Chicken Meat-Associated Enterococci: Influence of Agricultural Antibiotic Use and Connection to the Clinic

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ABSTRACT Industrial farms are unique, human-created ecosystems that provide the perfect setting for the development and dissemination of antibiotic resistance. Agricultural antibiotic use amplifies naturally occurring resistance mechanisms from soil ecologies, promoting their spread and sharing with other bacteria, including those poised to become endemic within hospital environments. To better understand the role of enterococci in the movement of antibiotic resistance from farm to table to clinic, we characterized over 300 isolates of *Enterococcus* cultured from raw chicken meat purchased at U.S. supermarkets by the Consumers Union in 2013. *Enterococcus faecalis* and *Enterococcus faecium* were the predominant species found, and antimicrobial susceptibility testing uncovered striking levels of resistance to medically important antibiotic classes, particularly from classes approved by the FDA for use in animal production. While nearly all isolates were resistant to at least one drug, bacteria from meat labeled as raised without antibiotics had fewer resistances, particularly for *E. faecium*. Whole-genome sequencing of 92 isolates revealed that both commensal- and clinical-isolate-like enterococcal strains were associated with chicken meat, including isolates bearing important resistance-conferring elements and virulence factors. The ability of enterococci to persist in the food system positions them as vehicles to move resistance genes from the industrial farm ecosystem into more human-proximal ecologies.

IMPORTANCE Bacteria that contaminate food can serve as a conduit for moving drug resistance genes from farm to table to clinic. Our results show that chicken meat-associated isolates of *Enterococcus* are often multidrug resistant, closely related to pathogenic lineages, and harbor worrisome virulence factors. These drug-resistant agricultural isolates could thus represent important stepping stones in the evolution of enterococci into drug-resistant human pathogens. Although significant efforts have been made over the past few years to reduce the agricultural use of antibiotics, continued assessment of agricultural practices, including the roles of processing plants, shared breeding flocks, and probiotics as sources for resistance spread, is needed in order to slow the evolution of antibiotic resistance. Because antibiotic resistance is a global problem, global policies are needed to address this threat. Additional measures must be taken to mitigate the development and spread of antibiotic resistance elements from farms to clinics throughout the world.

KEYWORDS agriculture, antibiotic resistance, antimicrobial resistance, *Enterococcus*, food safety, molecular epidemiology, poultry, resistance, virulence

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Antibiotics have been widely used in industrial livestock production for the last 60 years, both to treat infections and to promote animal growth (1). The latter, which entails feeding animals subtherapeutic doses of sometimes medically important antibiotics, leads to the emergence and spread of bacteria that are resistant to these antibiotics (2). The spread of drug resistance is further promoted among livestock through animal crowding and can eventually pass to humans through the introduction of resistant bacteria into human ecologies, either through direct contact with farm workers or on meat products that are contaminated during processing.

Increasingly, studies have linked antibiotic use in agricultural settings with drug-resistant bacteria isolated from humans. These include studies showing connections between methicillin-resistant *Staphylococcus aureus* isolates in swine, pig farmers, family members of farmers, and clinical infections (3–8). Similar links have been established for enterococci. Widespread use of avoparcin, an antibiotic with a mode of action similar to that of the medically important drug vancomycin, led to selection for vancomycin resistance in animal-associated enterococci in the late 20th century (9), which subsided after agricultural avoparcin use was banned in Europe (10–12). In previous work, we showed that epidemic, multidrug-resistant, hospital-adapted *Enterococcus faecium* strains evolved from a common ancestor with animal-associated strains, rather than human commensal strains, highlighting the close association between enterococci in domesticated animals and the clinic (13, 14).

Pointing to a likely route from farm to clinic, previous studies have shown that drug-resistant bacteria often contaminate meat products that are sold to consumers. These studies have identified food-associated bacterial lineages with high similarity to clinical strains, including multidrug-resistant *Klebsiella pneumoniae*, *Escherichia coli*, and *S. aureus* (15–17). In 2013, Consumers Union scientists sampled over 300 raw chicken breasts purchased at supermarkets around the United States (18). A follow-up study of *E. coli* contamination in these samples revealed the presence of isolates closely related to pathogenic clones and containing numerous virulence and drug resistance genes (16). Enterococci were the most common bacterial contaminants identified on chicken meat from this study, being found in 75% to 84% of the 316 chicken breasts sampled across different brands (18), although the specific species found were not reported at the time. Furthermore, it was observed that more than a quarter of *Enterococcus* isolates were resistant to two or more classes of antibiotics and that high levels of antibiotic resistance were detected in meat labeled as raised without antibiotics or as organic (18).

Using genomic and molecular tools, as well as additional antibiotic susceptibility testing, we further characterized the Consumers Union collection of chicken-meat associated enterococci (CUE collection) (18) to investigate their drug resistance and genetic relationships, including phylogenetic and gene-based comparisons with known clinical isolates. We confirmed the finding published previously in *Consumer Reports* magazine (18) that isolates were frequently resistant to clinically relevant antibiotics and that, for *E. faecium* in particular, isolates from conventionally raised chicken meat were correlated with increased resistance, particularly to drugs used in animal production. We found that chicken meat-associated enterococci included both commensal- and clinical-strain-like isolates, with some isolates having close relationships to disease-causing strains and harboring multiple virulence factors. This suggested that these food-associated strains could represent stepping stones for entry into the clinic by both direct transmission and collection points for resistance for transmission into enterococci already present in human ecologies. Further, our comparisons of isolates derived from chicken meat from across the country revealed instances of very closely related isolates, both with and without geographic links, raising questions of the mechanisms of spread of resistant organisms and their genes in food production.

RESULTS

Enterococci isolated from chicken meat were primarily *Enterococcus faecalis* or *E. faecium*, regardless of geography or rearing practice. We applied 16S rRNA

FIG 1 CUE isolates were primarily *E. faecalis* and *E. faecium*. (A) Breakdown of all 333 CUE isolates by species (determined by 16S rRNA gene sequencing). See Table S1 for strain counts for each species. (B) Whole-genome phylogeny of 92 newly sequenced CUE isolates, in the context of additional previously published strains (13). Abx, antibiotics.

sequence typing to 333 *Enterococcus* isolates from the CUE collection isolated from supermarket raw chicken breasts (18) (see Materials and Methods). The majority of isolates were typed as either *E. faecalis* (50.1% [170 strains]) or *E. faecium* (41.7% [139 strains]) (Fig. 1A; see also Table S1 in the supplemental material). We also identified 11 strains of *Enterococcus cecorum* (3.3%), 11 isolates of *Enterococcus hirae* (3.3%), and one strain each of *Enterococcus durans* and *Enterococcus gallinarum* (0.3% for each). Both *E. faecalis* and *E. faecium* were broadly distributed among chicken meat raised either with or without antibiotics and from diverse purchase and processing locations (Tables S2 to S6).

E. cecorum isolates were only observed on meat processed in Georgia and Missouri, and both *E. gallinarum* and *E. durans* were observed in meat processed in Virginia and

FIG 2 Resistance to drugs that are medically relevant for humans was common. Drug names shown in red are part of antibiotic classes used in animal rearing, except tigecycline and chloramphenicol; tigecycline and chloramphenicol are not used in animal rearing, but drugs from the same classes as these are used in animal rearing. Abx, antibiotics.

sold in Maine. We also detected two nonenterococcal species, which likely resulted from their ability to grow on selective media used to enrich for enterococci (18) (see Materials and Methods). Analysis of a single-copy core-genome phylogeny (Fig. 1B) (see Materials and Methods) produced from sequencing and assembling the genomes of a diverse subset of 92 CUE strains, selected to represent diverse species, resistance profiles, state of purchase, and packaging claims (see Table S7), combined with data from previously published genomes representing the diversity of the *Enterococcus* genus (19), confirmed 16S rRNA-based predictions. The results from this analysis also showed that enterococci from chickens raised under different conditions of antibiotic use were not phylogenetically resolved based on source, i.e., isolates from products raised under both regimes were scattered throughout the phylogenetic tree regardless of antibiotic use.

High levels of resistance to antibiotics used in animal production observed in isolates from chicken meat. In order to obtain a more complete view of resistance across the CUE collection, we challenged the entire collection of 322 enterococci with a diverse panel of 15 compounds (12 of 14 used in the original study [18], plus virginiamycin, sulfamethazine, and lincomycin), based on their use either in chicken rearing or to treat humans (Table S8). In agreement with earlier results (18), these isolates displayed high levels of antibiotic resistance (Fig. 2 and Tables S1b and S9), with CUE strains exhibiting, on average, resistance to 4.8 ± 2.0 antibiotics (range, 0 to 11 resistances). The vast majority of the observed resistance was to drugs used in animal production, though many were also medically important and included three for which over half of the isolates were resistant, lincomycin (93% of isolates), tetracycline (61%), and erythromycin (53%).

For a subset of the medically relevant drugs, we were able to compare rates of resistance among CUE isolates with those observed among isolates from the SENTRY study, which examined resistance in almost 50,000 clinical *Enterococcus* isolates from 298 medical centers worldwide (20). Compared to the SENTRY study (see Materials and Methods), CUE isolates had lower rates of resistance to vancomycin (0.3% versus 21%), chloramphenicol (0.3% versus 10.3%), linezolid (0% versus 0.4%), and tigecycline (0% versus 0.8%) (Table S9), drugs not used in animal production. CUE isolates had similar rates of resistance to tetracycline (61% versus 75%), a common agricultural antibiotic. Surprisingly, CUE isolates had higher rates of resistance to daptomycin (2.5% versus 0.4%) despite daptomycin not being used in animal rearing. Daptomycin-resistant CUE

FIG 3 *E. faecium* isolates from chickens raised without antibiotics had significantly less phenotypic resistance than those from chickens raised conventionally. The difference was not significant for *E. faecalis*. The number of bacterial samples in each category is shown in parentheses. *P* values were computed using a two-sided Student *t* test.

isolates included seven *E. faecium* isolates and one *E. faecalis* isolate that were all found to have daptomycin MICs of 8 µg/ml, placing them just above the recommended breakpoint for nonsusceptibility.

***E. faecium* isolates were more drug resistant than were *E. faecalis* isolates, particularly when isolated from conventionally raised meat.** Overall, enterococci from meat raised without antibiotics had slightly less phenotypic drug resistance than enterococci from conventionally raised meat (4.6 ± 1.8 versus 5.0 ± 2.0 phenotypic resistances, respectively; Table S10). The *E. faecium* strains within our collection exhibited the most striking difference (4.5 ± 1.9 versus 6.0 ± 2.0 resistances, respectively; $P = 0.008$; Fig. 3). In contrast, there was very little difference in resistance for *E. faecalis* isolates based on antibiotic use.

The increased resistance among *E. faecium* strains from chickens raised conventionally was due to a broad increase in resistance to drugs used in animal production (either for treatment of infections or growth promotion), including seven drugs that are also medically important (Fig. 4), as follows: lincomycin, erythromycin, tetracycline, ciprofloxacin, gentamicin, kanamycin, and daptomycin (used in second-line treatment of patients). It was also notable that enterococci from chickens raised without antibiotics never showed resistance to daptomycin or vancomycin; in contrast, nine isolates (3%) from conventionally raised chickens carried resistance to these second-line drugs used to treat antibiotic-resistant human infection (Table S11, $P = 0.025$).

Most CUE isolates are not from genetic backgrounds commonly associated with hospital-adapted enterococci, but some show striking similarities to known clinical isolates. As previous sequence-based studies of commensal and hospital-adapted isolates have provided in-depth understanding of the genetics and epidemiology of hospital-adapted *E. faecium* and *E. faecalis* isolates (13, 19, 21, 22), we sought to place the CUE collection within this context by analyzing their phylogenetic placement and virulence gene content relative to isolates from these other studies (Fig. 5 and S1 and Table S12) (see Materials and Methods). To create the highest-resolution views of the relationships between previously sequenced enterococci and the CUE collection, we generated species-specific phylogenetic trees. The *E. faecalis* species phylogeny (Fig. 5A) revealed that CUE *E. faecalis* isolates were broadly distributed across the phylogeny, with the majority (25 of 32 isolates) occurring in sequence types (STs) that were absent or rare among previously sequenced isolates from the NCBI, which was biased toward problematic clinical strains. However, seven CUE isolates were

FIG 4 The increase in resistance in *E. faecium* from chickens grown conventionally is due to broadly higher resistance to drugs used in animal rearing, including seven medically important drugs (high-lighted in yellow). Antibiotics in red represent drugs used in animal rearing, and antibiotics with an asterisk represent those in the same class as drugs used in animal rearing.

located within STs and clonal complexes (CCs) associated with strains endemic to the hospital, including CC40, CC21, and ST16 (23–25). In fact, six CUE isolates differed by 34 to 100 single-nucleotide polymorphisms (SNPs) in the species single-copy core (SCC) genome (Table 1), indicating high genetic identity to clinical strains. Some of these CUE isolates were resistant to numerous drugs, including to clinically important drugs.

Consistent with their placement in lower virulence-associated STs, CUE *E. faecalis* isolates had fewer known virulence factors (26) than did previously sequenced *E. faecalis* isolates of both animal and clinical origins (1.3 ± 0.8 versus 2.6 ± 1.0 , respectively; $P < 0.001$) (see Materials and Methods; Tables S13b and S14 and Fig. S2). Of the five virulence factors that we searched for, four were identified among a subset of the 32 CUE *E. faecalis* isolates, with gelatinase, a zinc metalloprotease that contributes to virulence through the degradation of a number of host-associated substrates such as complement, collagen, fibrinogen, and LL-37, encoded within nearly all isolates (91%). The other three virulence factors were less frequent (Table S14b) but were sometimes found in combination. For instance, *E. faecalis* 262EA1, which exhibited a high degree of similarity to clinical strain T12 (Table 1), contained four virulence factors, including the adhesin PrgB, cytolysin, gelatinase, and the well-studied pathogenicity island (PAI) (27, 28), along with resistance to lincomycin, virginiamycin, sulfamethazine, and intermediate-level resistance to erythromycin.

The *E. faecium* species phylogeny (Fig. 5B) indicated that chicken meat-associated *E. faecium* isolates belonged primarily to the known animal-associated clade A2 (38 of 43 isolates; enrichment $P < 0.0001$, Fisher's exact test), and the closest core-genome SNP distance between a CUE strain and a previously sequenced clade A2 clinical strain was 183 SNPs. They were less frequently associated with the human commensal-associated

FIG 5 Expanded species-specific phylogenies. (A) *E. faecalis* phylogeny including 32 newly sequenced CUE isolates, together with 149 additional *E. faecalis* strains (Table S4). (B) *E. faecium* phylogeny, including 43 newly sequenced CUE isolates, together with 73 previously published strains reported in Lebreton et al. (13) (Table S4).

clade (clade B) and were never found within the known multidrug-resistant, hospital-adapted clade (clade A1). We observed no known *E. faecium* virulence factors in the sequenced CUE *E. faecium* isolates, whereas 13% of the NCBI *E. faecium* strains (primarily from clade A1) contained virulence factors (Table S13c).

Mobile element differences between closely related isolates indicate the swift effects of antibiotic exposure on gene content. Differences in the mobilome and resistome contents between closely related isolates can indicate the action of different selective pressures, such as exposure to antibiotics (29–31). Genome-wide comparisons

TABLE 1 CUE *E. faecalis* isolates closely related to clinical isolates^a

Label ^b	ST	CUE genome(s)	Closely related genome(s)	Origin of closely related sample	No. of SNPs ^c
A	21	2EA1, 76EA1	12107	Clinical	81–87
B	21	52EA1	Com_6	Commensal	70
C	/	72EA1, 182EA1	9 isolates from gentamicin-resistant outbreak of porcine origin (25), BM4539 (66), EFCS_D6_V2	Clinical and animal (pig)	56–92
D	355	294EA1 ^d	T12	Clinical	34

^aClosely related defined as <100 SNPs. /, no ST determination available.

^bIdentifier in Fig. 2.

^cNumber of SNPs differing within the single-species single-copy core.

^dIsolate from organic chickens raised without antibiotics.

TABLE 2 Pairs of closely related CUE isolates^a

Label ^b	Species	Isolate 1			Isolate 2			No. of SNPs ^c
		Name	State purchased	State processed	Name	State purchased	State processed	
E	<i>E. faecalis</i>	2EA1	MA	VA	76EA1	ME	VA	6
F	<i>E. faecium</i>	46EA1	TX	TX	79EA1	VA	DE	7
G	<i>E. faecium</i>	7EA1	PA	DE	86EA1	FL	GA	4
H	<i>E. faecium</i>	316EA1 ^d	CO	TX	306EA1	CO	TX	5
I	<i>E. faecium</i>	78EA1	VA	DE	205EA1	ME	DE	15
J	<i>E. faecium</i>	67EA1 ^e	MD	GA	163EA1 ^f	AR	MS	8

^aClosely related defined as ≤ 15 SNPs. Matching states of purchase or processing are shaded in gray.

^bIdentifier in Fig. 2 and 3.

^cNumber of SNPs differing within the single-species single-copy core.

^dChloramphenicol acetyltransferase (CAT) resistance gene present in 316EA1 but not in 306EA1.

^eIsolate from organic chickens raised without antibiotics.

^f163EA1 was isolated from meat raised using antibiotics, whereas 67EA1 was not. 163EA1 contains aminoglycoside nucleotidyltransferase (ANT), erythromycin (Erm), CAT, and tetracycline (Tet) resistance genes not present in 67EA1.

of sequenced CUE isolates with very small differences in their single-copy core genomic regions revealed notable differences in predicted plasmid content (see Materials and Methods; Table 2). Perhaps unsurprisingly, plasmids that varied between isolates harbored antibiotic resistance genes, predicted using the CARD database (Table S13a) (32). For example, isolates 163EA1 and 67EA1, while differing by only eight SNPs in their SCC, also differed by almost 200 kb in their predicted plasmid content. Isolate 163EA1 was cultured from conventionally raised meat and carried a predicted plasmid sequence, as well as an eight-gene *Tn4011*-related region inserted into its chromosome; together, these encoded resistance to four antibiotic classes (aminoglycosides, chloramphenicol, tetracycline, and macrolides), none of which were present in the genome of the very closely related isolate from meat raised without antibiotics, 67EA1 (Table 2). Based on whole-genome alignments (see Materials and Methods), we predicted that resistance was gained (or lost) due to changes in both plasmid content and transposable elements in the chromosome, and it happened on the time scale required for fewer than eight SNPs to accrue.

Closely related CUE isolates on meat processed in the same state. We noted six instances of very closely related pairs of CUE isolates from different meat samples that differed by only 4 to 15 SNPs in their core genome content, indicating that they emerged very recently from a common ancestor (Table 2). Based on recent estimated evolution rates for enterococci of approximately 0.5 to 16 SNPs/genome/year, (21, 33, 34), we predicted that these pairs diverged between 3 and 90 months ago. Three of the pairs were linked by the state in which the meat was processed, including two that were not linked by the state of purchase (2EA1/76EA1 and 78EA1/205EA1) (Table 2), indicating a common source of these highly related pairs on the farm or site of processing, rather than site of purchase. For the other three pairs, including 67EA1/163EA1 and 46EA1/79EA1, there was no geographic link for either purchase or processing location.

DISCUSSION

In this study, we conducted an in-depth analysis of *Enterococcus* bacteria isolated from supermarket chicken meat collected by the Consumers Union in 2013 (18). We found that most of the isolates cultured from chicken meat belonged to *E. faecalis* and *E. faecium*, the two species most commonly implicated in human enterococcal infections (35). Bird-associated enterococcal species, including *E. gallinarum*, *E. casseliflavus*, *E. avium*, and *E. cecorum*, were rarer among supermarket chicken meat, though this might be explained by the fact that (i) both *E. faecalis* and *E. faecium* can be found in many animal species, including birds (19); (ii) domesticated chickens raised on industrial farms, sourced from antibiotic-treated eggs, and fed standardized feed may lack a “natural” avian microbiota where these species would be expected; or (iii) *E. faecium* and *E. faecalis* are better able to survive harsh conditions than are other species (19), which could enrich for their presence on grocery chicken meat. Regardless of the

reason, *E. faecalis* and *E. faecium* are, by far, the two enterococcal species most commonly associated with human infection, so it is of note that these are also widespread on our food.

Our work adds to the growing literature examining the movement of bacteria from farm to table via commercially purchased meat. Prior studies have shown these connections in detail, in both Gram-negative and Gram-positive species, including enterococci (7, 9, 15–17). In a recent study of *E. coli* isolated from the same chicken breasts as our study (16), roughly 10% of chicken meat-associated *E. coli* isolates contained extraintestinal pathogenic virulence genes, and 20% of these belonged to a highly drug-resistant and unusually pathogenic lineage. The authors found no differences in virulence gene contents comparing *E. coli* isolates from meats raised with and without antibiotics, but they did show that labeling indicating that chickens were raised without antibiotics predicted less antibiotic resistance. As in the *E. coli* study, we also observed (i) no connection between virulence gene content and the rearing practice used to create the meats from which the enterococci were isolated and (ii) significantly more antibiotic resistance in enterococci cultured from chicken meats raised without antibiotic-free claims (including organic), particularly for *E. faecium*. The impact of rearing practice was particularly striking in a comparison of the whole genomes of two very closely related isolates of *E. faecium* cultured from meat with different antibiotic use claims. Despite a difference of only eight SNPs in their core genomes, the isolate from conventionally raised meat had greater plasmid and antibiotic resistance gene content, which suggests that agricultural antibiotics are a strong selective pressure driving rapid adaptation toward increasingly resistant organisms.

While it is well known that enterococci are intrinsically resistant to a wide variety of commonly used antibiotics (36), a large number of CUE isolates were also resistant to high levels of aminoglycosides, as well as lincosamide, macrolide, sulfonamide, and tetracycline antibiotics. All of these antibiotic classes are currently approved for use in food-producing animals (37). We observed significantly more resistance to these drugs among *E. faecium* strains isolated from conventional farms than in those claiming not to use antibiotics, as well as more resistance in general among the *E. faecium* isolates than with isolates of *E. faecalis*. This is in agreement with previous studies (38–40) which have shown that resistance against antibiotics used solely for growth promotion was more prevalent in *E. faecium* than in *E. faecalis*. Fortunately, we observed little resistance to second-line clinical agents reserved for treating antibiotic-resistant enterococcal infection, except for daptomycin resistance, which was 6-fold higher than that in hospitals (2.5% of strains), or to other drugs not used in agriculture, which may be a risk, as shared resistance genes can be linked on mobile genetic elements (29). No isolate exhibited resistance to linezolid or tigecycline, and the only isolate testing nonsusceptible to vancomycin was an *E. gallinarum* isolate, which harbored the *vanC* operon that confers low-level resistance.

Although chicken-derived enterococci had less overall resistance than did clinical isolates from the SENTRY study, the resistant isolates derived from chicken meat included some that were closely related to hospital-adapted clinical isolates, including shared high virulence factor content. Thus, circulating among chicken meat-associated isolates are strains with the potential to become problematic hospital-adapted strains, highlighting that antibiotic use in agricultural settings can foster the proliferation of strains of potential hazard to human health. Their presence at the consumer end of the retail food chain putting them in frequent, close proximity to humans heightens this risk.

Agricultural practices need to be reviewed in light of the growing body of data on the evolution and spread of antibiotic resistance elements and resistant strains from farms to consumers and clinics throughout the world. Many details remain unknown regarding how farm practices contribute to the movement of bacteria and drug resistance genes between agricultural and human ecologies. We observed examples of *E. faecium* isolates with very close relationships originating in meat from the same state, highlighting the possible involvement of processing plants in the widespread distribution of resistant bacteria by cross-contaminating meats from different farms. Additional

geographic similarities in the processing locations of less commonly isolated *Enterococcus* species also point to the possibility of processing plants as a source of bacterial contamination (Fig. S1). It should be noted, however, that we do not know the identities of the processing plants other than the state where they were located. Nonetheless, in agreement with the hypothesis that processing plants could play a role in contamination, a 2015 study of *Campylobacter jejuni* contamination in poultry slaughterhouses found that animal-processing practices heavily contaminate the slaughterhouse environment, leading to possible cross-contamination (41). Routine decontamination of processing facilities may be insufficient to eradicate enterococci, which are known to be extremely hardy and tolerant of desiccation, starvation, and disinfectants (19). Thus, a broad commitment to limiting or ending antibiotic use in food animal rearing is needed to eliminate the risk of wide dissemination of antibiotic resistance in consumer foods.

Additional work is needed to elucidate the origins of closely related isolates from our study that were not linked by geographic proximity of supermarkets or state of processing. Besides contamination at the processing facility, additional explanations for the observed close relationships between isolates include the possibility that chickens were purchased from common chicken vendors that distribute fertilized eggs, which in turn originate from common breeding flocks that are often treated with antibiotics (42). The use of commercial probiotics in chicken rearing may also have a role, particularly since it has been noted that *E. faecium* strains used as probiotics for agriculture can contain antimicrobial resistance (AMR) genes (43). Finally, we do not know the locations where the chickens were farmed, and it is possible that additional links exist between the geographies of the source farms. One of the main limitations of our study is that we focused only on enterococci isolated from chicken meat and were not able to assess species distributions or levels of antibiotic resistance in enterococci isolated directly from chicken manure on farms or from the processing plants themselves. An examination of additional isolates from these sources would help elucidate the origins and movement of the bacteria and resistance elements we observed among chicken meat-associated isolates.

Increasing levels of drug resistance among bacterial isolates causing infections in humans have raised concerns that we could soon enter a period where there are no effective antibiotics available for many life-threatening infections. In response to this threat, the U.S. Food and Drug Administration has implemented new rules aimed at curtailing the use of antibiotics for growth promotion in livestock in the United States (44) and has recently put together a 5-year plan to further this goal (45). As antibiotic use is changing in the United States, it would be worthwhile to reassess the amount of antibiotic resistance in enterococci or other clinically important bacterial species cultured from supermarket meat after these new rules have been put into effect. While new regulations should lead to a reduction in antibiotic use in the United States, antibiotic resistance is a global problem. Additional measures need to be implemented to mitigate the development and spread of resistance elements from farms to tables and clinics throughout the world.

MATERIALS AND METHODS

Sampling of chicken meat. The Consumers Union anonymously purchased 316 packages of raw chicken from supermarkets over the course of 10 weekdays from Monday, 8 July 2013, to Tuesday, 23 July 2013, in 26 states geographically distributed throughout the country (AL, AR, AZ, CA, CO, IA, ID, IL, FL, KY, KS, LA, MA, MD, ME, MI, MN, NC, NJ, NY, OH, PA, TN, TX, VA, and WA). Chicken samples (including skinless boneless chicken breasts, thin-sliced breasts, breast tenderloins, and skin-on-bone-in breasts) were purchased at different store types (major national grocery chains, big box stores, and regional grocery stores) and from different brands (four major brands, including Tyson, Pilgrim's Pride, Sanderson Farms, and Perdue; nonmajor brands, including store brands and minor brands; as well as organic brands and brands that claimed to use no antibiotics). Samples were shipped overnight to the testing lab (18).

For this study, we had metadata on organic versus nonorganic and raised without antibiotics versus conventional for 234 of the isolates. Meat was classified as organic if it was labeled as "organic," "organic vegetarian feed," or "organic vegetarian diet." Meat was labeled as nonorganic if it had none of these labels. Meat was classified as raised without antibiotics if it was either organic or labeled as "no antibiotics ever," "USDA process verified no antibiotics ever," or "no antibiotics in the feed, the water, or the ova (egg)."

Isolation of enterococci. Isolation of enterococci was performed using methods similar to those previously published (40). Chicken parts were placed into a sterile bag with buffered peptone solution and vigorously shaken for 30 s. Fifty milliliters of double-strength Enterococcosel broth was added with 50 ml of rinsate, mixed thoroughly, and incubated at 45°C for 18 to 24 h. If no typical growth or blackening was observed in the flask, the sample was considered negative. If blackening of the broth was observed, a loopful was streaked for isolation onto an Enterococcosel agar plate (EAP) and incubated at 35°C for 18 to 24 h. If no typical growth was observed on the EAP, the sample was considered negative. If *Enterococcus*-like growth was present, one well-isolated colony was streaked for isolation onto a blood agar plate (BAP) and incubated at 35°C for 18 to 24 h in ambient air. Presumptive *Enterococcus* isolates were subsequently frozen at –70 to –80°C in brucella broth with 20% glycerol and shipped in cryovials on dry ice to the Food & Drug Administration Center for Veterinary Medicine (FDA/CVM). Upon arrival at the FDA/CVM, every isolate was streaked for purity on a BAP before being confirmed as *Enterococcus* using the Vitek 2 Compact microbial identification system (bioMérieux, Hazelwood, MO). Multiple isolates were obtained from 32 of the 316 chicken samples (10%). These are indicated in the naming scheme for samples listed in Table S1.

16S rRNA sequence typing. Species designations were obtained for 339 isolates using 16S rRNA typing. 16S rRNA typing was conducted using universal primers (46). Approximately 1,500 nucleotides of the 16S rRNA locus were amplified using NEB *Taq* polymerase (New England BioLabs, Ipswich, MA) and the primers 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-AAGGAGGTGATCCAGCCGCA-3'. An aliquot of each PCR product was run on an agarose gel to confirm amplification, and then the purified products were submitted for Sanger sequencing (Genewiz, South Plainfield, NJ). Sanger sequences were compared to a database of known 16S rRNA sequences using BLAST (blastn with default parameters) to identify the species of each isolate. Three hundred thirty-three of the 339 isolates were found to be *Enterococcus*; 6 isolates were found to be from other genera, including five *Vagococcus lutrae* isolates and one *Paenibacillus lactis* isolate.

Antibiotic resistance phenotypes. Antimicrobial susceptibility testing was performed using a modified version of the CLSI-recommended protocol (47). Briefly, chicken meat-associated *Enterococcus* isolates were first inoculated into cation-adjusted Mueller-Hinton broth. After overnight growth at 37°C, approximately 10⁵ bacteria were inoculated into fresh broth containing either one or two predefined concentrations of each antibiotic. Antibiotic concentrations were chosen based on established clinical breakpoints (47) or from a literature review (48). Growth was assessed after 24 h by both visual inspection and by using a Synergy 2 microplate reader (BioTek, Winooski, VT). Eleven enterococcal isolates were excluded from testing because they were duplicates, did not grow after 24 h in Mueller-Hinton broth, or were suspected to be contaminated. The remaining 322 isolates were assessed for growth in 15 drugs at concentrations chosen to distinguish between sensitive, intermediate, nonsusceptible, and resistant phenotypes. The following drugs were used: chloramphenicol (growth at 8 µg/ml, intermediate; growth at 16 µg/ml, resistant), ciprofloxacin (growth at 1 µg/ml, intermediate; growth at 2 µg/ml, resistant), daptomycin (growth at 4 µg/ml, nonsusceptible), erythromycin (growth at 0.5 µg/ml, intermediate; growth at 4 µg/ml, resistant), gentamicin (growth at 500 µg/ml, high-level resistant), kanamycin (growth at 1 mg/ml, high-level resistant), lincomycin (growth at 2 µg/ml, intermediate; growth at 4 µg/ml, resistant), linezolid (growth at 2 µg/ml, intermediate; growth at 4 µg/ml, resistant), penicillin (growth at 8 µg/ml, resistant), sulfamethazine (growth at 256 µg/ml, resistant), tetracycline (growth at 4 µg/ml, intermediate; growth at 8 µg/ml, resistant), tigecycline (growth at 0.25 µg/ml, resistant), tylosin (growth at 8 µg/ml, resistant), vancomycin (growth at 4 µg/ml, intermediate; growth at 16 µg/ml, resistant), and virginiamycin M1 (growth at 2 µg/ml, intermediate; growth at 4 µg/ml, resistant).

Sequencing, assembly, and annotation. A total of 92 Consumers Union samples were selected for sequencing at the Broad Institute (referred as Consumers Union Enterococci, or "CUE isolates"). These isolates were selected to represent diverse species, resistance profiles, states of purchase, and packaging claims. For Illumina sequencing, both jumping libraries and 180-bp paired fragment libraries were prepared. Jumping libraries were constructed following the approach summarized by Ribeiro and coauthors (49), and paired fragment libraries were constructed as previously described (50). Assemblies were generated using ALLPaths-LG (51). To ensure consistency and to reduce artefacts among genomes, all genomes, including those from GenBank, were reannotated in a uniform manner using the Broad Institute's prokaryotic pipeline with the *Enterococcus*-specific approach (13). Briefly, protein-coding genes were predicted with Prodigal (52) and filtered to remove genes with ≥70% overlap to tRNAs or rRNAs. The tRNAs were identified by tRNAscan-SE (53), and rRNA genes were predicted using RNAmmer (54). Gene product names were assigned based on top BLAST hits against the Swiss-Prot protein database (≥70% identity and ≥70% query coverage), and protein family profiles were searched against TIGRfam HMMER equivalents. Additional annotation analyses performed include Pfam (55), TIGRfam (56), KEGG (57), and Enzyme Commission (EC) numbers (58).

Additional *Enterococcus* genomes included in comparative analysis. An additional 259 enterococcal genomes representing clinical, animal, and commensal habitats were included for comparative analysis, including isolates from the *E. faecium* comparative analysis reported by Lebreton et al. (13), the genus-wide comparative analysis reported by Lebreton et al. (19), and additional *E. faecalis* strains sequenced as part of the EnteroGenome project. Additionally, two outgroups were included (*Carnobacterium* and *Vagococcus* outgroups were also included), as well as two *Melissococcus* spp., which are nested within the phylogeny of the *Enterococcus* genus (19).

Bioinformatic analysis. Orthogroup clustering was performed using SynergyTwo, available at <https://sourceforge.net/projects/synergystwo/> (59), and single-copy core orthogroups were concatenated to generate phylogenetic trees using RAxML (60), using a general time reversible (GTR) substitution

model. One thousand bootstrapping replicates were performed using RAxML's rapid bootstrapping algorithm. Orthogroup clustering and phylogenetic tree construction were first performed using all 92 CUE isolates plus 42 diverse *Enterococcus* isolates selected by Lebreton et al. (19) and from the literature, resulting in 548 single-copy core orthogroups. This phylogenetic tree was used to verify species designations.

We divided the isolates up into species-specific groups representing the six different species present in the CUE isolates (*E. faecium*, *E. faecalis*, *E. hirae*, *E. cecorum*, *E. gallinarum*, and *E. durans*) and performed orthogroup clustering and phylogenetic tree construction separately for each species. We used the following six species-specific sets of isolates (Table S12): (i) 43 *E. faecium* CUE isolates plus 73 *E. faecium* isolates reported by Lebreton et al. (13) and from the literature, resulting in 1,322 single-copy core orthogroups; (ii) 32 CUE *E. faecalis* isolates plus 149 *E. faecalis* isolates from the Broad Institute EnteroGenome project and from the literature, resulting in 1,668 single-copy core orthogroups; (iii) 10 CUE *E. hirae* isolates plus 2 *E. hirae* isolates from a study by Lebreton et al. (19) and the literature, resulting in 2,039 single-copy core genes; (iv) 5 CUE *E. cecorum* isolates plus one *E. cecorum* isolate from Lebreton et al. (19), resulting in 1,595 single-copy core orthogroups; (v) 1 CUE *E. gallinarum* isolate plus two *E. gallinarum* isolates from Lebreton et al. (19) and the literature, resulting in 2,615 single-copy core orthogroups; and (vi) 1 CUE *E. durans* isolate plus two *E. durans* strains from Lebreton et al. (19) and the literature, resulting in 2,247 single-copy core orthogroups.

For the *E. faecium* and *E. faecalis*-specific phylogenies, we removed recombination to generate improved phylogenies. We used the Threaded Blockset Aligner (TBA) (61) to generate a whole-genome alignment and then used BratNextGen (62) to remove recombined regions. We generated the phylogenetic tree with RAxML using a GTR model and 1,000 bootstrapping replicates using the rapid bootstrap methodology (60). We constructed the small phylogenies for *E. durans* and *E. gallinarum* using FastTree (63). Trees were midpoint rooted. Plasmids scaffolds were predicted using PlasmidFinder (64).

ANI calculations were performed as in reference 19. Antibiotic resistance genes were predicted using the CARD database (32). The *E. faecalis* PAI was identified by looking for the presence of PAI modules in between the direct repeat sequence TGACTCTTAA in each genome (65). The *E. faecium* PAI was identified by the presence of the ESP locus (28). Other virulence factors were identified using the Broad Institute's prokaryotic annotation pipeline with the *Enterococcus*-specific approach (13), as well as a BLAST comparison to known MMH594 and V583 virulence factors.

Comparison to resistance data from SENTRY study. The resistance rates in CUE isolates were compared to the North American resistance rates published from the SENTRY study (20). The resistance rates for chloramphenicol, linezolid, tigecycline, and vancomycin were taken from Table 4 of a study by Pfaller et al. (20). If available, data from the closest time period from the SENTRY study (2013 to 2016, including data for linezolid and tetracycline) were used. If data from 2013 to 2016 were not available, data from the closest available time period (2005 to 2008 for chloramphenicol and 1997 to 2016 for tigecycline and daptomycin) were used. The resistance rates for tigecycline and daptomycin were taken from Table 5 of the study by Pfaller et al. (20).

Statistical comparisons. Unless otherwise stated, a Student *t* test was used to compute all *P* values for comparisons between different groups, and a Bonferroni correction was used, where applicable. Fisher's exact test (Bonferroni corrected) was used to compare the number of strains with and without specific virulence factors, as well as the enrichment of CUE strains within clade A2.

Data availability. The genome assemblies have been deposited at GenBank under BioProject number PRJNA244756. The assembly statistics and accession numbers for these 92 isolates can be found in Table S7. The accession numbers for all 259 additional strains used for comparative analyses can be found in Table S12.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01559-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 4, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 5, XLSX file, 0.4 MB.

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REFERENCES

- Gilchrist MJ, Greko C, Wallinga DB, Beran GW, Riley DG, Thorne PS. 2007. The potential role of concentrated animal feeding operations in infectious disease epidemics and antibiotic resistance. *Environ Health Perspect* 115:313–316. <https://doi.org/10.1289/ehp.8837>.
- Marshall BM, Levy SB. 2011. Food animals and antimicrobials: impacts on human health. *Clin Microbiol Rev* 24:718–733. <https://doi.org/10.1128/CMR.00002-11>.
- Armand-Lefevre L, Ruimy R, Andremonet A. 2005. Clonal comparison of *Staphylococcus aureus* isolates from healthy pig farmers, human controls, and pigs. *Emerg Infect Dis* 11:711–714. <https://doi.org/10.3201/eid1105.040866>.
- Voss A, Loeffen F, Bakker J, Klaassen C, Wulf M. 2005. Methicillin-resistant *Staphylococcus aureus* in pig farming. *Emerg Infect Dis* 11:1965–1966. <https://doi.org/10.3201/eid1112.050428>.
- Schinasi L, Wing S, Augustino KL, Ramsey KM, Nobles DL, Richardson DB, Price LB, Aziz M, MacDonald PD, Stewart JR. 2014. A case control study of environmental and occupational exposures associated with methicillin resistant *Staphylococcus aureus* nasal carriage in patients admitted to a rural tertiary care hospital in a high density swine region. *Environ Health* 13:54. <https://doi.org/10.1186/1476-069X-13-54>.
- Smith TC, Gebreyes WA, Abley MJ, Harper AL, Forshey BM, Male MJ, Martin HW, Molla BZ, Sreevatsan S, Thakur S, Thiruvengadam M, Davies PR. 2013. Methicillin-resistant *Staphylococcus aureus* in pigs and farm workers on conventional and antibiotic-free swine farms in the USA. *PLoS One* 8:e63704. <https://doi.org/10.1371/journal.pone.0063704>.
- Wardyn SE, Forshey BM, Farina SA, Kates AE, Nair R, Quick MK, Wu JY, Hanson BM, O'Malley SM, Shows HW, Heywood EM, Beane-Freeman LE, Lynch CF, Carrel M, Smith TC. 2015. Swine farming is a risk factor for infection with and high prevalence of carriage of multidrug-resistant *Staphylococcus aureus*. *Clin Infect Dis* 61:59–66. <https://doi.org/10.1093/cid/civ234>.
- Wulf MW, Sorum M, van Nes A, Skov R, Melchers WJ, Klaassen CH, Voss A. 2008. Prevalence of methicillin-resistant *Staphylococcus aureus* among veterinarians: an international study. *Clin Microbiol Infect* 14: 29–34. <https://doi.org/10.1111/j.1469-0691.2007.01873.x>.
- Nilsson O. 2012. Vancomycin resistant enterococci in farm animals – occurrence and importance. *Infect Ecol Epidemiol* 2:16959. <https://doi.org/10.3402/iee.v2i0.16959>.
- Aarestrup FM, Seyfarth AM, Emborg HD, Pedersen K, Hendriksen RS, Bager F. 2001. Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal enterococci from food animals in Denmark. *Antimicrob Agents Chemother* 45:2054–2059. <https://doi.org/10.1128/AAC.45.7.2054-2059.2001>.
- Klare I, Badstubner D, Konstabel C, Bohme G, Claus H, Witte W. 1999. Decreased incidence of VanA-type vancomycin-resistant enterococci isolated from poultry meat and from fecal samples of humans in the community after discontinuation of avoparcin usage in animal husbandry. *Microb Drug Resist* 5:45–52. <https://doi.org/10.1089/mdr.1999.5.45>.
- van den Bogaard AE, Bruinsma N, Stobberingh EE. 2000. The effect of banning avoparcin on VRE carriage in The Netherlands. *J Antimicrob Chemother* 46:146–148. <https://doi.org/10.1093/jac/46.1.146>.
- Lebreton F, van Schaik W, McGuire AM, Godfrey P, Griggs A, Mazumdar V, Corander J, Cheng L, Saif S, Young S, Zeng Q, Wortman J, Birren B, Willems RJ, Earl AM, Gilmore MS. 2013. Emergence of epidemic multidrug-resistant *Enterococcus faecium* from animal and commensal strains. *mBio* 4:e00534–13. <https://doi.org/10.1128/mBio.00534-13>.
- Palmer KL, Godfrey P, Griggs A, Kos VN, Zucker J, Desjardins C, Cerqueira G, Gevers D, Walker S, Wortman J, Feldgarden M, Haas B, Birren B, Gilmore MS. 2012. Comparative genomics of enterococci: variation in *Enterococcus faecalis*, clade structure in *E. faecium*, and defining characteristics of *E. gallinarum* and *E. casseliflavus*. *mBio* 3:e00318–11. <https://doi.org/10.1128/mBio.00318-11>.
- Davis GS, Waits K, Nordstrom L, Weaver B, Aziz M, Gauld L, Grande H, Bigler R, Horwinski J, Porter S, Stegger M, Johnson JR, Liu CM, Price LB. 2015. Intermingled *Klebsiella pneumoniae* populations between retail meats and human urinary tract infections. *Clin Infect Dis* 61:892–899. <https://doi.org/10.1093/cid/civ428>.
- Johnson JR, Porter SB, Johnston B, Thuras P, Clock S, Crupain M, Rangan U. 2017. Extraintestinal pathogenic and antimicrobial-resistant *Escherichia coli*, including sequence type 131 (ST131), from retail chicken breasts in the United States in 2013. *Appl Environ Microbiol* 83:e02956–16. <https://doi.org/10.1128/AEM.02956-16>.
- Waters AE, Contente-Cuomo T, Buchhagen J, Liu CM, Watson L, Pearce K, Foster JT, Bowers J, Driebe EM, Engelthaler DM, Keim PS, Price LB. 2011. Multidrug-resistant *Staphylococcus aureus* in US meat and poultry. *Clin Infect Dis* 52:1227–1230. <https://doi.org/10.1093/cid/cir181>.
- Anonymous. 2014. The high cost of cheap chicken: 97 percent of the breasts we tested harbored bacteria that could make you sick. *Learn how to protect yourself. Consumer Rep* 79:30–36.
- Lebreton F, Manson AL, Saavedra JT, Straub TJ, Earl AM, Gilmore MS. 2017. Tracing the enterococci from Paleozoic origins to the hospital. *Cell* 169:849–861.e13. <https://doi.org/10.1016/j.cell.2017.04.027>.
- Pfaller MA, Cormican M, Flamm RK, Mendes RE, Jones RN. 2019. Temporal and geographic variation in antimicrobial susceptibility and resistance patterns of enterococci: results from the SENTRY Antimicrobial Surveillance Program, 1997–2016. *Open Forum Infect Dis* 6:S54–S62. <https://doi.org/10.1093/ofid/ofy344>.
- Raven KE, Reuter S, Gouliouris T, Reynolds R, Russell JE, Brown NM, Torok ME, Parkhill J, Peacock SJ. 2016. Genome-based characterization of hospital-adapted *Enterococcus faecalis* lineages. *Nat Microbiol* 1:15033. <https://doi.org/10.1038/nmicrobiol.2015.33>.
- Arredondo-Alonso S, Top J, Schürch AC, McNally A, Puranen S, Pesonen M, Pensar J, Marttinen P, Braat JC, Rogers MR, van Schaik W, Kaski S, Corander J, Willems RJ. 2019. Genomes of a major nosocomial pathogen *Enterococcus faecium* are shaped by adaptive evolution of the chromosome and plasmidome. *bioRxiv* <https://doi.org/10.1101/530725>.
- Ruiz-Garbajosa P, Bonten MJ, Robinson DA, Top J, Nallapareddy SR, Torres C, Coque TM, Canton R, Baquero F, Murray BE, del Campo R, Willems RJ. 2006. Multilocus sequence typing scheme for *Enterococcus faecalis* reveals hospital-adapted genetic complexes in a background of high rates of recombination. *J Clin Microbiol* 44: 2220–2228. <https://doi.org/10.1128/JCM.02596-05>.
- Kawalec M, Pietras Z, Daniłowicz E, Jakubczak A, Gniadkowski M, Hryniewicz W, Willems RJL. 2007. Clonal structure of *Enterococcus faecalis* isolated from Polish hospitals: characterization of epidemic clones. *J Clin Microbiol* 45:147–153. <https://doi.org/10.1128/JCM.01704-06>.
- Larsen J, Schonheyder HC, Lester CH, Olsen SS, Porsbo LJ, Garcia-Migura L, Jensen LB, Bisgaard M, Hammerum AM. 2010. Porcine-origin gentamicin-resistant *Enterococcus faecalis* in humans, Denmark. *Emerg Infect Dis* 16: 682–684. <https://doi.org/10.3201/eid1604.090500>.
- Jett BD, Huycke MM, Gilmore MS. 1994. Virulence of enterococci. *Clin Microbiol Rev* 7:462–478. <https://doi.org/10.1128/cmr.7.4.462>.
- Shankar N, Baghdadyan AS, Gilmore MS. 2002. Modulation of virulence within a pathogenicity island in vancomycin-resistant *Enterococcus faecalis*. *Nature* 417:746–750. <https://doi.org/10.1038/nature00802>.
- Leavis H, Top J, Shankar N, Borgen K, Bonten M, van Embden J, Willems RJ. 2004. A novel putative enterococcal pathogenicity island linked to the esp virulence gene of *Enterococcus faecium* and associated with epidemicity. *J Bacteriol* 186:672–682. <https://doi.org/10.1128/jb.186.3.672-682.2004>.
- Hegstad K, Mikalsen T, Coque TM, Werner G, Sundsfjord A. 2010. Mobile genetic elements and their contribution to the emergence of antimicrobial resistant *Enterococcus faecalis* and *Enterococcus faecium*. *Clin Microbiol Infect* 16:541–554. <https://doi.org/10.1111/j.1469-0691.2010.03226.x>.
- Manson JM, Hancock LE, Gilmore MS. 2010. Mechanism of chromosomal transfer of *Enterococcus faecalis* pathogenicity island, capsule, antimicrobial resistance, and other traits. *Proc Natl Acad Sci U S A* 107: 12269–12274. <https://doi.org/10.1073/pnas.1000139107>.
- Palmer KL, Kos VN, Gilmore MS. 2010. Horizontal gene transfer and the genomics of enterococcal antibiotic resistance. *Curr Opin Microbiol* 13:632–639. <https://doi.org/10.1016/j.mib.2010.08.004>.
- Jia B, Raphenya AR, Alcock B, Waghechani N, Guo P, Tsang KK, Lago BA, Dave BM, Pereira S, Sharma AN, Doshi S, Courtot M, Lo R, Williams LE, Frye JG, Elsayegh T, Sardar D, Westman EL, Pawlowski AC, Johnson TA, Brinkman FS, Wright GD, McArthur AG. 2017. CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. *Nucleic Acids Res* 45:D566–D573. <https://doi.org/10.1093/nar/gkw1004>.
- Duchêne S, Holt KE, Weill FX, Le Hello S, Hawkey J, Edwards DJ, Fourment M, Holmes EC. 2016. Genome-scale rates of evolutionary change

- in bacteria. *Microb Genom* 2:e000094. <https://doi.org/10.1099/mgen.0.000094>.
34. Howden BP, Holt KE, Lam MM, Seemann T, Ballard S, Coombs GW, Tong SY, Grayson ML, Johnson PD, Stinear TP. 2013. Genomic insights to control the emergence of vancomycin-resistant enterococci. *mBio* 4:e00412-13. <https://doi.org/10.1128/mBio.00412-13>.
 35. Agudelo Higuera NI, Huycke MM. 2014. Enterococcal disease, epidemiology, and implications for treatment. In Gilmore MS, Clewell DB, Ike Y (ed), *Enterococci: from commensals to leading causes of drug resistant infection*. Massachusetts Eye and Ear Infirmary, Boston, MA.
 36. Hollenbeck BL, Rice LB. 2012. Intrinsic and acquired resistance mechanisms in enterococcus. *Virulence* 3:421–433. <https://doi.org/10.4161/viru.21282>.
 37. U.S. Food and Drug Administration. 2016. 2016 summary report on antimicrobials sold or distributed for use in food-producing animals. U.S. Food and Drug Administration, Silver Spring, MD. <https://www.fda.gov/media/109457/download>.
 38. Butaye P, Devriese LA, Haesebrouck F. 2001. Differences in antibiotic resistance patterns of *Enterococcus faecalis* and *Enterococcus faecium* strains isolated from farm and pet animals. *Antimicrob Agents Chemother* 45: 1374–1378. <https://doi.org/10.1128/AAC.45.5.1374-1378.2001>.
 39. Usui M, Ozawa S, Onozato H, Kuge R, Obata Y, Uemae T, Ngoc PT, Heriyanto A, Chalemchaikit T, Makita K, Muramatsu Y, Tamura Y. 2014. Antimicrobial susceptibility of indicator bacteria isolated from chickens in Southeast Asian countries (Vietnam, Indonesia and Thailand). *J Vet Med Sci* 76:685–692. <https://doi.org/10.1292/jvms.13-0423>.
 40. Hayes JR, English LL, Carter PJ, Proescholdt T, Lee KY, Wagner DD, White DG. 2003. Prevalence and antimicrobial resistance of enterococcus species isolated from retail meats. *Appl Environ Microbiol* 69:7153–7160. <https://doi.org/10.1128/aem.69.12.7153-7160.2003>.
 41. Gruntau I, Biasizzo M, Kusar D, Pate M, Ocepik M. 2015. *Campylobacter jejuni* contamination of broiler carcasses: population dynamics and genetic profiles at slaughterhouse level. *Food Microbiol* 50:97–101. <https://doi.org/10.1016/j.fm.2015.03.007>.
 42. Philpott T. 2014. Wait, we inject antibiotics into eggs for organic chicken? Mother Jones. <https://www.motherjones.com/food/2014/01/organic-chicken-and-egg-antibiotics-edition/>.
 43. Amachawadi RG, Giok F, Shi X, Soto J, Narayanan SK, Tokach MD, Apley MD, Nagaraja TG. 2018. Antimicrobial resistance of *Enterococcus faecium* strains isolated from commercial probiotic products used in cattle and swine. *J Anim Sci* 96:912–920. <https://doi.org/10.1093/jas/sky056>.
 44. U.S. Department of Health and Human Services, U.S. Food and Drug Administration, Center for Veterinary Medicine. 2013. CVM GFI #213. New animal drugs and new animal drug combination products administered in or on medicated feed or drinking water of food-producing animals: recommendations for drug sponsors for voluntarily aligning product use conditions with GFI #209. U.S. Food and Drug Administration, Silver Spring, MD. <https://www.fda.gov/regulatory-information/search-fda-guidance-documents/cvm-gfi-213-new-animal-drugs-and-new-animal-drug-combination-products-administered-or-medicated-feed>.
 45. FDA Center for Veterinary Medicine. 2018. Supporting antimicrobial stewardship in veterinary settings: goals for fiscal years 2019–2023. U.S. Food and Drug Administration, Silver Spring, MD. <https://www.fda.gov/files/animal%20&%20veterinary/published/Supporting-Antimicrobial-Stewardship-in-Veterinary-Settings-Goals-for-Fiscal-Years-2019-2023.pdf>.
 46. Frank JA, Reich CI, Sharma S, Weisbaum JS, Wilson BA, Olsen GJ. 2008. Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. *Appl Environ Microbiol* 74:2461–2470. <https://doi.org/10.1128/AEM.02272-07>.
 47. CLSI. 2018. Performance standards for antimicrobial susceptibility, 29th ed. CLSI document M100-ED28. Clinical and Laboratory Standards Institute, Wayne, PA.
 48. Solway S, Vincent L, Tian N, Woodford N, Bendall R. 2003. Isolation of streptogramin-resistant *Enterococcus faecium* from human and non-human sources in a rural community. *J Antimicrob Chemother* 52: 707–710. <https://doi.org/10.1093/jac/dkg389>.
 49. Ribeiro FJ, Przybylski D, Yin S, Sharpe T, Gnerre S, Abouelleil A, Berlin AM, Montmayeur A, Shea TP, Walker BJ, Young SK, Russ C, Nusbaum C, MacCallum I, Jaffe DB. 2012. Finished bacterial genomes from shotgun sequence data. *Genome Res* 22:2270–2277. <https://doi.org/10.1101/gr.141515.112>.
 50. Fisher S, Barry A, Abreu J, Minie B, Nolan J, Delorey TM, Young G, Fennell TJ, Allen A, Ambrogio L, Berlin AM, Blumenstiel B, Cibulskis K, Friedrich D, Johnson R, Juhn F, Reilly B, Shammas R, Stalker J, Sykes SM, Thompson J, Walsh J, Zimmer A, Zwirko Z, Gabriel S, Nicol R, Nusbaum C. 2011. A scalable, fully automated process for construction of sequence-ready human exome targeted capture libraries. *Genome Biol* 12:R1. <https://doi.org/10.1186/gb-2011-12-r1>.
 51. Gnerre S, MacCallum I, Przybylski D, Ribeiro FJ, Burton JN, Walker BJ, Sharpe T, Hall G, Shea TP, Sykes S, Berlin AM, Aird D, Costello M, Daza R, Williams L, Nicol R, Gnirke A, Nusbaum C, Lander ES, Jaffe DB. 2011. High-quality draft assemblies of mammalian genomes from massively parallel sequence data. *Proc Natl Acad Sci U S A* 108:1513–1518. <https://doi.org/10.1073/pnas.1017351108>.
 52. Hyatt D, Chen GL, Locascio PF, Land ML, Larimer FW, Hauser LJ. 2010. Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics* 11:119. <https://doi.org/10.1186/1471-2105-11-119>.
 53. Lowe TM, Eddy SR. 1997. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Res* 25:955–964. <https://doi.org/10.1093/nar/25.5.955>.
 54. Lagesen K, Hallin P, Rodland EA, Staerfeldt HH, Rognes T, Ussery DW. 2007. RNAmmer: consistent and rapid annotation of ribosomal RNA genes. *Nucleic Acids Res* 35:3100–3108. <https://doi.org/10.1093/nar/gkm160>.
 55. Finn RD, Tate J, Misty J, Coghill PC, Sammut SJ, Hotz HR, Ceric G, Forslund K, Eddy SR, Sonnhammer EL, Bateman A. 2008. The Pfam protein families database. *Nucleic Acids Res* 36:D281–D288. <https://doi.org/10.1093/nar/gkm960>.
 56. Haft DH, Selengut JD, White O. 2003. The TIGRFAMs database of protein families. *Nucleic Acids Res* 31:371–373. <https://doi.org/10.1093/nar/gkg128>.
 57. Ogata H, Goto S, Sato K, Fujibuchi W, Bono H, Kanehisa M. 1999. KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res* 27:29–34. <https://doi.org/10.1093/nar/27.1.29>.
 58. Tian W, Arakaki AK, Skolnick J. 2004. EFICAz: a comprehensive approach for accurate genome-scale enzyme function inference. *Nucleic Acids Res* 32:6226–6239. <https://doi.org/10.1093/nar/gkh956>.
 59. Wapinski I, Pfeffer A, Friedman N, Regev A. 2007. Automatic genome-wide reconstruction of phylogenetic gene trees. *Bioinformatics* 23: i549–i558. <https://doi.org/10.1093/bioinformatics/btm193>.
 60. Stamatakis A. 2006. RAXML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22:2688–2690. <https://doi.org/10.1093/bioinformatics/btl446>.
 61. Blanchette M, Kent WJ, Riemer C, Elnitski L, Smit AF, Roskin KM, Baertsch R, Rosenbloom K, Clawson H, Green ED, Haussler D, Miller W. 2004. Aligning multiple genomic sequences with the threaded blockset aligner. *Genome Res* 14:708–715. <https://doi.org/10.1101/gr.1933104>.
 62. Marttinen P, Hanage WP, Croucher NJ, Connor TR, Harris SR, Bentley SD, Corander J. 2012. Detection of recombination events in bacterial genomes from large population samples. *Nucleic Acids Res* 40:e6. <https://doi.org/10.1093/nar/gkr928>.
 63. Price MN, Dehal PS, Arkin AP. 2009. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. *Mol Biol Evol* 26:1641–1650. <https://doi.org/10.1093/molbev/msp077>.
 64. Carattoli A, Zankari E, García-Fernández A, Voldby Larsen M, Lund O, Villa L, Møller Aarestrup F, Hasman H. 2014. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. *Antimicrob Agents Chemother* 58:3895–3903. <https://doi.org/10.1128/AAC.02412-14>.
 65. McBride SM, Coburn PS, Baghdadyan AS, Willems RJ, Grande MJ, Shankar N, Gilmore MS. 2009. Genetic variation and evolution of the pathogenicity island of *Enterococcus faecalis*. *J Bacteriol* 191:3392–3402. <https://doi.org/10.1128/JB.00031-09>.
 66. Depardieu F, Kolbert M, Pruul H, Bell J, Courvalin P. 2004. VanD-type vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis*. *Antimicrob Agents Chemother* 48:3892–3904. <https://doi.org/10.1128/AAC.48.10.3892-3904.2004>.