylogenetic trees were constructed using the maximum likelihood method implemented in RAxML (v.1.0.0) (53). Island Viewer (54) was used to predict genomic islands.

**Identification of single nucleotide polymorphisms and indels present in S. Heidelberg isolates.** Alignment of raw FASTQ reads to S. Heidelberg (GenBank accession no. CP016573) was done using BWA (v.0.7.17) (55), and SNPs/indels were called using the Genome Analysis Toolkit (56). Variant call format (VCF) files of SNPs/indels and the script used are available in the Dryad Digital Repository (https://doi.org/10.5061/dryad.cf661fg6c).

**16S rRNA gene community analysis.** The V4 hypervariable region of the 16S rRNA gene was sequenced using the paired-end (250 × 2) method on the Illumina MiSeq platform. The cecal (n = 59; 30 and 29 chicks from fresh litter and reused litter, respectively) and litter (n = 22; 11 samples each from fresh and reused litter) samples were part of a larger sequencing run and processed together with other samples. Thus, detailed sequence processing parameters are described in reference 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 5,000 reads were removed from the data set before further analysis.

**Statistical analysis of microbial communities was performed in the R environment using the packages “phyloseq,” “Amplisv2,” and “vegan.” Alpha diversity indices were calculated with a data set rarefied to the minimum sample size (8,740 sequences) and compared using the Wilcoxon signed-rank test. Principal-coordinate analysis (PCoA) based on Bray-Curtis distances was performed with no initial data transformation and after removing ASVs present with a relative abundance of less than 0.1% in any sample. The core microbiome was determined as ASVs with 0.1% relative abundance and present in at least 80% of samples.

**Cecal metagenome sequencing.** Two hundred fifty milligrams of cecal slurry from chicks cloacally challenged with S. Heidelberg and raised on fresh litter (n = 2) or reused litter (n = 2) was used to generate the shotgun library and Hi-C DNA library. For the shotgun library, cecal DNA was extracted with the Qiagen DNeasy PowerSoil DNA kit (Hilden, Germany), and the Nextera XT library preparation kit was used to make the library. The Hi-C library was made by the Phase Genomics (Seattle, WA, USA) ProxiMeta Hi-C microbiome kit following the manufacturer’s instructions. Libraries were sequenced by the Novogene Corporation (Sacramento, CA, USA) on the Illumina HiSeq platform using 150-bp paired-end reads. Two libraries were sequenced per HiSeq flow cell lane, resulting in a total of ~111 million shotgun reads, with ~213 million Hi-C reads per sample. Quality-controlled shotgun reads were classified using Kraken2 (v.2.0.8 beta) (43) to create a count profile of the metagenome. We used cumulative sum scaling implemented in metagenomeSeq to normalize read counts (57).
Metagenome-assembled genomes and associated plasmids and ARGs. The details of the methods used for proximity-guided metagenome assembly and deconvolution have been described before (7). Briefly, shotgun metagenomics sequence reads were filtered and trimmed for quality and normalized before assembly with metaSPAdes (58) using default options. Hi-C sequence reads were then aligned to the assembly following the ProxiMeta Hi-C kit manufacturer’s recommendations (https://phasegenomics.github.io/2019/09/19/hic-alignment-and-qc.html). Metagenome deconvolution was performed with ProxiMeta (59), resulting in the creation of putative genome and genome fragment clusters. Hi-C clusters were assessed for quality using CheckM (60) and assigned taxonomic classifications with Mash v.2.1.1 (61), resulting in the metagenome-assembled genomes (MAGs). We used PlasmidFinder (v.2.1.1) (62) to identify plasmids present in MAGs.

To view AMR gene connections to hosts, AMR genes in the metagenomic assembly were annotated using NCBI’s AMRFinderPlus software (version: v.3.10.5) with the –plus option specified and all other options set to defaults. The AMR assembly annotations were then used in combination with the MAG taxonomic annotations and the mobile element to host association matrices generated by ProxiMeta to annotate which AMR genes are present in each MAG, and specify whether the gene originated from a genomic, viral or plasmid contig. This was done by making an NxM matrix, C, where $\text{MAG} = |\text{AMR}|$ and $M = |\text{MAG}|$, where MAG = {set of individual metagenomic bins} and $\text{AMR} = \{x \in \text{AMR} : x \in \text{assembly}\}$, where AMR = {set of all AMR genes in database}. The matrix (C) was then filled as follows:

$$C_{ij} = \begin{cases} 
0 & \text{if } \text{AMR} \notin \text{MAG}, \\
S & \text{if } \text{AMR} \in \text{MAG}, \\
1 & \text{if } \text{AMR} \in \text{AMRg} \\
2 & \text{if } \text{AMR} \in \text{AMRv} \\
3 & \text{if } \text{AMR} \in \text{AMRp} 
\end{cases}$$

and where $\text{AMRg} = \{x \in \text{AMR} : x \in \text{genomic contigs}\}$, $\text{AMRv} = \{x \in \text{AMR} : x \in \text{viral contigs}\}$, $\text{AMRp} = \{x \in \text{AMR} : x \in \text{plasmid contigs}\}$. The matrix, C, was then filtered to remove rows and columns with only 0 values and plotted using a color-encoding system indicating whether the AMR gene was of genomic (cyan) or plasmid (gold) origin.

Functional pathway analysis. Both shotgun sequences and ProxiMeta-deconvoluted genomes were also utilized to assess enrichment of functional pathways. Shotgun metagenomic sequences were aligned to the Hi-C genomic contigs by using the BWA-MEM algorithm as implemented in BWA (v.0.7.17) (55). The Hi-C genomic contigs were then annotated using Prokka (v.1.13) (63). 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 (57). Enzyme commission identifiers were then mapped to KEGG functional pathways according to the KEGG ontology. For each pathway, an adjacency graph was reconstructed from the KEGG XML files, and connected components were determined using depth-first search (64). Z-scores were calculated for pathways and connected components for each sample, and statistical testing was then performed by comparing scores to the normal distribution. Details of this analysis are described in the methods in the supplemental material.

E. coli isolation from ceca. The method used for screening frozen fecal cecal contents has been reported previously (7). Briefly, two fecal slurries from challenged chicks raised on fresh litter and reused litter were spread plated on CHROMagar plates supplemented with or without gentamicin (8 ppm) and tetracycline (8 ppm) or ampicillin (16 ppm) and cefoxitin (16 ppm) (Thermo Fisher, Waltham, MA). After 24 h of incubation, blue-green and blue-cream colonies were counted as presumptive E. coli, and 4 to 5 colonies from each spread plate were picked and restruck for colony purification. No colonies grew on CHROMagar plates supplemented with ampicillin and cefoxitin. Colonies were preserved in LB broth containing glycerol and frozen before they were used for inclusion of a solid agar competition experiment.

Determination of plasmid copy number of IncI1. To compare the copy number of IncI1 harbored by S. Heidelberg strain (SH-16-0-5B) from reused litter to the copy number of IncI1 in three SH-IncI1-FL isolates from fresh litter, we exposed each isolate to acidified filter sterilized cecal extract (CE). CE was prepared from the cecal content of 2-week-old broiler chickens (see methods in the supplemental material). Cecal extracts were prerduced by covering tubes with a gas-permeable paper strip and incubating them overnight under microaerophilic conditions (5% O₂, 10% CO₂, and 85% N₂) at 42°C. Three single colonies of each strain were selected from overnight cultures grown on sheep blood agar and transferred to a microcentrifuge tube containing 900 μL of prerduced CE (pH 6.52): i.e., one tube for each SH-IncI1-FL strain and three tubes for SH-16-0-5B.

After transfer, tubes were vortexed, covered with a gas-permeable paper strip, and incubated at 41°C under microaerophilic conditions for 2 h. After incubation, tubes were vortexed and 100 μL of the suspension was transferred to a microcentrifuge tube containing 900 μL of CE (with the pH of CE adjusted to 2.52 using 1 M HCl [Spectrum Chemical Mfg. Corp., CA, USA] and 1 M NaOH [Fisher Chemical, NJ, USA]). Afterwards, tubes were vortexed, and incubation continued for another 24 h. To determine the copy number of IncI1, one replicate tube per strain was removed at time points 2 h for pH 6.5 and 0.5, 4, and 24 h for pH 2.5 and used for DNA extraction. DNA was extracted from 500 to 800 μL of CE samples using FastDNA Spin kit for soil. Calibration curves for gapA and incRNAI
were generated using genomic and plasmid DNA extracted from ancestral S. Heidelberg (GenBank accession no. CP066851) and one SH-IncI-FL isolate, respectively. The plasmid copy number of IncI was determined as the copy ratio of IncRNA to gapA (49).

**Statistical analyses.** Continuous variables were log transformed before any statistical tests were performed. Moreover, continuous variables did not meet the assumption of a normal distribution; therefore, nonparametric 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. Additionally, a generalized linear model with a binomially distributed outcome and a log link function was performed as described earlier (65) to determine if there are significant differences in *Salmonella* prevalence (presence/absence) and the litter used for raising broiler chicks. The significance of the model was established using a likelihood ratio test (R function ANOVA with argument test set to “Chisq”). Multiple comparisons of means (i.e., regression coefficients for route of inoculation and litter age/type) were done using the multcomp package in R. The mcp function was used to specify linear hypotheses, and the ght function was used to make Tukey contrasts. Statistical analyses were performed using R (v.4.0.3).

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

**Data availability.** All raw short and long FASTQ reads for *S. Heidelberg* are publicly available under NCBI accession no. PRJNA683658, while *E. coli* FASTQ reads are available under NCBI accession no. PRJNA684578. Shotgun and Hi-C reads from fresh litter and reused litter cecal samples are publicly available under NCBI accession no. PRJNA688069, and 16S rRNA gene sequences are available under NCBI accession no. PRJNA669215. The complete genome assemblies for *E. coli* strains Ec-FL1-1X and Ec-FL1-2X were previously published and are available under GenBank accession no. CP066836 and JAFCTRDR000000000. The complete genome of *E. coli* strain Ec-RL2-1X has been made available under GenBank accession no. CP066839. Variant call formats (VCF) files of identified SNPs/indels and the Linux/Unix shell script used have been deposited in the Dryad Digital Repository (https://doi.org/10.5061/dryad.c866t1g6c).

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**: XLSX file, 0.02 MB.

**SUPPLEMENTAL FILE 2**: PDF file, 1.8 MB.

**SUPPLEMENTAL FILE 3**: XLSX file, 0.02 MB.

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A.O. and Z.A. designed the study. A.O., G.Z., N.A.C., and C.R. performed live broiler chicken studies. M.J.R. Jr contributed to the design of experiments performed with *E. coli* strains. 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.L., J.R.G., C.W., J.C.T. IV, S.M.L., and R.W. performed bioinformatic analyses and data curation. A.O., J.L., and D.C. performed the solid agar mating experiment. A.O. and B.Z. performed statistical analyses. A.O., Z.A., N.A.C., B.Z., R.W., M.O.P., J.R.G., S.M.L., G.Z., and J.L. drafted the manuscript, which was reviewed and edited by all authors. A.O., Z.A., and S.E.A. supervised the study.
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