



Microbiome-Informed Food Safety and Quality: Longitudinal Consistency and Cross-Sectional Distinctiveness of Retail Chicken Breast Microbiomes

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ABSTRACT Microorganisms and their communities on foods are important determinants and indicators of food safety and quality. Despite growing interests in studying food and food-related microbiomes, how effective and practical it is to glean various food safety and quality information from food commodity microbiomes remains underinvestigated. Microbiomes of retail chicken breast from 4 processing establishments in 3 major U.S. broiler production states displayed longitudinal consistency over 7 months and cross-sectional distinctiveness associated with individual processing environments. Packaging type and processing environment but not antibiotic usage and seasonality affected composition and diversity of the microbiomes. Low abundances of antimicrobial resistance genes were found on chicken breasts, and no significant resistome difference was observed between antibiotic-free and conventional products. Benchmarked by culture enrichment, shotgun metagenomics sequencing delivered sensitive and specific detection of *Salmonella enterica* from chicken breasts.

IMPORTANCE Chicken has recently overtaken beef as the most-consumed meat in the United States. The growing popularity of chicken is accompanied by frequent occurrences of foodborne pathogens and increasing concerns over antibiotic usage. Our study represents a proof-of-concept investigation into the possibility and practicality of leveraging microbiome-informed food safety and quality. Through a longitudinal and cross-sectional survey, we established the chicken microbiome as a robust and multifaceted food microbiology attribute that could provide a variety of safety and quality information and retain systematic signals characteristic of overall processing environments.

KEYWORDS chicken, microbiome, shotgun metagenomics, antimicrobial resistance, *Salmonella*

Microorganisms and their communities on foods are important determinants and indicators of food safety and quality. There have been growing interests in studying food and food-related microbiomes. Deep sequencing of food and environment microbiomes allowed the detection of multiple foodborne pathogens and the monitoring of their relative abundances throughout the beef production chain (1). Microbiome analyses of meat and seafood products provided a microbiological dissection of food spoilage to study microbial origin and population dynamics of the complex process (2). Besides targeted investigations of pathogenic and spoilage microorganisms, overall food microbiome profiles have been investigated to suggest how agricultural and food processing practices affect resident food microbial communities in the context of food safety and spoilage (3–5). In addition to taxonomic compositions, particular microbiome constituents such as antimicrobial resistance genes (ARGs), or

Citation Li S, Mann DA, Zhang S, Qi Y, Meinersmann RJ, Deng X. 2020. Microbiome-informed food safety and quality: longitudinal consistency and cross-sectional distinctiveness of retail chicken breast microbiomes. mSystems 5:e00589-20. <https://doi.org/10.1128/mSystems.00589-20>.

Editor Paul D. Cotter, Teagasc Food Research Centre

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 A longitudinal and cross-sectional survey established chicken microbiome as a robust and multifaceted food attribute that could provide various safety and quality information and retain systematic signals characteristic of overall processing environments.

Received 29 June 2020

Accepted 20 August 2020

Published 8 September 2020

resistomes, have been profiled to indicate the ecological impact of antimicrobial usage at various stages of beef (6) and poultry (7, 8) production.

While characterizing food microbiomes has the potential to inform food safety and quality in a multifaceted and integrative manner, its application and practicality remain understudied. Several technical challenges and knowledge gaps are yet to be addressed before making the routine use of food microbiome analysis possible. For targeted detection and identification of particular microorganisms such as foodborne pathogens, the performance of the microbiome approach needs to be benchmarked by the gold standard of culture enrichment. To evaluate the overall microbiome profile as a holistic and informative attribute of food, the consistency, reproducibility, and distinctiveness of food microbiomes with respect to different production establishments and environments need to be measured. For any type of food microbiome application, it is important to determine a sufficient and logistically viable depth of sequencing coverage, which likely varies among different food commodities.

Raw chicken meat is a staple food of relatively high microbial loads ($\sim 4.5 \log_{10}$ CFU/ml total aerobic counts in chicken rinsates [9]). The consumption of chicken has been steadily increasing worldwide since the 1960s (10). In the United States, chicken overtook beef as the most consumed meat in 2013 (11). The high demands for chicken make it important to ensure the microbiological safety and quality of raw chicken meat, which has been a long-lasting challenge. In the United States from 1998 to 2012, poultry was the most common food in outbreaks caused by known pathogens such as *Salmonella enterica* (12). According to the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture, 22.2% of half-chicken carcasses and 24.0% of raw chicken parts in the United States in 2012 were *S. enterica* positive (9). Besides the frequent occurrence of pathogens, the rapid spread of antimicrobial resistance in poultry-associated pathogenic bacteria, such as *Salmonella*, has been noticed (13–15). The widespread use of antimicrobials for growth promotion in livestock production has been associated with the rise of antimicrobial resistance (16). In addition to microorganisms of safety concerns, spoilage bacteria can also render chicken meat unacceptable for human consumption. While many different bacterial populations can contribute to microbial spoilage, a fraction of species and strains outcompete others during meat storage and release undesired metabolites (17). The selection for dominant organisms during spoilage largely depends on storage temperature and packaging atmosphere (availability of oxygen).

In this study, we chose retail chicken breasts as a model food matrix to explore the possibility of using microbiomes to inform multiple food safety and quality characteristics. We hypothesized (i) that chicken breast microbiomes display longitudinal consistency over time and cross-sectional distinctiveness among different production conditions and (ii) that the microbiomes can be robustly characterized through shotgun metagenomics sequencing on a commonly used sequencing platform to allow ARG profiling and *Salmonella* detection. To test these hypotheses, we sampled 8 chicken breast products from 4 processing establishments under 5 brands over 7 months (from July 2017 to January 2018) (Table 1). The processing establishments were located in the top 3 broiler-producing states in the United States (Georgia, Arkansas, and North Carolina), which accounted for more than a third of national production in 2017 (18). The samples represented different production and processing practices, including “No Antibiotics Ever” versus conventional (no label regarding antibiotic usage) products and vacuum versus ordinary, air-permeable packages.

RESULTS

Sequencing coverage of chicken breast microbiomes. By multiplexing 10 to 12 samples in a single sequencing run on a MiSeq sequencer, an average of 5.3 million reads per sample were collected by pooling trimmed and filtered reads from two biological replicates of the same sample sequenced in two separate runs (Table 2). An average of 2.5 million reads per sample were classified as bacteria, corresponding to an average of 49.9% of the total sequencing output per sample (Table 2). Estimated by

TABLE 1 Chicken breast samples for microbiome survey

Product	Label	Brand	Processing establishment	Antibiotic usage	Package	Location	Season	Quantity ^a
1	A-I-CONV	A	I	CONV	Air permeable	Georgia	Summer ^b	6
2	B-II-CONV	B	II	CONV	Air permeable	Arkansas	Summer	6
3	C-III-CONV	C	III	CONV	Air permeable	North Carolina	Summer	5
4	C-III-ABF	C	III	ABF ^c	Air permeable	North Carolina	Winter ^d	3
5	D-III-ABF-V	D	III	ABF	Vacuum	North Carolina	Summer	6
6	D-III-ABF	D	III	ABF	Air permeable	North Carolina	Winter	3
7	E-IV-ABF	E	IV	ABF	Air permeable	North Carolina	Summer	6
8 ^e	D-III-ABF-V_V/A	D	III	ABF	Vacuum	North Carolina	Summer	2

^aTwo biological replicates were collected for each sample.

^bSummer samples were collected from July 2017 to September 2017 except for product 8, which was collected in June 2018.

^cChicken products with "No Antibiotic Ever" claim.

^dWinter samples were collected from November 2017 to January 2018.

^eThe two samples (D-III-ABF-V_V and D-III-ABF-V_A) were collected in June 2018 to confirm the effect of vacuum packaging on chicken breast microbiome.

Nonpareil (62), the sequencing coverage of chicken breast microbiomes ranged from 22.5% to 93.8%. Two samples that had a coverage below 75% were considered insufficiently sequenced and excluded from subsequent analyses. The coverages of the remaining 35 samples ranged from 75.6% to 93.8%, with a mean of 83.7% (Table 2). The average aerobic plate count (APC) of these samples ranged from 4.2 to 7.0 log₁₀ CFU/g, with a mean of 5.9 log₁₀ CFU/g (Table 2). There was no significant difference in APC between vacuum-packing and air-packaging samples ($P = 0.90$, t test). According to redundancy-based coverage projections by Nonpareil, ~5.6 million bacterial reads (~1.1 billion bases) are required for 95% abundance-weighted average coverage of the chicken breast bacterial communities (see Fig. S1 in the supplemental material).

Longitudinal consistency and cross-sectional distinctiveness of chicken breast microbiomes. Principal coordinates and hierarchical clustering analyses based on overall microbiome similarities revealed consistent clustering of chicken breast microbiomes over the sampling period ranging from 6 weeks up to 3 months (Fig. 1). Four major clusters were identified (C1 through C4), of which C2 was most distinctive and exclusively composed of vacuum-packaged samples. Clustering of samples by processing establishments was observed in other clusters, of which C1 and C3 consisted of samples from only processing establishments I and IV, respectively. C4 was further divided into 2 subclusters, C4-1 and C4-2. C4-1 contained all the samples from processing establishment II, and C4-2 included all the air-permeable (nonvacuum) samples from establishment III and a single establishment II sample (Fig. 1b). C4-2 was the only cluster or subcluster that had samples under different brands (B, C, and D). It is worth noting that brand D samples in C2 (D-III-ABF-V) and C4 (D-III-ABF) were both antibiotic-free (ABF) products processed at the same establishment, differing only by package types (vacuum versus air permeable). Two additional samples (D-III-ABF-V_A and D-III-ABF-V_V) were sampled 11 months later after the original D-III-ABF-V samples to confirm the packaging effect on the chicken breast microbiome as detailed below.

Packaging type and processing environment but not antibiotic usage and seasonality affected composition and diversity of chicken breast microbiomes. Differences in microbial compositions among chicken breast microbiomes were demonstrated by examining relative abundances of bacterial genera (Fig. 2a). It was apparent that the dominant bacteria on vacuum-packaged products were more diverse, including genera such as *Aeromonas* (20.7% ± 12.2%; mean ± SD), *Buttiauxella* (11.0% ± 8.3%), *Carnobacterium* (22.9% ± 18.8%), *Enterobacter* (2.9% ± 2.1%), *Hafnia* (8.4% ± 4.6%), *Lactococcus* (6.0% ± 4.3%), *Pseudomonas* (3.9% ± 3.2%), *Serratia* (4.3% ± 1.4%), *Shewanella* (4.8% ± 3.7%), and *Yersinia* (2.1% ± 1.2%). In comparison, air-permeable samples were dominated by *Pseudomonas* (87.3% ± 18.2%) (Fig. 2a; Table S1).

Vacuum-packaged samples had significantly higher alpha diversities than air-permeable samples (Shannon index, Fig. 2b; Simpson index, Fig. S2a). Group comparison of beta diversity (Bray-Curtis dissimilarity) also showed a significant discrimination between vacuum-packaged and air-permeable products (Table 3). To confirm the impact of

TABLE 2 Summary statistics of shotgun metagenomics sequencing

Sample ^a	APC ^b (log ₁₀ CFU/g)	Estimated sequencing coverage (%) ^c	Total passed reads (paired) ^d	Bacterial reads (paired) ^e	Bacterial sequences (%) ^f
A-I-CONV_1	6.4	84.3	5,481,182	3,056,476	52.5
A-I-CONV_2	4.5	86.1	6,526,130	2,518,460	38.6
A-I-CONV_3	4.2	82.4	5,448,486	1,107,782	19.4
A-I-CONV_4	5.1	87.3	4,941,042	3,800,622	79.6
A-I-CONV_5	5.6	84.8	4,900,690	3,400,858	68.9
A-I-CONV_6	4.3	88.1	4,657,918	2,717,628	59.0
B-II-CONV_1	5.8	81.8	1,869,964	1,468,654	76.9
B-II-CONV_2	5.9	82.2	5,749,824	2,679,654	49.9
B-II-CONV_3	6.7	76.4	2,006,446	1,620,716	79.5
B-II-CONV_4	6.7	77.6	4,859,556	3,358,930	70.2
B-II-CONV_5	5.4	79.4	5,175,854	2,127,380	41.4
B-II-CONV_6	5.4	79.8	5,908,820	3,072,276	50.0
C-III-CONV_1	6.0	78.2	4,172,702	2,747,400	71.0
C-III-CONV_2	6.6	86.0	5,642,726	4,634,768	82.4
C-III-CONV_3	6.9	84.3	5,520,640	4,554,954	83.1
C-III-CONV_4	6.0	86.1	6,421,858	5,290,498	82.9
C-III-CONV_5	6.5	87.2	5,689,396	4,953,032	87.1
C-III-ABF_1	6.8	88.8	7,313,912	6,111,836	84.0
C-III-ABF_2	6.4	84.9	3,857,934	3,340,052	86.6
C-III-ABF_3	6.0	87.6	6,822,072	3,162,742	50.5
D-III-ABF_1	6.3	82.2	4,410,610	2,188,946	48.5
D-III-ABF_2	6.0	80.6	3,316,508	1,134,484	35.2
D-III-ABF_3	5.4	86.0	4,839,180	2,073,838	39.8
D-III-ABF-V_1	6.1	80.5	6,965,766	923,962	14.3
D-III-ABF-V_2	6.2	78.4	6,311,318	1,381,234	22.5
D-III-ABF-V_3	6.2	80.9	6,708,452	1,505,780	24.8
D-III-ABF-V_4	6.0	77.9	12,562,666	2,380,342	18.8
D-III-ABF-V_5 ^g	6.5	22.5	5,698,708	115,450	2.0
D-III-ABF-V_6	6.2	75.6	4,121,128	1,019,544	25.1
E-IV-ABF_1	6.5	85.5	3,795,540	2,259,584	58.1
E-IV-ABF_2	6.2	93.8	5,706,956	3,353,044	62.2
E-IV-ABF_3	6.0	87.7	5,524,596	2,874,490	53.5
E-IV-ABF_4	5.7	80.6	5,481,604	1,373,962	26.0
E-IV-ABF_5 ^g	5.3	55.5	4,299,662	692,456	16.5
E-IV-ABF_6	6.2	89.9	4,852,236	1,481,336	32.4
D-III-ABF-V_A	7.0	85.2	2,936,008	1,300,570	45.2
D-III-ABF-V_V	5.2	90.2	7,193,950	701,334	9.3

^aAll results were reported as the averages from two replicates.

^bAerobic plate count.

^cSequencing coverage was estimated by using Nonpareil.

^dNumber of sequencing reads that passed quality control by Trimmomatic.

^eNumber of sequencing reads that were classified as bacteria by KrakenUniq.

^fPercentage of bacterial reads among all the sequencing reads.

^gThe sample was excluded from subsequent analyses, as the estimated sequencing coverage was lower than 75%.

vacuum packaging on the chicken breast microbiome, we compared the microbiomes of two additional vacuum-packaged samples by unwrapping one sample and exposing it to air (D-III-ABF-V_A) while keeping the other one's vacuum packaging intact (D-III-ABF-V_V) during storage. An apparent shift in microbiome composition was observed between the 2 samples, resulting in a *Pseudomonas*-dominated microbiome on the unwrapped sample (Fig. 1 and 2a). The 2 additional samples were collected 11 months after the original D-III-ABF-V samples and had a different packaging barcode and design; their overall microbiome profiles diverged from the earlier D-III-ABF-V samples.

Because of the significant impact of vacuum packaging on chicken breast microbiomes, vacuum-packaged samples were excluded from evaluating whether other production factors affected the microbiomes, including overall processing environments (different processing establishments), antibiotic usage (ABF versus CONV products), and seasonality (July 2017 to September 2017 versus November 2017 to January 2018).

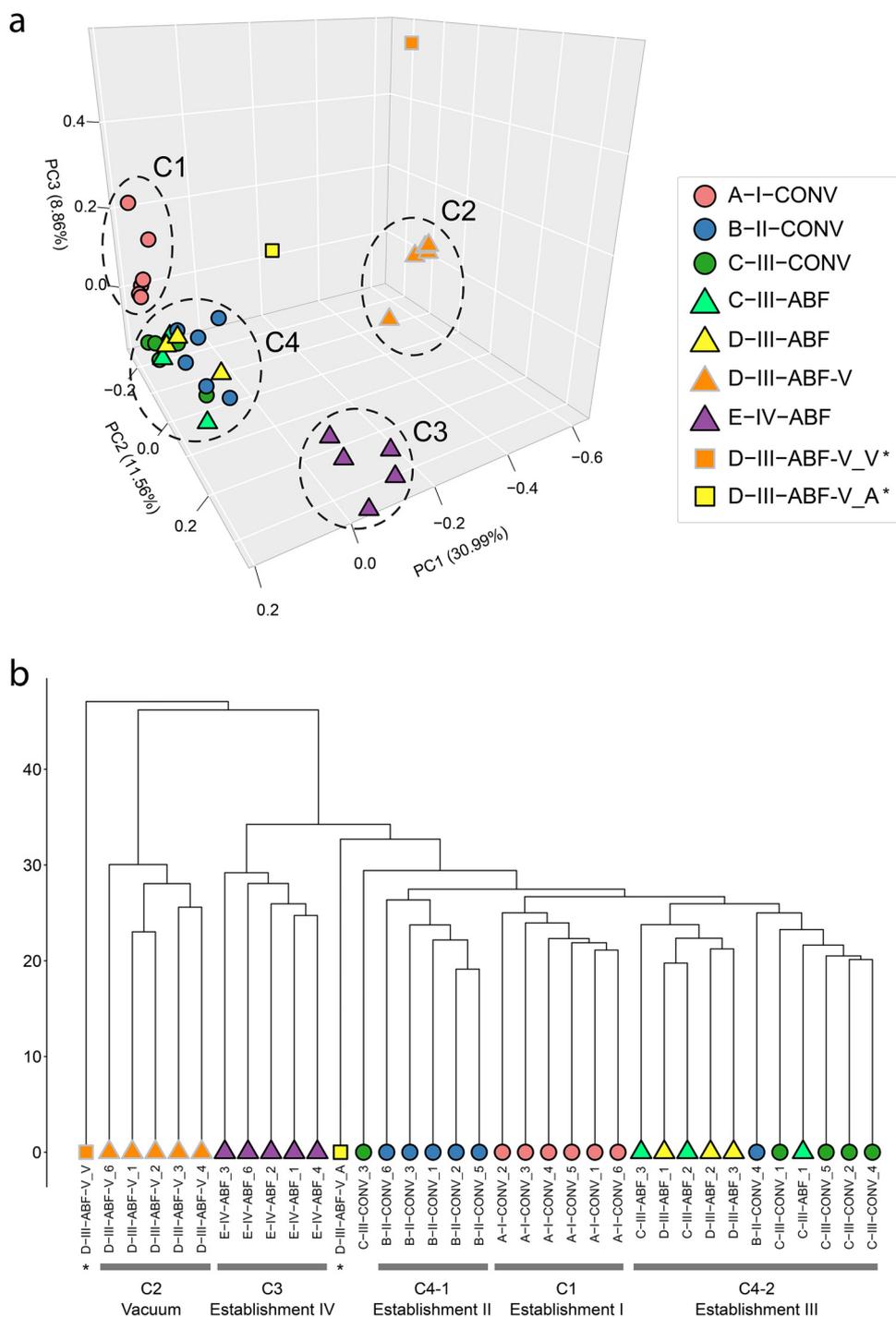


FIG 1 Clustering of chicken breast microbiomes based on Bray-Curtis dissimilarities. (a) Principal-coordinate analysis (PCoA). (b) Hierarchical clustering analysis. Samples were colored by products. Circles represent CONV samples, and triangles represent ABF samples. Squares (*) indicate two additional vacuum-packaged samples, of which D-III-ABF-V_A was unwrapped and exposed to air and D-III-ABF-V_V was kept intact during storage.

Consistent with overall microbiome distinctiveness associated with processing establishments according to Bray-Curtis dissimilarities (Fig. 1), significant compositional differences were observed among microbiomes from different establishments as measured by both alpha (Shannon index, Fig. 2b; Simpson index, Fig. S2a) and beta (Table 3) diversities. However, the numbers of detected genera were not significantly different among these microbiomes (Fig. S2b), suggesting that the observed microbiome differences were caused by genus diversity instead of genus richness.

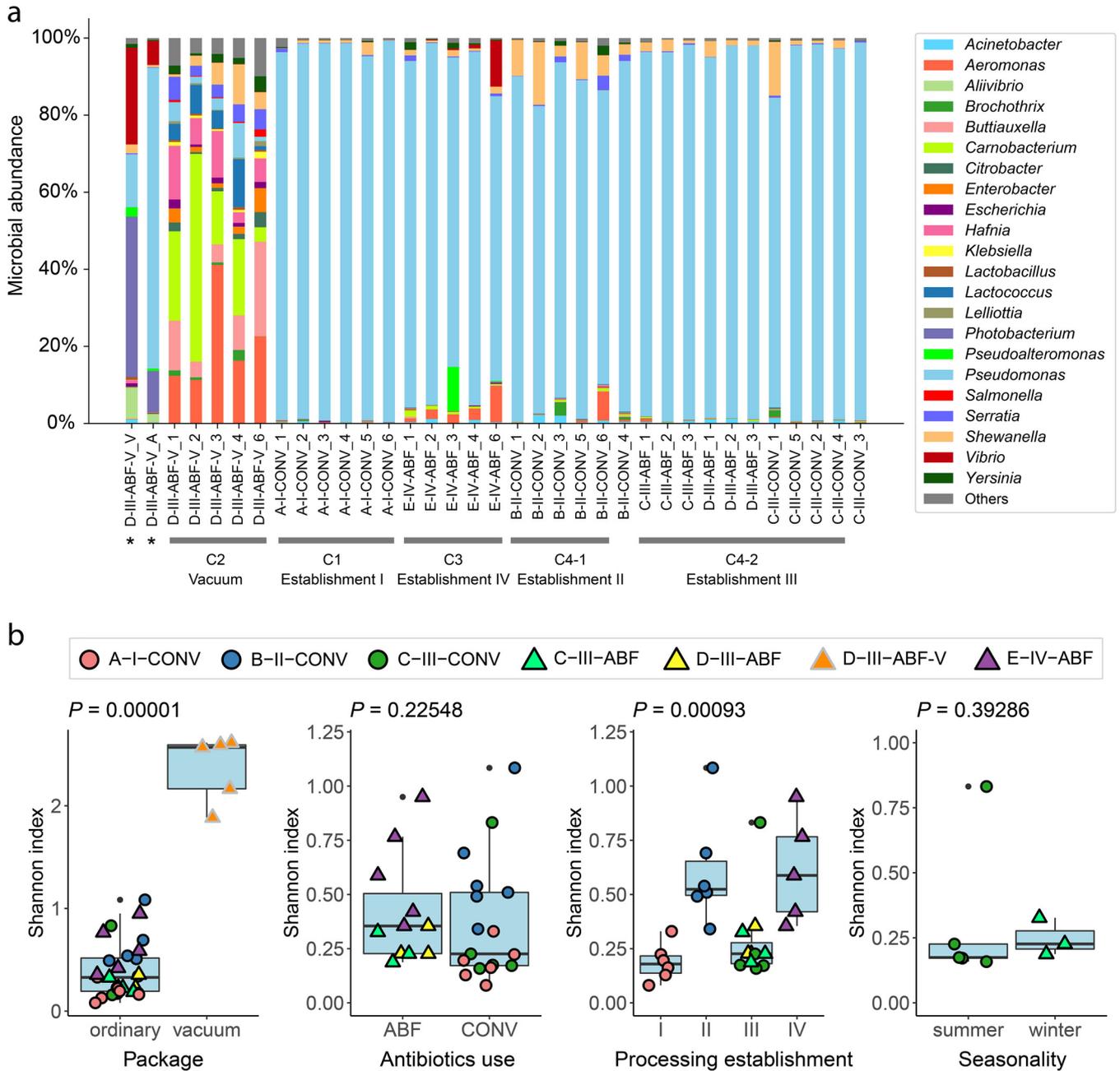


FIG 2 Comparison of taxonomic compositions among chicken breast microbiomes. (a) Relative abundances of microbial genera in chicken microbiomes showing compositional differences among chicken breast samples. “*” indicates two additional vacuum-packaged samples, of which D-III-ABF-V_A was unwrapped and exposed to air and D-III-ABF-V_V was kept intact during storage. (b) Comparisons of bacterial alpha diversities (Shannon index) based on genus profiles showing the differences between packaging types, antibiotic usage, processing establishments, and seasonality. Samples were colored by products. Circles represent CONV samples, and triangles represent ABF samples.

Compared with packaging type and processing environment, antibiotic usage and seasonality did not appear to significantly affect the composition of chicken breast microbiomes. No significant difference in alpha (Shannon index, Fig. 2b; Simpson index, Fig. S2a) and beta (Table 3) diversities was observed between ABF and CONV samples as well as between brand C samples processed at the same establishment during summer (from July 2017 to September 2017, C-III-CONV) and during winter (from November 2017 to January 2018, C-III-ABF). The monthly average temperatures at the location of the North Carolina processing establishment were 26.9°C, 25.3°C, and 22.4°C from July 2017 to September 2017 and 10.6°C, 6.1°C, and 3.2°C from November 2017 to

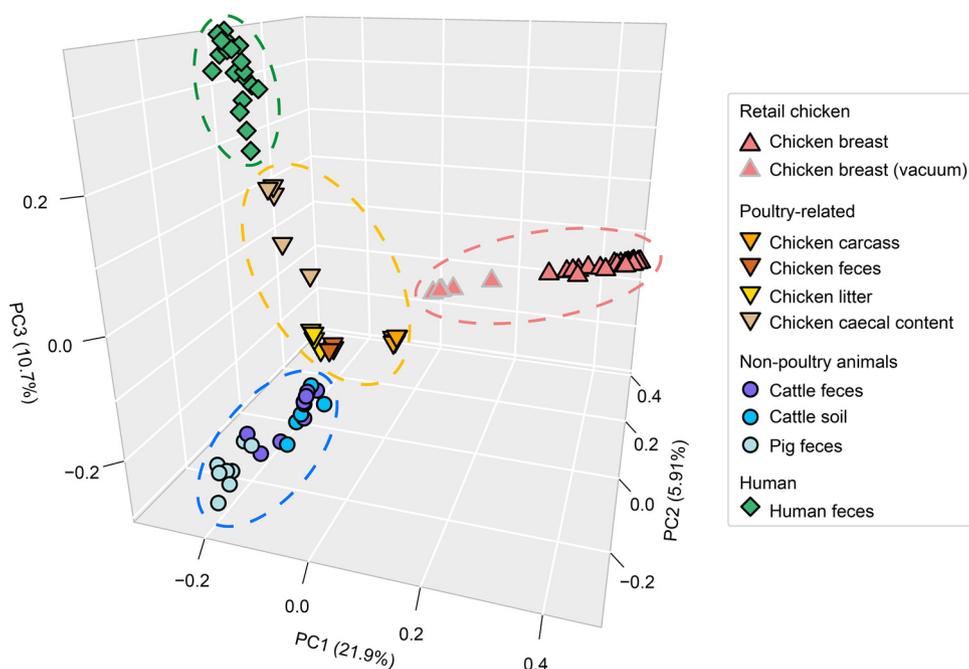
TABLE 3 Comparison of Bray-Curtis dissimilarities between microbiomes under different conditions using permutational multivariate analysis of variance (PERMANOVA)

Condition	Degrees of freedom	F	R ²	P
Package	1,32	180.45	0.8534	0.001
Antibiotic use	1,27	1.2975	0.0475	0.275
Processing establishment	3,27	3.9497	0.3305	0.002
Seasonality	1,7	0.3206	0.0507	0.979

January 2018 (<https://www.wunderground.com/>). According to FSIS, all poultry should be chilled immediately after processing and stored at 40°F (4.4°C) or less before packaging and shipping (19).

Chicken breast microbiomes were distinct from other poultry-related microbiomes. We compared chicken breast microbiomes with other poultry-related microbiomes including those of whole chicken carcasses, chicken litter, and chicken feces. Also included in this comparison were fecal microbiomes from nonpoultry animals and humans. Principal-coordinate analysis (PCoA) of the microbiomes based on Bray-Curtis dissimilarities identified 4 major clusters including chicken breasts, other poultry-related sources, nonpoultry animal sources, and human feces (Fig. 3). Notably, the average pairwise Bray-Curtis dissimilarity between chicken breasts and other poultry-related sources (0.12 ± 0.03) was smaller than that between chicken breasts and nonpoultry animal sources (0.18 ± 0.11) and that between chicken breasts and human feces (0.32 ± 0.06).

Low abundances of chicken breast resistomes. The average normalized ARG abundance of chicken breast samples (copies of ARG per prokaryotic cell) was 0.16 ± 0.16 (mean \pm SD), significantly lower ($P < 0.05$, *t* test) than that of other samples analyzed except a U.S. soil microbiome (0.04 ± 0.01) (Fig. 4; Tables S2 and S3). In comparison, the average normalized ARG abundance was 5.52 ± 3.12 for other poultry-related microbiomes (cecal content, carcass wash, feces, and litter), 2.56 ± 1.36 for cattle-related microbiomes (cattle feces and cattle soil), 6.06 ± 1.91 for pig feces,

**FIG 3** Principal-coordinate analysis (PCoA) of chicken breast, poultry-related, and nonpoultry microbiomes based on Bray-Curtis dissimilarities. Four major clusters were identified, including chicken breasts (red dashed oval), other poultry-related sources (yellow dashed oval), nonpoultry animal sources (blue dashed oval), and human feces (green dashed oval).

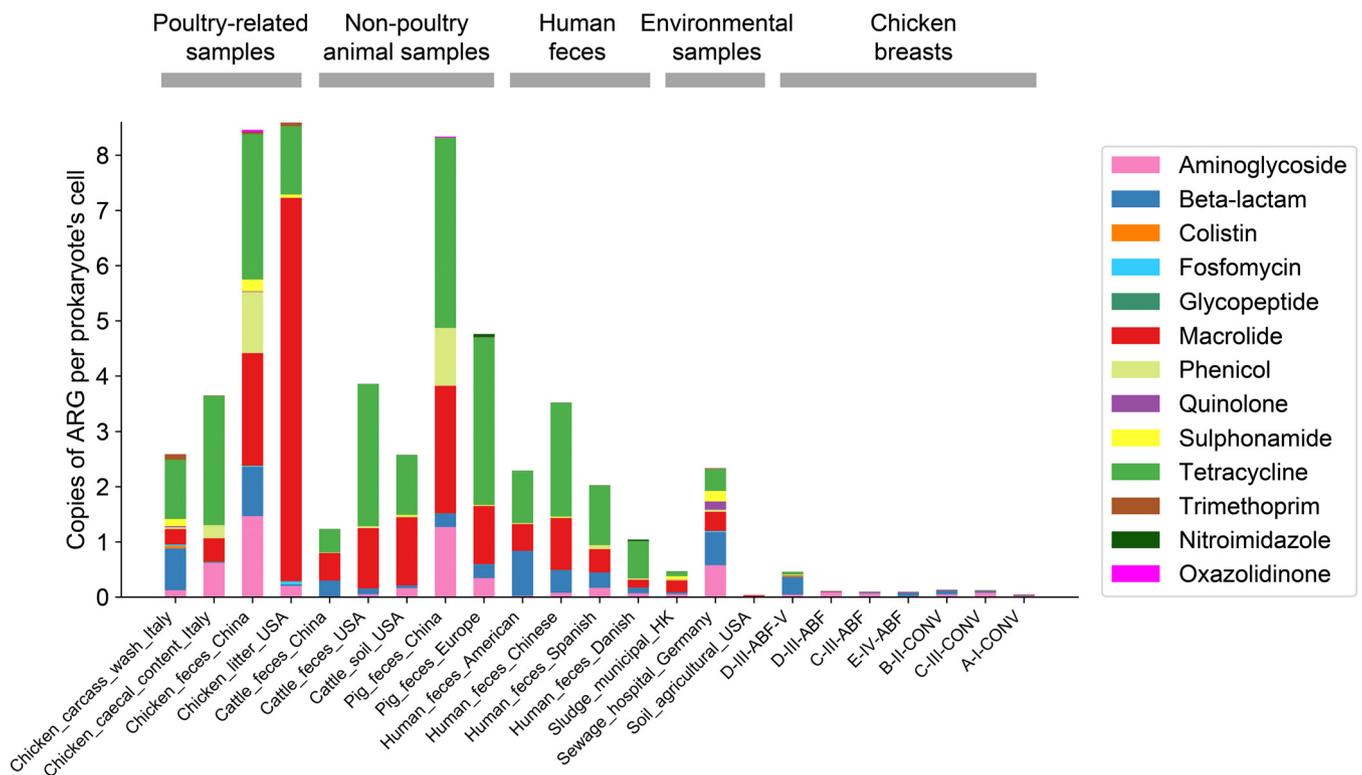


FIG 4 Normalized ARG abundances in the microbiomes of chicken breasts and other samples from various sources. The abundance of each ARG class was calculated as the sum of normalized abundances of ARGs belonging to the class. Stacked bars are colored by ARG classes. The abundances of ARGs were normalized by prokaryotic cell counts.

2.21 ± 1.11 for human feces, and 1.50 ± 1.01 for sludge and sewage (Fig. 4). ARGs belonging to antimicrobial classes of tetracyclines, aminoglycosides, and macrolides had significantly lower normalized abundances ($P < 0.05$, t test) in chicken breast samples than those in other poultry-related samples, cattle-related samples, and pig feces (except aminoglycosides in cattle feces samples) (Fig. 4). These antibiotics represented the largest volume of domestic sales of medically important antimicrobial drugs in food-producing animals in 2017 (20). The average normalized ARG abundance of tetracyclines, aminoglycosides, and macrolides in chicken breast samples was 0.01 ± 0.02 , 0.05 ± 0.03 , and 0.0004 ± 0.0004 , respectively (Fig. 5a; Table S3). ARGs belonging to the aminoglycoside class were detected in all samples, with *ant(3'')-Ia* being the only ARG that was present in every sample (Fig. 5b; Table S2). Low abundances of mobilized colistin resistance genes (*mcr-3*, *mcr-4*, *mcr-7*, and *mcr-9*) were sporadically found in 15 samples (3 A-I-CONV samples, 2 B-II-CONV samples, 2 C-III-CONV samples, 1 C-III-ABF sample, 5 D-III-ABF-V samples, and 2 E-IV-ABF samples) (Fig. 5b; Table S2). The average normalized abundance of *mcr* genes in these samples was 0.0007 ± 0.001 . Sequencing reads ($n = 163$) that were aligned to *mcr* genes (>80% similarity) were fragments of *mcr-3* variants (*mcr-3.6* to *mcr-3.25*, $n = 86$), *mcr-4* variants (*mcr-4.3* to *mcr-4.6*, $n = 14$), *mcr-7* variant (*mcr-7.1*, $n = 32$), and *mcr-9* variant (*mcr-9*, $n = 31$). The average length of these reads was 203.7 ± 55.4 bp. *mcr-3*-positive samples ($n = 7$) were found to have a significantly higher relative abundance of *Aeromonas* ($P = 0.04$, t test) than *mcr-3*-negative samples ($n = 26$).

Packaging type had greater impact on chicken breast resistomes than antibiotic usage. In total, 132 ARGs of 10 antibiotic classes were detected from chicken breast microbiomes (Table S2 and Fig. S3). The majority (53.8%) of the detected ARGs belonged to the classes of aminoglycosides ($n = 14$) and beta-lactams ($n = 57$) (Table S2).

Resistome comparison between vacuum-packaged and air-permeable samples showed significant differences in ARG abundance and composition (Fig. 5a). The

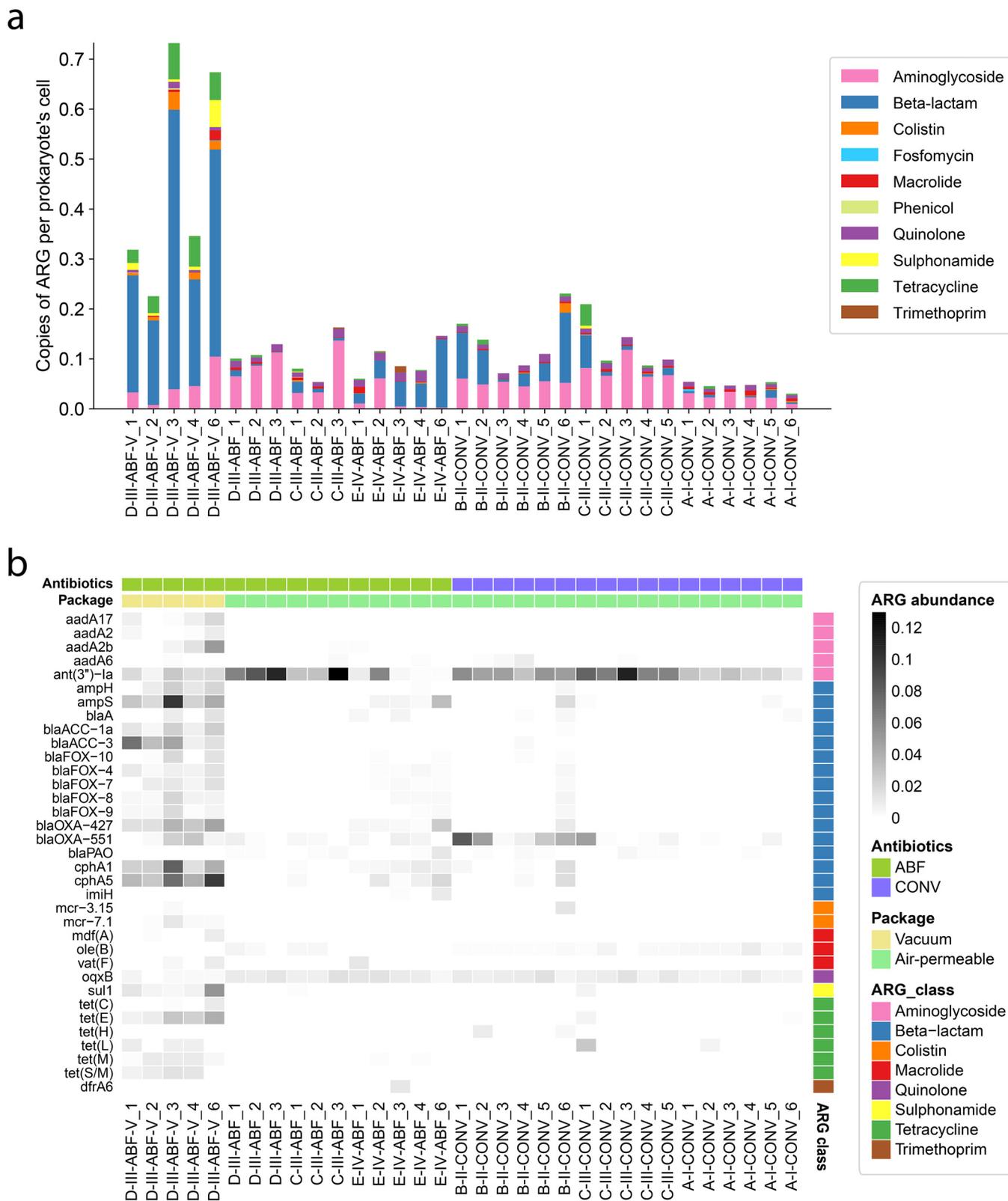


FIG 5 Normalized ARG abundances in chicken breast microbiomes. (a) Normalized abundance of ARGs in each sample. The abundance of each ARG class was calculated as the sum of normalized abundances of ARGs belonging to the class. Stacked bars were colored by ARG classes. (b) Normalized abundances of 35 major ARGs ($>1.0 \times 10^{-2}$ copies of ARG per prokaryote cell in at least one sample) in chicken breast samples. The abundances of ARGs were normalized by prokaryotic cell counts. The two sets of horizontal annotation blocks were colored by antibiotic usage and packaging types, respectively. The vertical annotation blocks were colored by ARG classes.

average normalized ARG abundance of the vacuum-packaged samples ($n = 5$) was 4.5 times higher than that of air-permeable samples ($n = 28$) (Fig. 5a; Table S3). Significant differences in alpha diversity (Shannon index) ($P = 0.000$, Mann-Whitney U test) of detected ARGs were found between vacuum-packaged and air-permeable samples. The average Shannon index was 3.33 ± 0.27 for vacuum-packaged samples and 1.74 ± 0.48 for air-permeably packaged samples (Table S4). The resistomes of vacuum-packaged samples were dominated by beta-lactam ARGs, with an average relative abundance of $69.6\% \pm 7.4\%$ (mean \pm SD). In comparison, aminoglycoside ARGs appeared to be more abundant in air-permeable samples ($50.4\% \pm 24.8\%$), accounting for the majority of the resistome in 21 out of 28 samples ($61.3\% \pm 16.5\%$) (Fig. 5a and b).

Compared with packaging type, the impact of antibiotic usage on chicken breast resistomes, if any, was much milder. There was no significant difference in normalized ARG abundances ($P = 0.97$, t test) between ABF samples (mean abundance = 0.10) and CONV samples (mean abundance = 0.10) with air-permeable packaging. Similarly, when comparing the diversity of ARGs, no significantly different Shannon indices ($P = 0.834$, Mann-Whitney U test) were found between CONV and ABF samples (excluding vacuum-packaged samples). The average Shannon index was 1.76 ± 0.58 for ABF samples and 1.72 ± 0.42 for CONV samples (Table S4).

Cooccurrence of ARGs and taxa in chicken breast microbiomes. Comparison between microbial diversity and ARG diversity (Shannon index) in chicken breast microbiomes identified a significant positive correlation ($\rho = 0.79$, $P = 0.000$, Spearman test) in between (Table S4). Further network analysis revealed certain taxa that strongly cooccurred with some beta-lactam ARGs, including *Aeromonas* (e.g., *cphA1*, *cphA5*, and *bla*_{OXA-427}) and *Shewanella* (*bla*_{OXA-551}) (Fig. 6). Cooccurrence of certain beta-lactam ARGs was also observed, including that of *ampH*, *ampS*, *bla*_{FOX-4}, *bla*_{FOX-7}, *bla*_{FOX-8}, *bla*_{OXA-427}, *cphA1*, and *cphA5* (Fig. 6). The correlation between the two genera and beta-lactam ARGs was confirmed by analyzing assembled metagenome contigs (Table S5). The 3 beta-lactam ARGs (*cphA1*, *cphA5*, and *bla*_{OXA-427}) that cooccurred with *Aeromonas* were commonly found on contigs that were determined as chromosomal sequences of *Aeromonas*. Similarly, *bla*_{OXA-551} was exclusively found on contigs that were considered *Shewanella* chromosomal sequences (Table S5).

Network analysis also identified cooccurrence of certain taxa. Facultatively anaerobic bacteria, including *Yersinia*, *Serratia*, *Hafnia*, *Enterobacter*, *Citrobacter*, *Klebsiella*, *Buttiauxella*, *Raoultella*, *Cronobacter*, *Salmonella*, *Cedecea*, *Erwinia*, *Kosakonia*, *Lelliottia*, and *Pluralibacter*, were found to cooccur and be abundant in vacuum-packaged samples (Fig. 6). Obligately or facultatively aerobic bacteria, such as *Burkholderia*, *Cupriavidus*, *Achromobacter*, *Paraburkholderia*, *Azotobacter*, *Halomonas*, *Marinobacter*, and *Xenorhabdus* cooccurred commonly in air-permeable products (Fig. 6). Notably, correlation analysis on normalized abundances of all detected microbial taxa identified negative associations between the facultatively anaerobic bacteria and *Pseudomonas* (Fig. S4), which was the dominant taxon in air-permeable samples (Fig. 2a).

Sensitive and specific detection of *Salmonella* by shotgun metagenomic sequencing. *Salmonella* was detected by culture enrichment in 4 samples of 2 products (B-II-CONV_4, B-II-CONV_6, C-III-ABF_1, and C-III-ABF_3) (Table 4). The *Salmonella*-positive rate among the 33 chicken breast samples was 12.1% (2 samples sequenced later for evaluating vacuum packaging effect were not included for *Salmonella* detection), which was similar to *Salmonella* prevalence on retail skinless chicken breasts (12.3%) that were surveyed spatio-temporally close to the samples used in the current study (Atlanta metropolitan area, Georgia, in 2017) (21). *Salmonella* detection in metagenomic DNA by real-time PCR yielded negative results for all samples (Table 4).

Salmonella detection was also carried out by identifying *Salmonella* sequences in shotgun sequencing data. According to MetaPhlan2 (76) identification using reference marker genes, no *Salmonella* sequence was detected in any sample. Based on *Salmonella*-specific k -mers detected by KrakenUniq, 17 samples were positive for *Salmonella* sequences (Table 4). Notably, the metagenomes of vacuum-packaged sam-

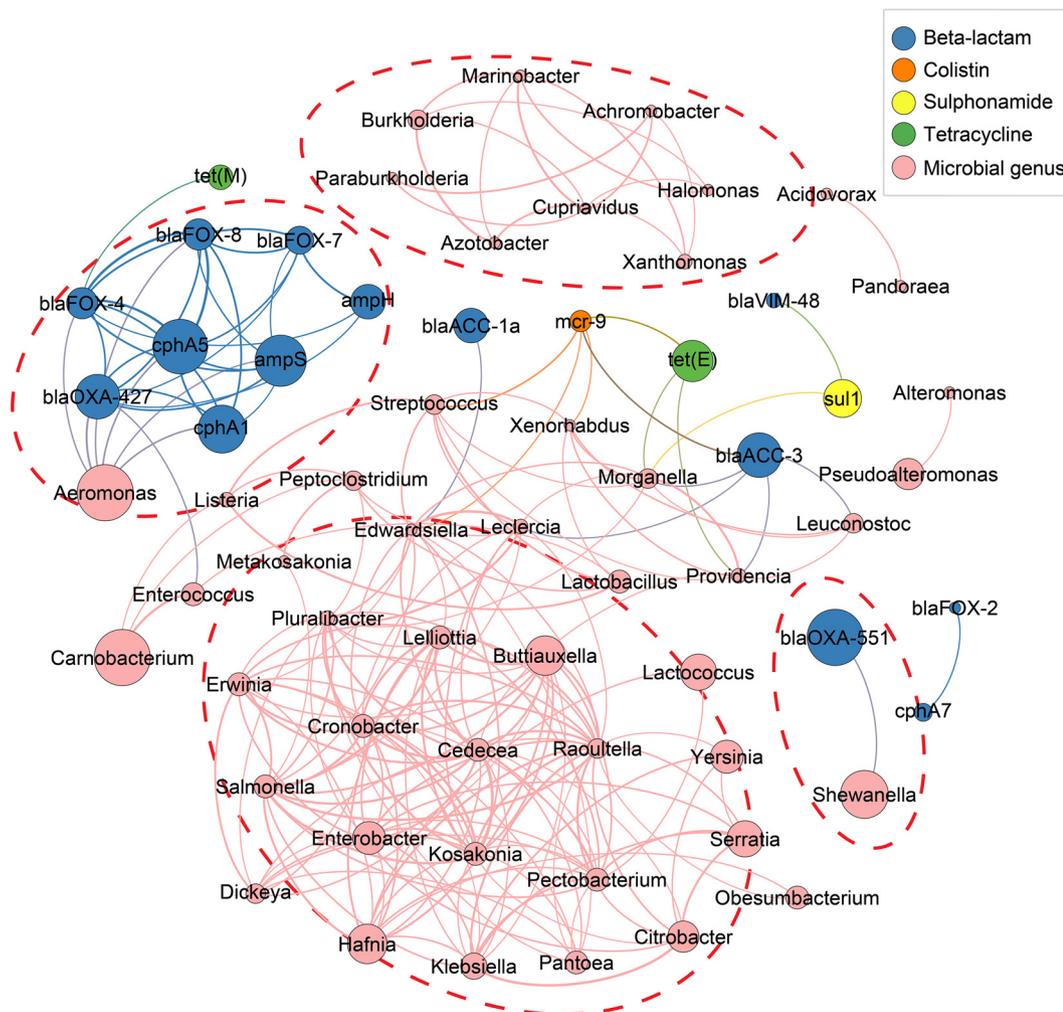


FIG 6 Network analysis showing cooccurrences among ARGs and microbial taxa. The red dashed ovals indicate cooccurrence patterns of interest. Links were drawn when Spearman's correlation coefficient (ρ) between two nodes (ARG or microbial genus) was larger than 0.8 ($P < 0.01$). The width of each link is proportional to the value of the correlation coefficient. The size of each node is proportional to the relative abundance of ARG or microbial genus.

ples were particularly abundant in unique k -mers classified by KrakenUniq (60) as *Salmonella* (Table 4). After filtering out sequencing reads that were likely of plasmid or phage origin by PPR_Meta, 5 samples remained as *Salmonella* positive, 3 of which were *Salmonella* positive also by culture enrichment (Table 4).

Using *Salmonella* detection by culture enrichment as a benchmark, we evaluated the sensitivity and specificity of *Salmonella* detection from shotgun sequencing data (Table 4). The best overall performance was delivered by reads classification followed by mobile genetic elements (MGE) filtering, with a sensitivity of 0.75 and specificity of 0.93. In comparison, reads classification by KrakenUniq alone delivered a sensitivity of 0.75 and specificity of 0.52 for *Salmonella* detection. Both real-time PCR and MetaPhlan2 analyses of the microbiomes yielded a sensitivity of zero by detecting *Salmonella* in none of the samples.

DISCUSSION

Microbial populations on chicken experience drastic shifts in composition and abundance over multiple stages during production including live production, poultry processing, retail display, and storage. While microbiome characterization throughout the "farm-to-fork" continuum is possible and has been attempted (22), a routine practice of microbiome monitoring, if justified, would require a more targeted ap-

TABLE 4 Comparison of *Salmonella* detection results by metagenomic sequencing and culture enrichment

Sample ^a	No. of reads ^b	No. of unique <i>k</i> -mers ^c	Culturing ^d	Real-time PCR	MetaPhlAn2 ^e	KrakenUniq ^f	KrakenUniq + PPR_Meta ^g	No. of screened reads ^h
A-I-CONV_1	122	2,189	– ⁱ	–	–	+	–	0
A-I-CONV_2	37	592	–	–	–	–	–	0
A-I-CONV_3	15	332	–	–	–	–	–	0
A-I-CONV_4	63	731	–	–	–	–	–	0
A-I-CONV_5	95	2,080	–	–	–	+	–	0
A-I-CONV_6	30	538	–	–	–	–	–	0
B-II-CONV_1	15	405	–	–	–	–	–	0
B-II-CONV_2	41	662	–	–	–	–	–	0
B-II-CONV_3	26	743	–	–	–	–	–	0
B-II-CONV_4	168	3,861	+ ^j	–	–	+	+	2
B-II-CONV_5	32	748	–	–	–	–	–	0
B-II-CONV_6	322	6,009	+	–	–	+	+	1
C-III-CONV_1	81	1,606	–	–	–	+	–	0
C-III-CONV_2	77	1,415	–	–	–	+	–	0
C-III-CONV_3	134	2,260	–	–	–	+	–	0
C-III-CONV_4	64	1,268	–	–	–	+	–	0
C-III-CONV_5	78	1,893	–	–	–	+	–	0
C-III-ABF_1	81	1,825	+	–	–	+	+	8
C-III-ABF_2	37	700	–	–	–	–	–	0
C-III-ABF_3	56	640	+	–	–	–	–	0
D-III-ABF-V_1	1,258	18,338	–	–	–	+	–	0
D-III-ABF-V_2	684	13,048	–	–	–	+	–	0
D-III-ABF-V_3	924	20,307	–	–	–	+	+	2
D-III-ABF-V_4	1,849	22,405	–	–	–	+	+	3
D-III-ABF-V_6	2,293	24,645	–	–	–	+	–	0
D-III-ABF_1	29	586	–	–	–	–	–	0
D-III-ABF_2	13	300	–	–	–	–	–	0
D-III-ABF_3	42	499	–	–	–	–	–	0
E-IV-ABF_1	142	3,164	–	–	–	+	–	0
E-IV-ABF_2	29	712	–	–	–	–	–	0
E-IV-ABF_3	60	1,183	–	–	–	+	–	0
E-IV-ABF_4	17	325	–	–	–	–	–	0
E-IV-ABF_6	112	403	–	–	–	–	–	0

^aTwo biological replicates were collected for each sample.

^bNumber of *S. enterica* reads reported by KrakenUniq. The results were reported as the averages from two replicates.

^cNumber of unique *k*-mers identified for *S. enterica* by KrakenUniq. The results were reported as the averages from two replicates.

^d*Salmonella* detection results by culturing. Sample was considered positive when a *Salmonella* colony was detected in either of the two replicates.

^e*Salmonella* detection results by MetaPhlAn2. Sample was considered positive when an *S. enterica* taxon was identified in either of the two replicates.

^f*Salmonella* detection results by KrakenUniq. Sample was considered positive when the number of unique *k*-mers for *S. enterica* was $\geq 1,000$ in either of the two replicates.

^g*Salmonella* detection results by KrakenUniq plus PPR_Meta. Sample was considered positive when at least one chromosomal read classified as *S. enterica* was detected in either of the two replicates.

^hNumbers of chromosomal reads classified as *S. enterica* after MGE filtering by PPR_Meta.

–, *Salmonella*-negative result.

+^j, *Salmonella*-positive result.

proach that is scientifically effective and logistically viable. Our study captured post-processing and preconsumption snapshots of chicken breast microbiomes. Our results suggest that chicken microbiomes at this junction allow robust metagenomics characterization through shotgun sequencing, retain sufficient information for microbiological evaluation of poultry processing, and provide multiple food safety and quality characteristics such as the presence of *Salmonella* in the final products that directly matter to consumers.

Overall, we observed distinct and temporally consistent chicken breast microbiomes associated with individual processing establishments. The observation indicates that variation in processing practices may lead to quantifiable and stable characteristics in finished product microbiomes. We envision that microbiome monitoring of poultry products can help establish a baseline of the regular chicken microbiome, which may be characteristic of individual processing establishments and potentially useful in signaling irregularities or perturbations to normal processing routines, such as change or failure of antimicrobial agents or protocols. As we have shown that poultry micro-

biomes inform the presence of *Salmonella* and the composition of spoilage organisms among other potential metagenomics markers of food safety and quality, the accumulation of baseline and irregular microbiome data may help classify or rate poultry microbiomes according to these markers, which may eventually lead to the evaluation of processing practices and environments.

Shotgun metagenomics sequencing promises unbiased and comprehensive characterization of food microbiomes unmatched by 16S rRNA amplicon sequencing. However, its efficiency is challenged by food matrices that have high ratios of food to microbial cells. In such cases, large proportions of sequencing output are food DNA sequences uninformative to food safety and quality. For instance, in a recent study, over 99% of shotgun sequencing data of beef microbiomes came from the bovine genome, leaving less than 1% of sequencing data attributed to bacteria (6). Chicken breast rinsates sampled in this study were much less contaminated by animal cells, resulting in an average bacterial yield being about half of the sequencing output. The relatively high efficiency of chicken microbiome sequencing allowed the coverage of the vast majority of microbial taxa in each microbiome sample by sequencing the equivalent of 5 or 6 samples in a MiSeq sequencing run (after combing reads from 2 replicate samples). These results suggest that sufficient characterization of chicken breast microbiome is possible through multiplexed shotgun sequencing, which can be relatively cost-effective and potentially practical for industrial applications.

In this study, we synchronized chicken breast microbiomes by holding samples under refrigeration until their designated “sell by” dates. During the holding storage, the microbiomes shifted from their earlier states off processing lines as indicated by increasing APC over time (chicken breast APC before “sell by” dates not shown). Despite the shift, overall microbiome profiles clustered by their association with particular processing establishments, and the clustering largely persisted throughout the sampling period with products up to 3 months apart. Such longitudinal consistency and cross-sectional distinctiveness suggest that chicken breast microbiomes on “sell by” dates carry over and retain sufficient metagenomic information that was reflective and characteristic of the processing environment at individual establishments.

Poultry processing converts live birds to finished products in multiple steps, including prescald brushing, scalding, defeathering, evisceration, rehang, on- and off-line reprocessing, carcass washing, chilling, and postchill treatment. Such steps can have substantial impact on the microbial ecology of processing environments and chicken meats. Some examples include microbial contamination due to gut rupture and gut content spillage during evisceration (23) and antimicrobial treatment during equipment spraying (24) and carcass washing (25). Complete microbial monitoring of all these steps can therefore be complex and logistically challenging for routine practices. Alternatively, after chicken meats go through the processing steps and pick up environmental influences along the way, the chicken meat microbiome constitutes an endpoint sample that represents the eventual consequences of processing environments on the finished products. It is possible that microbiome characterization of chicken meats right off processing provides closer indicators of microbial ecology of poultry processing. However, obtaining sufficient metagenomic DNA for shotgun sequencing from samples immediately after processing remains a challenge as we experienced in the current study. Such samples were much less abundant in microorganisms than samples held until “sell by” dates. According to our preliminary analysis of 3 brands of chicken breast, their APC ranged from 0.9 to 2.2 log₁₀ CFU/g when assayed on the same day of purchase. Similarly, APCs of beef trimmings in the United States are routinely between 2 and 3 log₁₀ CFU/g (26). The low microbial load on beef likely also contributed to the low output of microbial DNA in beef metagenomes (less than 1% compared with a mean of 49.9% in this study) as previously mentioned (6).

Interestingly, microbiomes of ABF samples under the same brand, from the same processing establishment, but collected 11 months apart diverged from each other (D-III-ABF-V_V versus D-III-ABF-V, Fig. 1). Different packaging barcodes and designs indicate different products. It is unknown whether any changes in the processing of the

later product occurred. In contrast, samples of the same product collected in warm and cold seasons from a North Carolina processing establishment did not show any significant difference in microbial composition. This finding was inconsistent with the seasonal differences observed in 16S amplicon sequencing-characterized chicken microbiomes from South Korea (27). In the South Korean study, seasonal samples were collected from months of maximum and lowest rates of processing (July and January), while year-round broiler production in the United States does not feature noticeable seasonality (28). It is unclear whether the disparity in production volumes, any other structural difference in the production system, or actual climatic seasonality caused the seasonal differences found in South Korean chicken microbiomes.

Our study also demonstrated the significant influence of vacuum packaging on chicken breast microbiomes. Microbiomes of vacuum-packaged samples were more diverse and characteristic of a variety of facultative anaerobic bacteria, compared with those of air-permeable samples that were dominated by *Pseudomonas*. Similarly, *Pseudomonas* was found to become dominant on chicken cuts under modified atmosphere packaging (MAP) when there was a higher percentage of oxygen in the MAP (29) and on beef chops during refrigerated storage in air (30). While inhibited, *Pseudomonas*, an obligate aerobe, was still present on vacuum-packaged samples, which is consistent with a previous observation that *Pseudomonas* was still able to grow on foal meat under refrigerated vacuum storage (30) and suggests the presence of residual oxygen in vacuum packages. *Pseudomonas* and *Shewanella*, which were dominant on chicken breast samples with air-permeable packaging, are major psychrotrophic bacteria that cause meat spoilage under aerobic storage conditions (31). On the other hand, *Aeromonas*, *Buttiauxella*, *Carnobacterium*, *Hafnia*, and *Serratia*, which were found to be more abundant on vacuum-packaged samples, commonly occur during chilled storage of raw meat in MAP or vacuum packaging (32). Our data suggest the potential of food microbiome characterization in evaluating and developing packaging technologies for extending product shelf life against spoilage microorganisms.

Excessive use of antibiotics in food animal production is perceived to be an important contributor to the rise of antibiotic resistance throughout food production environments (33). Shotgun metagenomics has been suggested to help quantitative risk assessment of antibiotic resistance in the food supply chain (33–35). Our resistome comparison between CONV and ABF samples identified no significant difference in ARG abundance and composition between the two production practices. Furthermore, ARG abundance of the chicken breast samples was considerably lower than that of any other livestock samples and all but one (U.S. soil) environmental samples analyzed in the current study. These results indicate a low risk of ARG accumulation on chicken breasts regardless of antibiotic usage in live production. Our finding is consistent with antimicrobial resistance surveys in beef production. It was reported that no ARG was detected on beef products and that interventions during slaughtering and beef processing might reduce the risk of ARG transmission to consumers (6). Similar levels of antimicrobial resistance were measured between conventional and “Raised Without Antibiotics” ground beef products (36) and beef cattle (37), which led to the doubt on the assumed impact of antimicrobial use in U.S. beef production and whether significant reductions in antimicrobial resistance could be yielded by reducing antimicrobial use. Although a recent study identified differences in resistomes between farms with different practices regarding antibiotic use (conventional versus “Raised Without Antibiotics”), the authors did not conclude a cause-effect relationship between antimicrobial use and the resistome differences because other factors (e.g., location, cattle source, management practices, and diet) could have affected the resistomes (38). It should be noted that our findings regarding the resistomes of ABF versus CONV samples were limited to finished chicken products. It remains to be investigated whether resistome differences related to antibiotic usage exist in any production environments, and whether such differences, if any, can be largely neutralized during poultry processing, similar to cattle processing (6).

Between chicken breasts, the resistomes of vacuum-packaged products had higher

normalized ARG abundances than the resistomes of air-permeable samples, largely due to the higher levels of beta-lactam-class ARGs in the vacuum-adapted resistomes (Fig. 5). The higher ARG abundances were unrelated to antibiotic use because all the vacuum-packaged products were also ABF. The perceived resistome differences between vacuum-packaged and air-permeable samples were caused by different microbiome compositions as the consequence of oxygen availability. For example, *Aeromonas* was abundant on vacuum-packaged samples and outcompeted on air-permeable samples, whereas *Pseudomonas* was dominant on air-permeable samples and inhibited on vacuum-packaged samples (Fig. 2a). Notably, *Pseudomonas* species have been reported to be antagonistic against *Aeromonas* (39). Network analysis revealed the cooccurrence of *Aeromonas* and 3 beta-lactam ARGs (*cphA1*, *cphA5*, and *bla*_{OXA-427}) in chicken breast microbiomes, which was substantiated by the fact that *cphA* genes were commonly found on *Aeromonas* chromosomes (40) and confirmed by ARG analysis of metagenome contigs (see Table S5 in the supplemental material). Therefore, metagenomics profiling of food resistome needs to be interpreted with caution when it comes to evaluating product safety, quality, and the impact of antibiotic usage during production. ARGs naturally occur; their presence and even elevated abundance in food microbiomes do not necessarily indicate selection by antibiotics or occurrence of multidrug-resistant organisms. As we showed, normalized ARG abundances (ARG copies per prokaryotic cell) vary among different bacterial populations, with densely populated populations such as livestock soil and gut (fecal) microbiomes displaying higher abundances. Abundant ARGs on food samples may be associated with certain production environments naturally rich in ARGs, which does not directly translate into higher food safety or quality risks. Therefore, food ARG abundances should not be interpreted in isolation but considered along with their bacterial hosts and environmental origins. While source attribution of ARGs using short-read metagenomics data is challenging, cooccurrence identification by network analysis as shown in the current and previous studies (41) can help reveal prominent associations.

Low abundances of *mcr* genes (variants of *mcr-3*, *mcr-4*, *mcr-7*, and *mcr-9*) were identified in 15 out of 33 retail chicken microbiomes. Notably, *mcr-3*-positive microbiomes ($n = 7$) had significantly higher relative abundances of *Aeromonas* than *mcr-3*-negative microbiomes ($n = 26$), indicating that *Aeromonas* might be a source of *mcr* genes in chicken microbiomes. Indeed, chromosome-mediated *mcr-3* variants have been found in *Aeromonas* isolates from chicken in China (42, 43). *Aeromonas* has also been speculated as a reservoir of *mcr-7* (44). Other bacterial hosts of *mcr* genes have also been identified in our chicken microbiomes, including *Klebsiella* (45) and *Shewanella* (46). Together, our results provide metagenomics evidence for the prevalence of *mcr* genes in poultry production (47), although we were not able to determine whether the *mcr* genes were plasmid-borne and/or carried by common foodborne pathogens such as *Escherichia coli* and *Salmonella*.

Finally, we showed that shotgun metagenomics data supported sensitive and specific detection of *S. enterica* from chicken breasts as benchmarked by the gold standard of culture enrichment. Shotgun metagenomics considerably outperformed real-time PCR in detecting *S. enterica* from the same DNA samples; the latter method failed to detect the pathogen in any of the 4 culture-confirmed chicken breast samples. These results suggest that taxonomic classification of sequencing reads increased detection sensitivity by allowing comprehensive screening for *S. enterica* signals that may come from multiple loci of the pathogen's genome. In comparison, real-time PCR targeted only a single locus and was less sensitive in detecting *S. enterica* when only fractional *S. enterica* genomes were present in DNA samples extracted from chicken breast rinsates.

On the other hand, the specificity of taxonomic classification was compromised by mobile genetic elements such as plasmids and phages. Using *k*-mer-based taxonomic classification alone, 17 out of the 33 samples were called *S. enterica* positive. A similar issue was observed in metagenomics detection of *S. enterica* from cattle feces and attributed to the presence of plasmid sequences in the reference database (48). In our

study, we found that exclusion of reads of likely plasmid and phage origins by PPR_Meta substantially increased detection specificity (from 0.52 to 0.93, Table 4). This method was particularly effective in correcting false-positive calls on vacuum-packaged samples by sole taxonomic classification, which yielded large quantities of *S. enterica*-like reads and unique *k*-mers for these samples (Table 4). It is possible that vacuum-packaged samples, whose microbiome compositions were distinct from those of air-permeable samples, contained non-*Salmonella* bacteria that shared plasmids or phages with *S. enterica*.

Sequencing coverage of metagenome can affect the sensitivity of *Salmonella* detection. In our study, except for 2 outliers that were excluded from analysis due to exceedingly low metagenomics coverage (22.5% and 55.5%), the estimated coverage of samples ranged from 75.6% to 93.8% (mean = 83.7%) (Table 2). These samples yielded a sensitivity of 75% in detecting *S. enterica* using the metagenomics approach. The results suggest that an estimated metagenomics coverage above 80% (~5.3 million reads) has the potential to detect *S. enterica* from the vast majority of contaminated chicken breast microbiomes. Higher coverages may improve detection sensitivity, but it cannot be statistically determined in our study due to the limited sample size ($n = 35$), and especially the small number of *Salmonella*-positive samples ($n = 4$). In fact, the only false-negative sample by metagenomics detection had a higher coverage (87.6% for CIII-ABF_3) than 2 of the 3 true-positive samples (77.6% for B-II-CONV_4 and 79.8% for B-II-CONV_4), suggesting stochastic coverage of a low-abundance organism in the microbiome. Future studies of larger scale may help determine optimal levels of metagenomics coverage that balance the sensitivity of pathogen detection and the demand for sequencing output.

The performance of metagenomics detection of *Salmonella* was benchmarked by culture enrichment that is commonly regarded as the gold standard for foodborne pathogen detection. However, the equivalency between metagenomics and culture detection may be skewed by the presence of *Salmonella* cells that are difficult or unable to culture. The so-called viable but nonculturable (VBNC) state of *Salmonella* cells can be induced by adverse environmental conditions such as chlorine treatment (49) and starvation (50). Dead pathogen cells whose DNA molecules are still present in food samples are considered to be a source of false-positive results by DNA-based detection methods incapable of distinguishing between signals from live and dead cells (51). In our study, *Salmonella* was detected by metagenomics sequencing in 2 samples that were *Salmonella* negative by culture enrichment, which could be attributed to difficult-to-culture or dead *Salmonella* cells. Further validation of metagenomics detection of foodborne pathogens should evaluate the likelihood that DNA from dead cells could be sequenced from food samples and whether removal of extracellular DNA during sample preparation could mitigate or avoid sequencing DNA from dead cells. While a *Salmonella*-positive chicken microbiome does not definitively suggest the presence of live and infectious *Salmonella* cells on the product, it indicates the occurrence of *Salmonella* in the processing environment. Recent advances in quantitative metagenomics analysis, such as the use of synthetic internal DNA standards (52), may allow robust qualification of pathogen or indicator DNA in food microbiomes, which could help develop probabilistic or quantitative risk assessment of microbial contamination in processing establishments.

Metagenomics detection of *Salmonella* has also been performed by 16S rRNA amplicon sequencing using universal primers for 16S rRNA genes on retail chicken products from France (29) and South Korea (8). Neither study detected *Salmonella*; it is not known whether the negative results reflected low *Salmonella* prevalence on retail chicken in these countries or a difference in *Salmonella* detection sensitivity between 16S rRNA amplicon and shotgun sequencing. A recent comparison between the two metagenomics approaches in detecting foodborne bacteria suggested superior performance by shotgun sequencing (53). Using a mock bacterial community, 16S rRNA amplicon sequencing based on universal primers showed a lack of consistency and missed several pathogenic species (53). Nonetheless, it is possible to use specific

primers for targeted amplicon sequencing to improve the detection of particular pathogens. 16S rRNA amplicon sequencing is relatively cost-effective and able to characterize communities with low DNA levels, which can have advantageous applications.

Conclusion. Our study represents a proof-of-concept investigation into the possibility and practicality of leveraging microbiome-informed food safety and quality. Through a longitudinal and cross-sectional survey of a household item, we established the chicken microbiome as a robust and multifaceted food microbiology attribute that could provide a variety of safety and quality information.

The methodology described here can apply to studying other food commodities. The diversity of foods and their microbiomes warrants expanding the investigation to more food matrices to better explore and evaluate metagenomics applications in food safety and quality.

MATERIALS AND METHODS

Sample collection and preparation. Eight different retail boneless and skinless chicken breast products under 5 brands (A, B, C, D, and E) were purchased from grocery stores in Griffin (Atlanta metropolitan area), Georgia. For 7 products, a total of 35 samples were collected between July 2017 and January 2018; these chicken samples were collected every other week for 3 to 6 months depending on their retail availability. Two more samples of an additional product were later collected in June 2018 to confirm the effect of vacuum packaging on the product microbiome. All 37 samples of the 8 products were traced back to 4 processing establishments (I, II, III, and IV) in Georgia, Arkansas, and North Carolina using the FSIS establishment numbers (54) on the packages. Five of the 8 products were considered antibiotic free (ABF) according to the “No Antibiotics Ever” package label. The other 3 without any antibiotic usage claim were classified as conventional products (CONV). Two products of 8 ABF samples under brand D and from processing establishment III were vacuum packaged (D-III-ABF-V and D-III-ABV-V_V/A); these products were sealed in thick oxygen-impermeable film. Other samples had air-permeable packaging. Detailed sample information is summarized in Table 1. For each sample, two packages of the same product were purchased and prepared as biological replicates. With the exception of sequencing coverage estimation, for any quantitative metagenomics measurement detailed below, each replicate was individually analyzed and the average of two measurements was taken. For the coverage estimation and the *k*-mer-based clustering analyses of chicken microbiomes, sequencing reads from the two replicates of each sample were pooled and analyzed.

Breast samples were stored at 4°C until their designated “sell by” dates. On the “sell by” date, each sample was inspected; no visual sign of spoilage and off-odor was detected. Each aliquot of 500 g meat was rinsed in 150 ml buffered peptone water (BPW; Acumedia, USA) by hand massage for 2 min. Thirty milliliters of the rinsate was collected for aerobic plate count and culture-based detection of *Salmonella*. One hundred milliliters of the rinsate was pelleted by a two-step centrifugation before DNA extraction. The rinsate was first centrifuged at $100 \times g$ for 10 min to remove heavier food particles. The supernatant was transferred to another tube and centrifuged at $5,000 \times g$ for 15 min to collect bacterial cells. Each cell pellet was washed by resuspension in 10 ml of $1 \times$ phosphate-buffered saline (PBS; Fisher Scientific, USA) followed by another round of centrifugation at $5,000 \times g$ for 15 min. Finally, the pellet was resuspended in 5 ml of $1 \times$ PBS and used for DNA extraction.

Aerobic plate count. Aerobic plate count was determined for each rinsate sample. Serial dilutions (10^{-1} to 10^{-4}) of the rinsates were prepared in BPW, and enumerations were performed on plate count agar (PCA; Becton, Dickinson, USA) according to the *Bacteriological Analytical Manual* (BAM) (55). Plate count agars were incubated at 35°C for 48 h, and single colonies were enumerated from the appropriate dilutions.

Culture-based detection of *Salmonella*. Culture-based detection of *Salmonella* was conducted according to the *Microbiology Laboratory Guidebook* (USDA-FSIS) (56) with modifications. Briefly, each 30-ml aliquot of BPW chicken rinsate was incubated at 37°C for 24 h. An 0.1-ml amount of each preenriched rinsate was transferred to 10 ml of Rappaport-Vassiliadis soya (RV) broth (Acumedia, USA) and incubated at 42°C for 24 h. After the selective enrichment in RV, a loopful of the enriched sample was streaked on xylose-lysine-Tergitol 4 agar (XLT4 agar; Becton, Dickinson, USA) and incubated at 37°C for 24 h. The presumptive *Salmonella* colonies from the XLT4 agar were plated on triple sugar iron agar (TSI; Becton, Dickinson, USA) and lysine iron agar (LIA; Acumedia, USA) and incubated at 37°C for 24 h, followed by confirmation using real-time PCR. Real-time PCR for the detection of *Salmonella* was conducted as previously described (57). Samples with a threshold cycle (C_t) value of ≥ 40 or higher than that of negative control were considered negative results.

DNA extraction and real-time PCR detection of *Salmonella* in DNA samples. Pellets of chicken rinsate samples were subjected to DNA extraction using the PowerSoil extraction kit (Mo Bio, USA) according to the manufacturer's instructions. DNA concentrations were determined using a Qubit BR double-stranded DNA (dsDNA) assay kit (Invitrogen, USA) and were diluted to 0.2 ng/ μ l for library preparation. Real-time PCR for *Salmonella* detection in DNA samples was performed as described above.

Shotgun metagenomic sequencing. DNA libraries were prepared according to the Illumina Nextera XT DNA library prep kit reference guide. Shotgun metagenomic sequencing was performed on an Illumina MiSeq platform with the paired-end sequencing strategy (2×250 bp). Initial quality inspection

of the sequencing data was conducted using FastQC (58). Low-quality reads were removed or trimmed by Trimmomatic (59). The leading three and the trailing three nucleotides were removed from the reads, and a 4-nucleotide sliding window was used to remove nucleotides from the 3' ends when the average Phred score dropped below 20. After trimming, reads that were shorter than 75 bp were discarded.

Taxonomic classification of sequencing reads. Trimmed and filtered reads were taxonomically classified using KrakenUniq (60) with the NCBI nucleotide collection (nt) database (as of August 2018), which yielded the highest average recall according to KrakenUniq's performance test (60). Reads classified as plasmid or phage sequences were discarded after taxonomic classification. The relative abundances of microbial genus and species in sequencing samples were then determined using Bracken (61).

Estimation of microbial diversity coverage by shotgun metagenomics sequencing. Reads classified as "Bacteria" by KrakenUniq were used for further microbiome analyses. Nonpareil (v3.0) (62) was used to estimate average sequencing coverage of bacterial diversity in each microbiome sample.

Similarity and clustering analyses of chicken breast microbiomes. Pairwise Bray-Curtis dissimilarity was calculated between shotgun metagenomics samples using Simka with default settings (63). Simka computed the dissimilarities based on *k*-mer counts from sequencing reads classified as bacteria. Hierarchical clustering and principal-coordinate analysis (PCoA) were performed using Bray-Curtis dissimilarities with the base R package (64).

Alpha and beta diversities of chicken breast microbiomes. Genus-level read counts obtained from analyses by KrakenUniq and Bracken were used for alpha and beta diversity calculations. Alpha diversities were calculated using the Shannon index in the base R package and the Simpson index in the Vegan R package (65). Beta diversities were calculated using the Bray-Curtis dissimilarities in the Vegan R package. Genus richness was also calculated using the Vegan R package.

ARG identification from chicken breast microbiomes. Trimmed and filtered sequencing reads were screened for ARGs using the ARGs-OAP v2.0 (66) pipeline with default settings except using a customized reference database. ARGs were identified by aligning reads against the ResFinder database (67) (as of June 2019) using BLAST+ blastn (68) with E value $<1 \times 10^{-7}$. A read was annotated as an ARG sequence if its best hit had $>80\%$ identity and the alignment length was >75 bp (66). ARG sequence abundances were normalized against a curated list of single-copy genes known as the universal essential single-copy marker genes available in the ARGs-OAP pipeline (66). The abundance of ARG was expressed as copies of ARG per prokaryote cell.

ARGs were also identified from assembled metagenome sequences. Metagenomes were *de novo* assembled using classified bacterial reads (including plasmid and phage reads) with MEGAHIT (69). Only contigs over 1 kb were kept for analysis and merged at 99% identity with CD-HIT-EST (70). The resulting contigs were assembled again using Minimus2 from the AMOS package (71). A median of 6,719 contigs was obtained for each sample, and the median N_{50} for these contigs was 2,751 bp (data not shown). These contigs were screened for ARGs against ResFinder (67) with ABRicate (72). Only hits with sequence identity $>80\%$ and query coverage $>80\%$ were kept. To determine the occurrence of ARGs in chromosomes or plasmids, we used BLAST+ blastn to align the metagenome contigs against the NCBI nt database. Hits with sequence identity $>70\%$ and query coverage $>70\%$ were kept.

Statistical and network analyses. Statistical comparisons of microbiome diversities were performed using Mann-Whitney *U* test (2 levels) or Kruskal-Wallis test (>2 levels) in the base R package. Permutational analysis of variance (PERMANOVA) was performed to compare community difference among different categories (e.g., ABF versus CONF) using the "adonis" function in the Vegan R package.

For network analysis of microbial and ARG abundances, we first screened 48 ARGs that occurred in at least 5 retail chicken samples, and 65 microbial genera that occurred in at least 10 retail chicken samples. This filtering step was performed to remove the poorly represented ARGs and microbial genera with limited occurrence across samples and thus reduce artificial association bias (41). We then constructed a correlation matrix by calculating all possible pairwise Spearman's rank correlations among ARGs and microbial genera. Only correlations with the Spearman correlation coefficient (ρ) >0.8 and the *P* value <0.01 were considered statistically robust (41). The *P* values were adjusted using the Benjamini-Hochberg method (73). The robust pairwise correlations were then used to build a cooccurrence network between ARGs and microbial genera. The correlation analysis was performed using the psych R package (74), and the cooccurrence network was visualized by Gephi (75).

Salmonella detection from chicken breast microbiomes. We compared different approaches for *Salmonella* detection from the metagenomic shotgun sequencing data. (i) Taxonomic classification of the sequencing reads was performed by MetaPhlan2 with its default marker database (76). Only samples with an assignment of "s_Salmonella_enterica" were considered positive results. (ii) Taxonomic classification was performed by KrakenUniq with the NCBI nucleotide collection (nt) database. Only samples with unique *k*-mer numbers $\geq 1,000$ for *Salmonella enterica* were considered positive results (60). (iii) To reduce the false-positive *Salmonella* detection caused by *Salmonella*-like mobile genetic elements, such as plasmids and phages, that were present in non-*Salmonella* organisms, reads classified as "*Salmonella enterica*" by KrakenUniq were analyzed by PPR_Meta with default settings (77) to determine whether these sequences came from chromosomes, plasmids, or phages. Only samples with at least one chromosomal read classified as *S. enterica* were considered *Salmonella* positive.

Other microbiomes. Publicly available shotgun metagenomics data of various poultry and non-poultry samples were analyzed in comparison with chicken breast microbiomes. These samples included chicken litter (USA, $n = 5$), chicken carcass wash (Italy, $n = 5$), chicken cecal content (Italy, $n = 5$), chicken feces (China, $n = 3$), cattle feces (USA, $n = 5$; China, $n = 5$), cattle soil (USA, $n = 5$), pig feces (China, $n = 3$; Europe $n = 5$), human feces (American, $n = 5$; Chinese, $n = 5$; Spanish, $n = 5$; Danish, $n = 5$), municipal

sludge (Hong Kong, $n = 6$), hospital sewage (Germany, $n = 6$), and agricultural soil (USA, $n = 5$). These microbiomes were summarized in Table S6 in the supplemental material.

Availability of data and materials. The sequencing data from this study are available in the NCBI Sequence Read Archive (SRA) under BioProject accession number [PRJNA612140](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA612140). Accession numbers for the publicly available shotgun metagenomics data used in the study are listed in Table S6.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 1.5 MB.

FIG S2, TIF file, 0.3 MB.

FIG S3, TIF file, 2.1 MB.

FIG S4, TIF file, 0.7 MB.

TABLE S1, XLSX file, 0.05 MB.

TABLE S2, XLSX file, 0.03 MB.

TABLE S3, XLSX file, 0.01 MB.

TABLE S4, XLSX file, 0.01 MB.

TABLE S5, XLSX file, 0.01 MB.

TABLE S6, XLSX file, 0.01 MB.

ACKNOWLEDGMENTS

This work was supported in part by a Hatch project (1006141) from the USDA National Institute of Food and Agriculture.

We thank Brian Fairchild, Harshavardha Thippareddi, and Mark Berrang for helpful discussion.

REFERENCE

- Yang X, Noyes NR, Doster E, Martin JN, Linke LM, Magnuson RJ, Yang H, Geornaras I, Woerner DR, Jones KL, Ruiz J, Boucher C, Morley PS, Belk KE. 2016. Use of metagenomic shotgun sequencing technology to detect foodborne pathogens within the microbiome of the beef production chain. *Appl Environ Microbiol* 82:2433–2443. <https://doi.org/10.1128/AEM.00078-16>.
- Chaillou S, Chaulet-Talmon A, Caekebeke H, Cardinal M, Christieans S, Denis C, Desmots MH, Dousset X, Feurer C, Hamon E, Joffraud JJ, La Carbona S, Leroi F, Leroy S, Lorre S, Mace S, Pilet MF, Prevost H, Rivollier M, Roux D, Talon R, Zagorec M, Champomier-Verges MC. 2015. Origin and ecological selection of core and food-specific bacterial communities associated with meat and seafood spoilage. *ISME J* 9:1105–1118. <https://doi.org/10.1038/ismej.2014.202>.
- Jarvis KG, Daquigan N, White JR, Morin PM, Howard LM, Manetas JE, Ottesen A, Ramachandran P, Grim CJ. 2018. Microbiomes associated with foods from plant and animal sources. *Front Microbiol* 9:2540. <https://doi.org/10.3389/fmicb.2018.02540>.
- Ottesen AR, Gonzalez Pena A, White JR, Pettengill JB, Li C, Allard S, Rideout S, Allard M, Hill T, Evans P, Strain E, Musser S, Knight R, Brown E. 2013. Baseline survey of the anatomical microbial ecology of an important food plant: *Solanum lycopersicum* (tomato). *BMC Microbiol* 13:114. <https://doi.org/10.1186/1471-2180-13-114>.
- Tan X, Chung T, Chen Y, Macarasin D, LaBorde L, Kovac J. 2019. The occurrence of *Listeria monocytogenes* is associated with built environment microbiota in three tree fruit processing facilities. *Microbiome* 7:115. <https://doi.org/10.1186/s40168-019-0726-2>.
- Noyes NR, Yang X, Linke LM, Magnuson RJ, Dettenwanger A, Cook S, Geornaras I, Woerner DE, Gow SP, McAllister TA, Yang H, Ruiz J, Jones KL, Boucher CA, Morley PS, Belk KE. 2016. Resistome diversity in cattle and the environment decreases during beef production. *Elife* 5:e13195. <https://doi.org/10.7554/eLife.13195>.
- Handley JA, Park SH, Kim SA, Ricke SC. 2018. Microbiome profiles of commercial broilers through evisceration and immersion chilling during poultry slaughter and the identification of potential indicator microorganisms. *Front Microbiol* 9:345. <https://doi.org/10.3389/fmicb.2018.00345>.
- Lee SI, Choi J, Daeschel DZ, Park SH. 2019. Microbiome characterization of poultry products based on the poultry part and production label. *FEMS Microbiol Lett* 366:fnz092. <https://doi.org/10.1093/femsle/fnz092>.
- USDA FSIS. 2012. The nationwide microbiological baseline data collection program: raw chicken parts survey, January 2012–August 2012. Food Safety and Inspection Service, US Department of Agriculture, Washington, DC. https://www.fsis.usda.gov/shared/PDF/Baseline_Data_Raw_Chicken_Parts.pdf. Accessed 28 February 2020.
- Ritchie H, Roser M. 2019. Meat and dairy production. <https://ourworldindata.org/meat-production>. Accessed 28 February 2020.
- USDA ERS. 2019. Per capita availability of chicken higher than that of beef. Economic Research Service, US Department of Agriculture, Washington, DC. <https://www.ers.usda.gov/data-products/chart-gallery/gallery/chart-detail?chartId=58312>. Accessed 28 February 2020.
- Chai SJ, Cole D, Nisler A, Mahon BE. 2017. Poultry: the most common food in outbreaks with known pathogens, United States, 1998–2012. *Epidemiol Infect* 145:316–325. <https://doi.org/10.1017/S0950268816002375>.
- Gyles CL. 2008. Antimicrobial resistance in selected bacteria from poultry. *Anim Health Res Rev* 9:149–158. <https://doi.org/10.1017/S1466252308001552>.
- FDA. 2017. 2015 NARMS integrated report. US Food and Drug Administration, Washington, DC. <https://www.fda.gov/animal-veterinary/national-antimicrobial-resistance-monitoring-system/2015-narms-integrated-report>. Accessed 28 February 2020.
- FDA. 2019. 2016–2017 NARMS integrated summary. US Food and Drug Administration, Washington, DC. <https://www.fda.gov/animal-veterinary/national-antimicrobial-resistance-monitoring-system/2016-2017-narms-integrated-summary>. Accessed 28 February 2020.
- 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>.
- Casaburi A, Piombino P, Nychas GJ, Villani F, Ercolini D. 2015. Bacterial populations and the volatolome associated to meat spoilage. *Food Microbiol* 45:83–102. <https://doi.org/10.1016/j.fm.2014.02.002>.
- USDA NASS. 2019. Poultry - production and value 2018 summary. National Agricultural Statistics Service, US Food and Drug Administration, Washington, DC. https://www.nass.usda.gov/Publications/Todays_Reports/reports/plva0519.pdf. Accessed 28 February 2020.
- USDA FSIS. 2014. FSIS compliance guide: modernization of poultry slaughter inspection: chilling requirements. Food Safety and Inspection Service, US Food and Drug Administration, Washington, DC. <https://www.fsis.usda.gov/wps/wcm/connect/7a0a728e-3b29-49e9-9c1b-ec55f2f04887/Chilling-Requirements-1014.pdf?MOD=AJPERES>. Accessed 21 June 2020.

20. FDA CVM. 2018. 2017 summary report on antimicrobials sold or distributed for use in food-producing animals. Center for Veterinary Medicine, US Food and Drug Administration, Washington, DC. <https://www.fda.gov/media/119332/download>. Accessed 28 February 2020.
21. Guran HS, Mann D, Alali WQ. 2017. Salmonella prevalence associated with chicken parts with and without skin from retail establishments in Atlanta metropolitan area, Georgia. *Food Control* 73:462–467. <https://doi.org/10.1016/j.foodcont.2016.08.038>.
22. Oakley BB, Morales CA, Line J, Berrang ME, Meinersmann RJ, Tillman GE, Wise MG, Siragusa GR, Hiett KL, Seal BS. 2013. The poultry-associated microbiome: network analysis and farm-to-fork characterizations. *PLoS One* 8:e57190. <https://doi.org/10.1371/journal.pone.0057190>.
23. Gill C. 2005. Sources of microbial contamination at slaughtering plants, p 231–243. In Sofos JN (ed), *Improving the safety of fresh meat*. Woodhead Publishing Ltd, Cambridge, United Kingdom.
24. Bailey J, Thomson J, Cox N, Shackelford A. 1986. Chlorine spray washing to reduce bacterial contamination of poultry processing equipment. *Poult Sci* 65:1120–1123. <https://doi.org/10.3382/ps.0651120>.
25. Kemp GK, Aldrich ML, Waldroup AL. 2000. Acidified sodium chlorite antimicrobial treatment of broiler carcasses. *J Food Prot* 63:1087–1092. <https://doi.org/10.4315/0362-028x-63.8.1087>.
26. USDA FSIS. 2011. National prevalence estimate of pathogens in domestic beef manufacturing trimmings (trim). Food Safety and Inspection Service, US Food and Drug Administration, Washington, DC. https://www.fsis.usda.gov/wps/wcm/connect/f07f5e1d-63f2-4ec8-a83a-e1661307b2c3/Baseline_Data_Domestic_Beef_Trimmings_Rev.pdf?MOD=AJPERES. Accessed 21 June 2020.
27. Kim H-E, Lee J-J, Lee M-J, Kim B-S. 2019. Analysis of microbiome in raw chicken meat from butcher shops and packaged products in South Korea to detect the potential risk of foodborne illness. *Food Res Int* 122:517–527. <https://doi.org/10.1016/j.foodres.2019.05.032>.
28. USDA ERS. 2019. Livestock, dairy, and poultry outlook. Economic Research Service, US Department of Agriculture, Washington, DC. <https://www.ers.usda.gov/publications/pub-details/?pubid=92608>. Accessed 28 February 2020.
29. Rouger A, Moriceau N, Prevost H, Remenant B, Zagorec M. 2018. Diversity of bacterial communities in French chicken cuts stored under modified atmosphere packaging. *Food Microbiol* 70:7–16. <https://doi.org/10.1016/j.fm.2017.08.013>.
30. Ercolini D, Ferrocino I, Nasi A, Ndagijimana M, Vernocchi P, La Stora A, Laghi L, Mauriello G, Guerzoni ME, Villani F. 2011. Monitoring of microbial metabolites and bacterial diversity in beef stored under different packaging conditions. *Appl Environ Microbiol* 77:7372–7381. <https://doi.org/10.1128/AEM.05521-11>.
31. Nychas G-JE, Skandamis PN, Tassou CC, Koutsoumanis KP. 2008. Meat spoilage during distribution. *Meat Sci* 78:77–89. <https://doi.org/10.1016/j.meatsci.2007.06.020>.
32. Doulgeraki AI, Ercolini D, Villani F, Nychas GJ. 2012. Spoilage microbiota associated to the storage of raw meat in different conditions. *Int J Food Microbiol* 157:130–141. <https://doi.org/10.1016/j.ijfoodmicro.2012.05.020>.
33. Bengtsson-Palme J. 2017. Antibiotic resistance in the food supply chain: where can sequencing and metagenomics aid risk assessment? *Curr Opin Food Sci* 14:66–71. <https://doi.org/10.1016/j.cofs.2017.01.010>.
34. Oniciuc EA, Likotrafti E, Alvarez-Molina A, Prieto M, Santos JA, Alvarez-Ordóñez A. 2018. The present and future of whole genome sequencing (WGS) and whole metagenome sequencing (WMS) for surveillance of antimicrobial resistant microorganisms and antimicrobial resistance genes across the food chain. *Genes* 9:268. <https://doi.org/10.3390/genes9050268>.
35. De Filippis F, Parente E, Ercolini D. 2018. Recent past, present, and future of the food microbiome. *Annu Rev Food Sci Technol* 9:589–608. <https://doi.org/10.1146/annurev-food-030117-012312>.
36. Vikram A, Miller E, Arthur TM, Bosilevac JM, Wheeler TL, Schmidt JW. 2018. Similar levels of antimicrobial resistance in U.S. food service ground beef products with and without a “Raised without Antibiotics” claim. *J Food Prot* 81:2007–2018. <https://doi.org/10.4315/0362-028X.JFP-18-299>.
37. Vikram A, Rovira P, Agga GE, Arthur TM, Bosilevac JM, Wheeler TL, Morley PS, Belk KE, Schmidt JW. 2017. Impact of “Raised without Antibiotics” beef cattle production practices on occurrences of antimicrobial resistance. *Appl Environ Microbiol* 83:e01682-17. <https://doi.org/10.1128/AEM.01682-17>.
38. Rovira P, McAllister T, Lakin SM, Cook SR, Doster E, Noyes NR, Weinroth MD, Yang X, Parker JK, Boucher C, Booker CW, Woerner DR, Belk KE, Morley PS. 2019. Characterization of the microbial resistome in conventional and “Raised Without Antibiotics” beef and dairy production systems. *Front Microbiol* 10:1980. <https://doi.org/10.3389/fmicb.2019.01980>.
39. Das BK, Samal SK, Samantaray BR, Sethi S, Pattnaik P, Mishra BK. 2006. Antagonistic activity of cellular components of *Pseudomonas* species against *Aeromonas hydrophila*. *Aquaculture* 253:17–24. <https://doi.org/10.1016/j.aquaculture.2005.01.028>.
40. Massidda O, Rossolini GM, Satta G. 1991. The *Aeromonas hydrophila* cphA gene: molecular heterogeneity among class B metallo-beta-lactamases. *J Bacteriol* 173:4611–4617. <https://doi.org/10.1128/jb.173.15.4611-4617.1991>.
41. Li B, Yang Y, Ma L, Ju F, Guo F, Tiedje JM, Zhang T. 2015. Metagenomic and network analysis reveal wide distribution and co-occurrence of environmental antibiotic resistance genes. *ISME J* 9:2490–2502. <https://doi.org/10.1038/ismej.2015.59>.
42. Ling Z, Yin W, Li H, Zhang Q, Wang X, Wang Z, Ke Y, Wang Y, Shen J. 2017. Chromosome-mediated mcr-3 variants in *Aeromonas veronii* from chicken meat. *Antimicrob Agents Chemother* 61:e01272-17. <https://doi.org/10.1128/AAC.01272-17>.
43. Shen Y, Xu C, Sun Q, Schwarz S, Ou Y, Yang L, Huang Z, Eichhorn I, Walsh TR, Wang Y, Zhang R, Shen J. 2018. Prevalence and genetic analysis of mcr-3-positive *Aeromonas* species from humans, retail meat, and environmental water samples. *Antimicrob Agents Chemother* 62:e00404-18. <https://doi.org/10.1128/AAC.00404-18>.
44. Liu D, Song H, Ke Y, Xia J, Shen Y, Ou Y, Hao Y, He J, Li X, Zhou Y, Fu J, Wang Y, Lv Z, Wu C. 2020. Co-existence of two novel phosphoethanolamine transferase gene variants in *Aeromonas jandaei* from retail fish. *Int J Antimicrob Agents* 55:105856. <https://doi.org/10.1016/j.ijantimicag.2019.11.013>.
45. Yang YQ, Li YX, Lei CW, Zhang AY, Wang HN. 2018. Novel plasmid-mediated colistin resistance gene mcr-7.1 in *Klebsiella pneumoniae*. *J Antimicrob Chemother* 73:1791–1795. <https://doi.org/10.1093/jac/dky111>.
46. Carattoli A, Villa L, Feudi C, Curcio L, Orsini S, Luppi A, Pezzotti G, Magistrali CF. 2017. Novel plasmid-mediated colistin resistance mcr-4 gene in *Salmonella* and *Escherichia coli*, Italy 2013, Spain and Belgium, 2015 to 2016. *Euro Surveill* 22:30589. <https://doi.org/10.2807/1560-7917.ES.2017.22.31.30589>.
47. Nang SC, Li J, Velkov T. 2019. The rise and spread of mcr plasmid-mediated polymyxin resistance. *Crit Rev Microbiol* 45:131–161. <https://doi.org/10.1080/1040841X.2018.1492902>.
48. Doster E, Rovira P, Noyes NR, Burgess BA, Yang X, Weinroth MD, Linke L, Magnuson R, Boucher C, Belk KE, Morley PS. 2019. A cautionary report for pathogen identification using shotgun metagenomics; a comparison to aerobic culture and polymerase chain reaction for *Salmonella enterica* identification. *Front Microbiol* 10:2499. <https://doi.org/10.3389/fmicb.2019.02499>.
49. Leriche V, Carpentier B. 1995. Viable but nonculturable *Salmonella typhimurium* in single- and binary-species biofilms in response to chlorine treatment. *J Food Prot* 58:1186–1191. <https://doi.org/10.4315/0362-028X-58.11.1186>.
50. Chmielewski RA, Frank JF. 1995. Formation of viable but nonculturable *Salmonella* during starvation in chemically defined solutions. *Lett Appl Microbiol* 20:380–384. <https://doi.org/10.1111/j.1472-765x.1995.tb01326.x>.
51. González-Escalona N, Hammack TS, Russell M, Jacobson AP, De Jesús AJ, Brown EW, Lampel KA. 2009. Detection of live *Salmonella* sp. cells in produce by a TaqMan-based quantitative reverse transcriptase real-time PCR targeting invA mRNA. *Appl Environ Microbiol* 75:3714–3720. <https://doi.org/10.1128/AEM.02686-08>.
52. Hardwick SA, Chen WY, Wong T, Kanakamedala BS, Deveson IW, Ongley SE, Santini NS, Marcellin E, Smith MA, Nielsen LK, Lovelock CE, Neilan BA, Mercer TR. 2018. Synthetic microbe communities provide internal reference standards for metagenome sequencing and analysis. *Nat Commun* 9:3096. <https://doi.org/10.1038/s41467-018-05555-0>.
53. Grutzke J, Malorny B, Hammerl JA, Busch A, Tausch SH, Tomaso H, Deneke C. 2019. Fishing in the soup—pathogen detection in food safety using metabarcoding and metagenomic sequencing. *Front Microbiol* 10:1805. <https://doi.org/10.3389/fmicb.2019.01805>.
54. USDA FSIS. 2020. Meat, poultry and egg product inspection directory. Food Safety and Inspection Service, US Food and Drug Administration,

- Washington, DC. <https://origin-www.fsis.usda.gov/wps/portal/fsis/topics/inspection/mpi-directory>. Accessed 28 February 2020.
55. Maturin L, Peeler JT. 2001. Bacteriological analytical manual—chapter 3 aerobic plate count. US Food and Drug Administration, Washington, DC. <https://www.fda.gov/food/laboratory-methods-food/bam-aerobic-plate-count>. Accessed 1 July 2017.
 56. USDA FSIS. 2017. Microbiology laboratory guidebook—isolation and identification of Salmonella from meat, poultry, pasteurized egg, and siluriformes (fish) products and carcass and environmental sponges. Food Safety and Inspection Service, US Food and Drug Administration, Washington, DC. <https://www.fsis.usda.gov/wps/wcm/connect/700c05fe-06a2-492a-a6e1-3357f7701f52/mlg-4.pdf?MOD=AJPERES>. Accessed 1 July 2017.
 57. Hyeon JY, Deng X. 2017. Rapid detection of Salmonella in raw chicken breast using real-time PCR combined with immunomagnetic separation and whole genome amplification. *Food Microbiol* 63:111–116. <https://doi.org/10.1016/j.fm.2016.11.007>.
 58. Andrews S. 2010. FastQC: a quality control tool for high throughput sequence data. Babraham Bioinformatics, Babraham Institute, Cambridge, United Kingdom. <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>. Accessed 1 January 2018.
 59. Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30:2114–2120. <https://doi.org/10.1093/bioinformatics/btu170>.
 60. Breitwieser FP, Baker DN, Salzberg SL. 2018. KrakenUniq: confident and fast metagenomics classification using unique k-mer counts. *Genome Biol* 19:198. <https://doi.org/10.1186/s13059-018-1568-0>.
 61. Lu J, Breitwieser FP, Thielen P, Salzberg SL. 2017. Bracken: estimating species abundance in metagenomics data. *PeerJ Comput Sci* 3:e104. <https://doi.org/10.7717/peerj-cs.104>.
 62. Rodriguez RL, Gunturu S, Tiedje JM, Cole JR, Konstantinidis KT. 2018. Nonpareil 3: fast estimation of metagenomic coverage and sequence diversity. *mSystems* 3:e00039-18. <https://doi.org/10.1128/mSystems.00039-18>.
 63. Benoit G, Peterlongo P, Mariadassou M, Drezen E, Schbath S, Lavenier D, Lemaitre C. 2016. Multiple comparative metagenomics using multiset k-mer counting. *PeerJ Comput Sci* 2:e94. <https://doi.org/10.7717/peerj-cs.94>.
 64. R Core Team. 2013. R: a language and environment for statistical computing. <http://www.R-project.org>. Accessed 1 January 2018.
 65. Dixon P. 2003. VEGAN, a package of R functions for community ecology. *J Veg Sci* 14:927–930. <https://doi.org/10.1111/j.1654-1103.2003.tb02228.x>.
 66. Yin X, Jiang XT, Chai B, Li L, Yang Y, Cole JR, Tiedje JM, Zhang T. 2018. ARGs-OAP v2.0 with an expanded SARG database and Hidden Markov Models for enhancement characterization and quantification of antibiotic resistance genes in environmental metagenomes. *Bioinformatics* 34:2263–2270. <https://doi.org/10.1093/bioinformatics/bty053>.
 67. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM, Larsen MV. 2012. Identification of acquired antimicrobial resistance genes. *J Antimicrob Chemother* 67:2640–2644. <https://doi.org/10.1093/jac/dks261>.
 68. Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden TL. 2009. BLAST+: architecture and applications. *BMC Bioinformatics* 10:421. <https://doi.org/10.1186/1471-2105-10-421>.
 69. Li D, Liu CM, Luo R, Sadakane K, Lam TW. 2015. MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics* 31:1674–1676. <https://doi.org/10.1093/bioinformatics/btv033>.
 70. Fu L, Niu B, Zhu Z, Wu S, Li W. 2012. CD-HIT: accelerated for clustering the next-generation sequencing data. *Bioinformatics* 28:3150–3152. <https://doi.org/10.1093/bioinformatics/bts565>.
 71. Treangen TJ, Sommer DD, Angly FE, Koren S, Pop M. 2011. Next generation sequence assembly with AMOS. *Curr Protoc Bioinformatics Chapter 11:Unit 11.8*. <https://doi.org/10.1002/0471250953.bi1108s33>.
 72. Seemann T. 2019. Mass screening of contigs for antimicrobial and virulence genes. <https://github.com/tseemann/abricate>. Accessed 1 March 2019.
 73. Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc B* 57:289–300. <https://doi.org/10.1111/j.2517-6161.1995.tb02031.x>.
 74. Revelle WR. 2017. psych: procedures for personality and psychological research. <https://CRAN.R-project.org/package=psych>. Accessed 1 January 2018.
 75. Bastian M, Heymann S, Jacomy M. 2009. Gephi: an open source software for exploring and manipulating networks, p 361–362. *In* Third international AAAI conference on weblogs and social media, San Jose, CA.
 76. Truong DT, Franzosa EA, Tickle TL, Scholz M, Weingart G, Pasolli E, Tett A, Huttenhower C, Segata N. 2015. MetaPhlan2 for enhanced metagenomic taxonomic profiling. *Nat Methods* 12:902–903. <https://doi.org/10.1038/nmeth.3589>.
 77. Fang Z, Tan J, Wu S, Li M, Xu C, Xie Z, Zhu H. 2019. PPR-Meta: a tool for identifying phages and plasmids from metagenomic fragments using deep learning. *Gigascience* 8:giz066. <https://doi.org/10.1093/gigascience/giz066>.