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On-Farm Anaerobic Digestion of Dairy Manure Reduces the Abundance of Antibiotic Resistance-Associated Gene Targets and the Potential for Plasmid Transfer

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ABSTRACT The present study investigated the impact of on-farm anaerobic digestion on the abundance of enteric bacteria, antibiotic resistance-associated gene targets, and the horizontal transfer potential of extended-spectrum β-lactamase (ESBL) genes. Samples of raw and digested manure were obtained from six commercial dairy farms in Ontario, Canada. Digestion significantly abated populations of viable coliforms in all six farms. Conjugative transfer of plasmids carrying β-lactamase genes from manure bacteria enriched overnight with buffered peptone containing 4 mg/liter cefotaxime into a β-lactam-sensitive green fluorescent protein (GFP)-labeled *Escherichia coli* recipient strain was evaluated in patch matings. Digestion significantly decreased the frequency of the horizontal transfer of ESBL genes. Twenty-five transconjugants were sequenced, revealing six distinct plasmids, ranging in size from 40 to 180 kb. A variety of ESBL genes were identified: $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-27}}$, $bla_{\text{CTX-M-55}}$, and $bla_{\text{PER-1}}$. $bla_{\text{CTX-M-15}}$ was the most prevalent ESBL gene detected on plasmids harbored by transconjugants. Various mobile genetic elements were found located proximal to resistance genes. Ten gene targets, including *sul1*, *str*(A), *str*(B), *erm*(B), *erm*(F), *intI1*, *aadA*, *incW*, bla_{PSE}, and $bla_{\text{OXA-20}}$, were quantified by quantitative PCR on a subset of 18 raw and 18 digested samples. Most targets were significantly more abundant in raw manure; however, *erm*(B) and *erm*(F) targets were more abundant in digested samples. Overall, on-farm digestion of dairy manure abated coliform bacteria, a number of antibiotic resistance-associated gene targets, and the potential for *in vitro* conjugation of plasmids conferring resistance to extended-spectrum β-lactams and other classes of antibiotics into *E. coli* CV601.

IMPORTANCE Using livestock manure for fertilization can entrain antibiotic-resistant bacteria into soil. Manure on some dairy farms is anaerobically digested before being land applied. Recommending the widespread implementation of the practice should be founded on understanding the impact of this treatment on various endpoints of human health concern. Although lab-scale anaerobic treatments have shown potential for reducing the abundance of antibiotic resistance genes, there are very few data from commercial farms. Anaerobic digestion of manure on six dairy farms efficiently abated coliform bacteria, *E. coli*, and a majority of antibiotic resistance-associated gene targets. In addition, the conjugation potential of plasmids carrying ESBL genes into introduced *E. coli* strain CV601 was reduced. Overall, anaerobic digestion abated coliform bacteria, the genes that they carry, and the potential for ESBL-carrying plasmid transfer.

KEYWORDS antibiotic resistance, dairy manure, anaerobic digestion, extended-spectrum β-lactamase genes, plasmid conjugation

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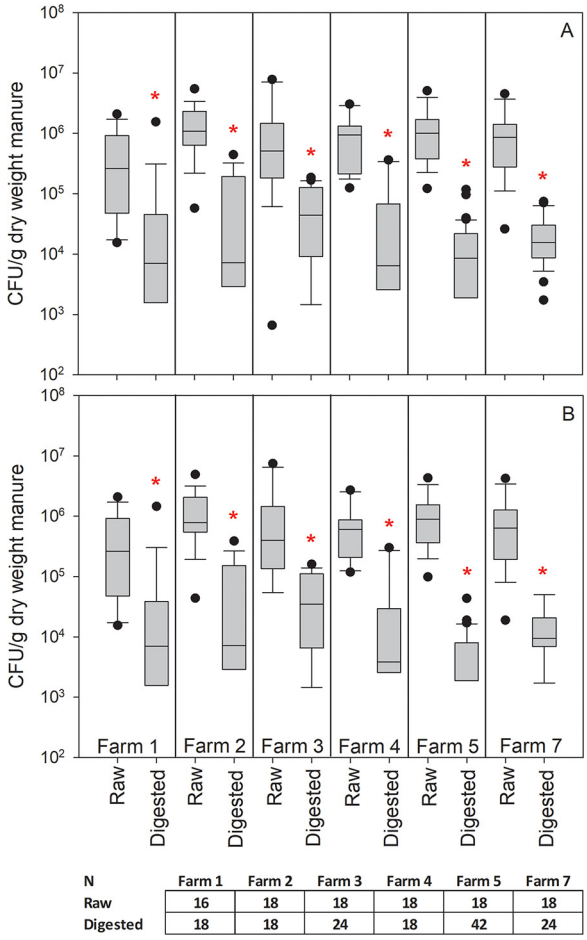
Manures from poultry, cattle, and swine farms are a valued source of nutrients and organic matter when used as fertilizers in crop production (1). However, fecal material can contain viral, bacterial, or parasitic pathogens that can threaten human health, and thus these amendments must be managed judiciously, particularly when used to grow crops destined for direct human consumption (2). Manures from animals that receive antibiotics are enriched in antibiotic-resistant bacteria and may contain excreted antibiotic residues (3, 4). There is a concern that contamination of agricultural soil with antibiotic-resistant bacteria and antibiotic residues will increase the reservoir of antibiotic resistance in crop production systems that are amended with manures (5, 6). Presumably, human exposure to this additional burden of resistant bacteria through consumption of contaminated crops represents a health risk (7).

Pretreatment of manures prior to land application to reduce the burden of antibiotic-resistant bacteria and destroy antibiotic residues should reduce this potential health risk (8). Treatment options commonly practiced on commercial farms consist of aerobic composting or anaerobic digestion at either mesophilic or thermophilic temperatures (9). Anaerobic digestion offers the advantage of generating biogas (methane, hydrogen, and carbon dioxide) that can be exploited for energy production, and thus this approach is widely practiced in many jurisdictions (10–13). The technology employed for anaerobic digestion ranges from the rudimentary to the quite sophisticated and thus has the advantage of being usable across a range of income settings (14).

The potential abatement of antibiotic-resistant bacteria in manure by anaerobic digestion and how this varies with process parameters are as yet not completely understood (15, 16). Process parameters, including temperature, pH, organic loading rate, and hydraulic retention time, are important factors for the efficient removal of pathogens, veterinary antibiotics, and antibiotic resistance genes while contributing to increased biogas production (15, 16). There have been several studies on the effect of temperature on the abundance of antibiotic resistance genes with findings that are often conflicting or inconsistent, indicating that other factors must be important (17, 18).

Antibiotic resistance and associated risk factors in bovine/dairy manure have been studied previously (19–21). Bovine manure not only improved the phosphorus and organic matter in soil because of its ideal C/N ratio (22/1) for soil microorganisms but also had lower antibiotic resistance gene diversity and abundance compared to those of other animal manures (22, 23). Dairy farm manure was previously found to contain extended-spectrum β-lactamase (ESBL)-/AmpC-encoding genes, including $bla_{\text{CTX-M-1, -2, -14, -15, -32, and -55}}$ and $bla_{\text{TEM-52}}$ (19, 21, 24, 25). Other antibiotic resistance genes detected in dairy manure confer resistance to tetracycline [*tet*(C), *tet*(G), *tet*(M), *tet*(Q), *tet*(X), *tet*(W)], sulfonamides (*sul1*, *sul2*), macrolides [*mef*(A), *erm*(B), *erm*(Q)], and aminoglycosides and fluoroquinolones [*aac(6′)-Ib-cr*] (20, 26). Mobile genetic elements such as plasmids, integrons, or transposons are normally associated with antibiotic resistance genes to facilitate the gene transfer among bacterial communities (26). Network analysis revealed the strong cooccurrence of mobile genetic elements (*intI1*, *intI2*, and IS*CR1*) and six antibiotic resistance genes (27).

In previous work with manure sourced from a commercial dairy farm, anaerobic digestion abated viable enteric bacteria but did not reduce the abundance of antibiotic resistance genes quantified by quantitative PCR (qPCR) (28). In the present study, manures prior to and after anaerobic digestion were sampled from six commercial dairy farms in Ontario, Canada, on a monthly basis over approximately 1 year. A detailed description of manure types as well as manure sampling methods can be found in the supplemental material (Text S1 and Fig. S2). Various enteric bacteria were quantified by viable plate count, and the abundance of selected gene targets associated with antibiotic resistance was quantified by qPCR. The potential for *in vitro* conjugal transfer of plasmids conferring resistance to β-lactam antibiotics from manure communities into an introduced GFP-tagged *Escherichia coli* recipient (CV601) was determined. Overall, results from the present study indicate that under commercial conditions, anaerobic digestion efficiently removed coliform bacteria and a majority of resistance genes.



N	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5	Farm 7
Raw	16	18	18	18	18	18
Digested	18	18	24	18	42	24

FIG 1 A comparison of coliform bacterial counts in raw and digested manure across the six participating farms: (A) total coliforms and (B) *E. coli*. Boxes represent the 25th to 75th percentile, whiskers indicate the 10th and 90th percentiles, and dots represent outliers outside the 10th and 90th percentiles. Asterisks indicate a statistically significant difference between treatments (Mann-Whitney U test, $P < 0.05$). N represents the number of samples collected for each treatment at each farm location.

However, it had little effect on Gram-positive bacteria and increased the abundance of the gene targets *ermB* and *ermF*.

RESULTS

Impact of anaerobic digestion on the abundance of viable enteric bacteria. A variety of bacteria in raw and in digested manure were enumerated by viable plate count. Total coliforms and *E. coli* colonies enumerated on Chromocult agar medium were consistently 1.1 $\log_{10}$ to 2.2 $\log_{10}$-fold lower in digested samples than in raw samples across all six participating farms (Fig. 1). In contrast, there was little impact of digestion on the abundance of Gram-positive bacteria, including *Enterococcus* spp., *Staphylococcus* spp., and presumptive *Clostridium perfringens* (Fig. 2).

***In vitro* conjugation frequency from raw and digested manure into *E. coli* CV601.** Transconjugants were identified as ESBL or non-ESBL based on the outcome of the ESBL confirmation test. Manure samples that yielded ESBL transconjugants were identified as carrying the ESBL phenotype, samples that yielded non-ESBL transconjugants were considered non-ESBL phenotype, and samples that yielded cefotaxime-resistant transconjugants of either or both phenotypes (ESBL, non-ESBL, or both) were considered extended-spectrum cephalosporinase (ESC) phenotype. Raw manure samples more frequently yielded transconjugants with the ESC, ESBL, and non-ESBL phenotypes than did digested manure samples (Table 1). However, raw samples only had significantly greater

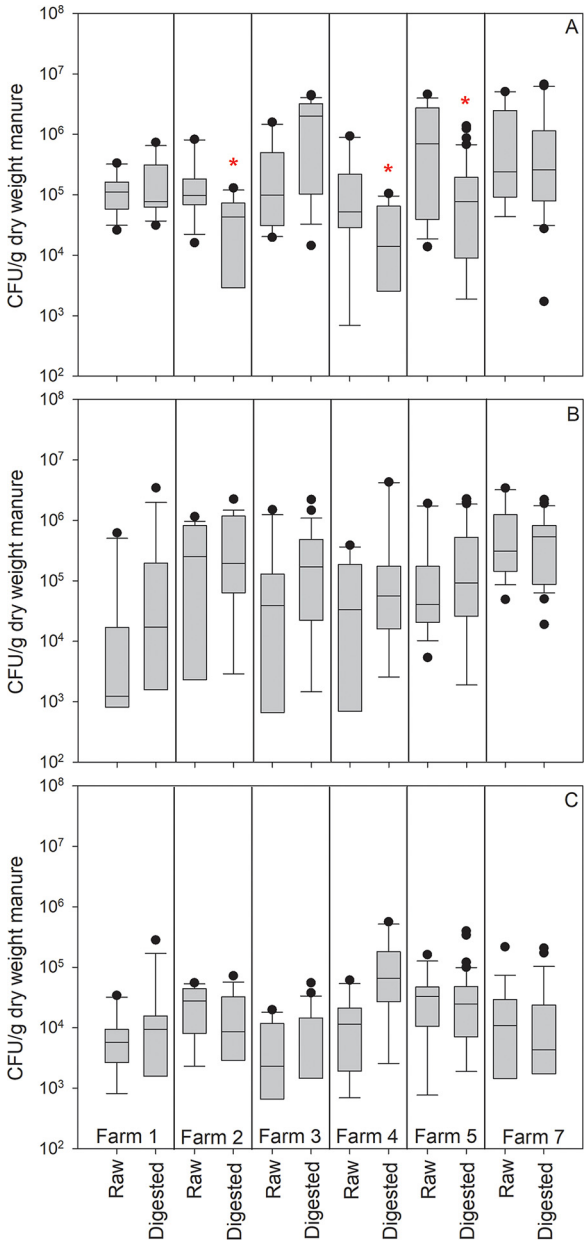


FIG 2 A comparison of selected Gram-positive viable counts in raw and digested manure across the six participating farms: (A) *Enterococcus* spp., (B) *Staphylococcus* spp., and (C) *Clostridium perfringens*. Box plot description and sample numbers per farm are indicated in Fig. 1. Asterisks indicate a statistically significant difference (Mann-Whitney U test, $P < 0.05$) between raw manure and digested manure.

odds of yielding transconjugants with the ESC and ESBL phenotypes compared to those of digested samples (Table 2). The mean ($\log_{10}$) and median transformed conjugation frequency were higher for raw samples than for digested samples (Table 1). The $\log_{10}$ transformed conjugation frequency was significantly greater for raw samples than for digested samples, and the $\log_{10}$ transformed conjugation frequency was significantly higher in samples collected in the fall and winter than in samples collected in the spring (Table 3). Based on the variance components in all the models, it appears that farm-level effects contribute to a substantial proportion of the variance in these outcomes (Tables 2 and 3). The assumption of homoscedasticity was met for the standardized residuals in the multilevel linear model and the best linear unbiased predictions (BLUPs) of all models. The normality

TABLE 1 Prevalence of samples that have ESC, ESBL, and non-ESBL phenotypes and mean (log_{10}) and median enhanced conjugation frequency estimated for all samples and when stratified by processing stage and season[a]

Stratification parameters	% of samples with phenotype (95% CI)			Mean log_{10} conjugation frequency (95% CI)	Conjugation frequency (interquartile range)
	ESC[b]	ESBL[c]	Non-ESBL[d]		
Processing stage					
Digested ($n = 63$)	57.1 (44.0, 69.5)	44.4 (31.9, 57.5)	19.0 (10.2, 30.9)	−7.6 (−8.3, −7.0)	0 (0, 2×10^{-6})
Raw ($n = 63$)	82.5 (70.9, 90.9)	60.3 (47.2, 72.4)	33.3 (22.0, 46.3)	−6.8 (−7.5, −6.1)	8.0×10^{-8} (0, 1.9×10^{-5})
Season					
Spring ($n = 50$)	56.0 (41.3, 70.0)	36.0 (22.9, 50.8)	24.0 (13.1, 38.2)	−7.8 (−8.5, −7.1)	0 (0, 7.5×10^{-7})
Summer ($n = 30$)	83.3 (65.3, 94.4)	66.7 (47.2, 82.7)	30.0 (14.7, 49.4)	−6.9 (−7.9, −6.0)	5.4×10^{-8} (0, 1.2×10^{-5})
Fall ($n = 12$)	75.0 (42.8, 94.5)	58.3 (27.7, 84.8)	33.3 (9.9, 65.1)	−7.1 (−8.6, −5.6)	1.2×10^{-7} (0, 1.7×10^{-5})
Winter ($n = 34$)	76.5 (58.8, 89.3)	61.8 (43.6, 77.8)	23.5 (10.7, 41.2)	−6.7 (−7.6, −5.7)	6.9×10^{-7} (0, 1.9×10^{-5})
Overall ($n = 126$)	69.8 (61.0, 77.7)	52.4 (43.3, 61.3)	26.2 (18.8, 34.8)	−7.2 (−7.7, −6.8)	2.3×10^{-9} (0, 8.0×10^{-6})

[a]Phenotypes of transconjugants were determined by ESBL confirmation assay and binned into either ESBL or non-ESBL phenotypes.
[b]ESC, samples yielding transconjugants of any phenotypes (ESBL, non-ESBL, or both phenotypes). CI, confidence interval.
[c]ESBL, samples yielding ESBL phenotype transconjugants.
[d]Non-ESBL, samples yielding non-ESBL phenotype transconjugants.

assumption for these residuals and BLUPs was either met or only symmetrical or mildly skewed. No outliers were identified in any of the multilevel models fitted.

Genotypic and phenotypic characterization of recovered plasmids. A total of 459 plasmids were isolated, digested with EcoRI, and binned into 10 distinct restriction enzyme (RE) profiles. Both raw and digested manures from a farm sampling appeared to share similar RE profile clusters where they both had the most prevalent plasmid (pT267A) (Table S1). Other less frequent plasmid RE profiles were detected in either raw manures (pT145A, pT247A, pT277A, pT308A) or digested manures (pT156A, pT224A, pT295A, pT476A).

Based on preliminary RE profiles, a subset of 25 transconjugants with the ESBL phenotype was selected and subjected to whole-genome sequencing on the Illumina MiSeq short-read sequencing platform. Eleven of these transconjugants were further sequenced on the MinION long-read sequencing platform. Hybrid assembly was used to completely close plasmid sequences. Key characteristics of the plasmids are presented in Table 4. Plasmid incompatibility groups detected by Mob-suite included IncI1, IncN, IncFIIA, IncFII, IncX1, and IncC (Table 4). The most common plasmid (~100 kb pT267A) carrying numerous antibiotic resistance genes was found across all participating farms in both raw and digested manure. Snippy analysis showed that there were between one and seven single nucleotide polymorphisms (SNPs) when

TABLE 2 The results of multilevel logistic regression models examining the associations between processing stage and season on the odds of samples having transconjugants that have ESC, ESBL, and non-ESBL phenotypes[a]

Stratification parameters	ESC[b]			ESBL[c]			Non-ESBL[d]		
	OR	95% CI	*P* value	OR	95% CI	*P* value	OR	95% CI	*P* value
Processing stage									
Digested	Referent			Referent			Referent		
Raw	9.05	2.12, 38.69	0.003	3.53	1.16, 10.73	0.026	2.52	0.97, 6.54	0.057
Variance components	Variance (VPC[e])	95% CI		Variance (VPC[e])	95% CI		Variance (VPC[e])	95% CI	
Farm	1.38 (15.3%)	0.10, 18.24		3.80 (34.0%)	0.56, 26.03		1.40 (28.9%)	0.23, 8.44	
Farm on date of sampling	4.33 (48.1%)	0.91, 20.58		4.08 (36.5%)	1.01, 16.53		0.16 (3.3%)	5.25×10^{-6}, 4.98×10^{3}	

[a]Phenotypes of transconjugants were determined by ESBL confirmation assay and binned into either ESBL or non-ESBL phenotypes. OR, odds ratio.
[b]ESC, samples yielding transconjugants of any phenotypes: ESBL, non-ESBL, or both.
[c]ESBL, samples yielding ESBL phenotype transconjugants.
[d]Non-ESBL, samples yielding non-ESBL phenotype transconjugants.
[e]Variance partition coefficients (VPC) estimated using the latent variable technique.

TABLE 3 The results of a multilevel linear regression model examining the associations between processing stage and season on the $\log_{10}$ conjugation frequency

Stratification parameters	β^a	95% CI	*P* value
Digestion stage			
Digested	Referent		
Raw	0.82	0.23, 1.41	0.006
Season			
Spring	Referent		
Summer	0.92	−0.09, 1.93	0.075
Fall	1.59	0.15, 3.03	0.030
Winter	1.13	0.16, 2.11	0.022
Variance components	Variance (VPC[b])	95% CI	
Farm	2.69 (40.8%)	0.77, 9.34	
Farm on date of sampling	1.07 (16.2%)	0.40, 2.82	
Sample	2.83 (42.9%)	2.00, 4.01	

[a]Model coefficients.
[b]Variance partition coefficients.

Illumina assemblies of transconjugants, likely carrying this common plasmid, were mapped against a reference genome with a closed plasmid sequence (Table S2).

Eleven complete closed plasmids were aligned and their sequence identity was compared (Fig. 3). On this basis, six likely identical plasmids which were captured in six individual transconjugants were identified and designated pT82A, pT101A, pT159A, pT270A, pT267A, and pT209A. Three plasmids, pT156A, pT224A, and pT257A, shared a majority of their sequence in common, while pT199A and pT247A were more unique in their sequences. For the most prevalent plasmid (pT267A) which was present in both raw and digested manure samples, the average GC content of areas containing resistance genes and mobile genetic elements was about 54%, whereas the rest of DNA plasmid had an average GC content of 37.5%. Maps of six distinct complete plasmids were constructed (Fig. 4). A variety of mobile genetic elements were found in areas surrounding resistance genes including Tn*3*, Tn*7*, IS*26*, *intI1*, *insA*, *insB*, IS*Ec63*, IS*Ec9*, IS*903B*, IS*5*, IS*91*, IS*5075*, and IS*Vsa3*. Five of the six plasmids carried IS*26*.

Putative β-lactamases and other antibiotic resistance genes carried by the 25 sequenced plasmids were identified using the StarAMR tool (Table 4). Six ESBL genes were detected: $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-55}}$, $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-27}}$, and $bla_{\text{PER-1}}$. The most common ESBL gene was $bla_{\text{CTX-M-15}}$, which was carried by 13 of the 25 plasmids. Other genes were associated with resistance to macrolides [*mph*(E), *msr*(E)], tetracyclines [*tet*(A), *tet*(C), *tet*(E), *tet*(X)], lincomycin [*lnu*(G)], florfenicol (*floR*), sulfonamides (*sul2*), chloramphenicol (*catB4*), trimethoprim (*dfrA1*), and aminoglycosides (*aac(6′)-Ib-cr*). AMR genes detected with the ABRicate tool were similar to those detected by starAMR (Table S3), except that ABRicate also reported genes with lower coverage than 50% and a different bla_{TEM} variant. ABRicate identified the gene as *blaTEM*-141, while starAMR identified it as the $bla_{\text{TEM-206}}$ gene. Both variants had the same coverage of 86.64% with our gene sequence.

Antibiotic susceptibility of the 25 transconjugants was determined phenotypically using the Sensititre Gram-negative susceptibility panels. They were all confirmed to possess ESBL phenotypes. Most of the antibiotic resistance phenotypes were consistent with their corresponding genotypes based on Clinical and Laboratory Standards Institute (CLSI) standards (99) except for gentamicin (Gen) and ciprofloxacin (Cip) (Table 5). In these cases, the plasmids carried the resistance gene *aac(6′)-Ib-cr* but did not confer phenotypic resistance. MICs in most transconjugants (i.e., T82A, T101A, T159A, and T209A) carrying this plasmid-mediated gene were slightly elevated (MIC_{Cip} = 0.06 to 0.12 mg/liter, MIC_{Gen} = 2 mg/liter) compared to that in the host *E. coli* CV601 alone (MIC_{Cip} = 0.03 mg/liter, MIC_{Gen} = 1 mg/liter).

Abundance of selected gene targets associated with antibiotic resistance or horizontal gene transfer. Most gene targets, including *aadA*, *int1*, bla_{PSE}, *str*(A), *str*(B), and *sul1*, were less abundant in digested manure than in raw manure (Fig. 5). In contrast,

TABLE 4 Plasmid characteristics and plasmid-borne resistance genes in 25 transconjugants using Miseq/MinION sequencing platforms

Plasmid ID	Resistance genes on the plasmid[b,c]	Farm/sample type	Inc group[d,e]	Plasmid size (bp)	Predicted mobility	Predicted resistance phenotype[f]
pT82A[a]	*aac (6′)-Ib-cr*, *aph(3′′)-Ib*, *aph(6)-Id*, ***bla*$_{CTX-M-15}$**, *bla*$_{OXA-1}$, *catB4*, *dfrA1*, *floR*, *lnu(G)*, *sul2*	Farm 4/raw	Inc−	104,812	Conjugative	Cip Gen Str Kan Amp Axo Tmp Chl Lin Sxz
pT101A[a]	*aac(6′)-Ib-cr*, *aph(3′′)-Ib*, *aph(6)-Id*, ***bla*$_{CTX-M-15}$**, *bla*$_{OXA-1}$, *catB4*, *dfrA1*, *floR*, *lnu(G)*, *sul2*	Farm 4/raw	Inc−	104,812	Conjugative	Cip Gen Str Kan Amp Axo Tmp Chl Lin Sxz
pT159A[a]	*aac(6′)-Ib-cr*, *aph(3′′)-Ib*, *aph(6)-Id*, ***bla*$_{CTX-M-15}$**, *bla*$_{OXA-1}$, *catB4*, *dfrA1*, *floR*, *lnu(G)*, *sul2*	Farm 1/digested	Inc−	104,812	Conjugative	Cip Gen Str Kan Amp Axo Tmp Chl Lin Sxz
pT209A[a]	*aac(6′)-Ib-cr*, *aph(3′′)-Ib*, *aph(6)-Id*, ***bla*$_{CTX-M-15}$**, *bla*$_{OXA-1}$, *catB4*, *dfrA1*, *floR*, *lnu(G)*, *sul2*	Farm 7/digested	Inc−	104,864	Conjugative	Cip Gen Str Kan Amp Axo Tmp Chl Lin Sxz
pT267A[a]	*aac(6′)-Ib-cr*, *aph(3′′)-Ib*, *aph(6)-Id*, ***bla*$_{CTX-M-15}$**, *bla*$_{OXA-1}$, *catB4*, *dfrA1*, *floR*, *lnu(G)*, *sul2*	Farm 5/raw	Inc−	104,864	Conjugative	Cip Gen Str Kan Amp Axo Tmp Chl Lin Sxz
pT270A[a]	*aac(6′)-Ib-cr*, *aph(3′′)-Ib*, *aph(6)-Id*, ***bla*$_{CTX-M-15}$**, *bla*$_{OXA-1}$, *catB4*, *dfrA1*, *floR*, *lnu(G)*, *sul2*	Farm 5/raw	Inc−	104,864	Conjugative	Cip Gen Str Kan Amp Axo Tmp Chl Lin Sxz
pT221A	*aac(6′)-Ib-cr*, *aph(3′′)-Ib*, *aph(6)-Id*, ***bla*$_{CTX-M-15}$**, *bla*$_{OXA-1}$, *catB4*, *dfrA1*, *floR*, *lnu(G)*, *sul2*	Farm 7/digested	Inc−	~96,702	Conjugative	Cip Gen Str Kan Amp Axo Tmp Chl Lin Sxz
pT286A	*aac(6′)-Ib-cr*, *aph(3′′)-Ib*, *aph(6)-Id*, ***bla*$_{CTX-M-15}$**, *bla*$_{OXA-1}$, *catB4*, *dfrA1*, *floR*, *lnu(G)*, *sul2*	Farm 2/raw	Inc−	~96,702	Conjugative	Cip Gen Str Kan Amp Axo Tmp Chl Lin Sxz
pT496A	*aac(6′)-Ib-cr*, *aph(3′′)-Ib*, *aph(6)-Id*, ***bla*$_{CTX-M-15}$**, *bla*$_{OXA-1}$, *catB4*, *dfrA1*, *floR*, *lnu(G)*, *sul2*	Farm 5/raw	Inc−	~96,702	Conjugative	Cip Gen Str Kan Amp Axo Tmp Chl Lin Sxz
pT304A	*aac(6′)-Ib-cr*, *aph(3′′)-Ib*, *aph(6)-Id*, ***bla*$_{CTX-M-15}$**, *bla*$_{OXA-1}$, *catB4*, *dfrA1*, *floR*, *lnu(G)*, *sul2*	Farm 3/raw	Inc−	~96,702	Conjugative	Cip Gen Str Kan Amp Axo Tmp Chl Lin Sxz
pT306A	*aac(6′)-Ib-cr*, *aph(3′′)-Ib*, *aph(6)-Id*, ***bla*$_{CTX-M-15}$**, *bla*$_{OXA-1}$, *catB4*, *dfrA1*, *floR*, *lnu(G)*, *sul2*	Farm 3/raw	Inc−	~96,702	Conjugative	Cip Gen Str Kan Amp Axo Tmp Chl Lin Sxz
pT409A	*aac(6′)-Ib-cr*, *aph(3′′)-Ib*, *aph(6)-Id*, ***bla*$_{CTX-M-15}$**, *bla*$_{OXA-1}$, *catB4*, *dfrA1*, *floR*, *lnu(G)*, *sul2*	Farm 7/digested	Inc−	~96,702	Conjugative	Cip Gen Str Kan Amp Axo Tmp Chl Lin Sxz
pT478A	*aac(6′)-Ib-cr*, *aph(3′′)-Ib*, *aph(6)-Id*, ***bla*$_{CTX-M-15}$**, *bla*$_{OXA-1}$, *catB4*, *dfrA1*, *floR*, *lnu(G)*, *sul2*	Farm 4/raw	Inc−	~96,650	Conjugative	Cip Gen Str Kan Amp Axo Tmp Chl Lin Sxz
pT145A	***bla*$_{CTX-M-15}$**	Farm 1/raw	IncI1	~85,051	Conjugative	Amp Axo
pT277A	***bla*$_{CTX-M-15}$**	Farm 1/raw	IncI1	~83, 223	Conjugative	Amp Axo
pT308A	***bla*$_{CTX-M-14}$**	Farm 3/raw	IncI1	~94,724	Conjugative	Amp Axo
pT247A[a]	***bla*$_{CTX-M-14}$**	Farm 2/raw	IncI1	91,905	Conjugative	Amp Axo
pT199A[a]	***bla*$_{CTX-M-1}$**	Farm 7/raw	IncN	42,578	Conjugative	Amp Axo
pT257A[a]	***bla*$_{CTX-M-27}$**	Farm 2/digested	IncFIIA, IncFII	66,581	Conjugative	Amp Axo
pT455A	***bla*$_{CTX-M-27}$**	Farm 2/digested	IncN	~41,970	Conjugative	Amp Axo
pT295A[a]	*aph(3′′)-Ib*, *aph(6)-Id*, ***bla*$_{CTX-M-27}$**, *bla*$_{TEM-1B}$, *dfrA14*, *sul2*, *tet*(A)	Farm 2/digested	IncN, IncX1	77,311	Conjugative	Str Kan Amp Axo Tmp Sxz Tet
pT476A	***bla*$_{CTX-M-55}$**, *bla*$_{TEM-1B}$	Farm 7/digested	IncX1	~40,307	Conjugative	Amp Axo
pT224A[a]	***bla*$_{CTX-M-55}$**, *bla*$_{TEM-206}$, *fosA3*	Farm 7/digested	IncFIIA, IncFII	70,625	Conjugative	Amp Axo Fos
pT156A[a]	***bla*$_{CTX-M-55}$**, *bla*$_{TEM-206}$	Farm 1/digested	IncFIIA, IncFII	61,659	Conjugative	Amp Axo
pT413A[a]	*aadA2*, ***bla*$_{PER-1}$**, *sul1*[g], *tet*(E), *tet*(X), *mph*(E), *msr*(E), *aph(3′)-Ia*, *tet*(C)	Farm 5/raw	IncC	187,012	Conjugative	Str Amp Axo Ery Azi

[a]Samples were sequenced on both Miseq and MinION platforms, followed by hybrid assembly. These plasmids had complete closed sequences of precise sizes.
[b]Genes in bold were identified as ESBL genes.
[c]Resistance genes were identified by starAMR tool.
[d]Inc group, plasmid size, and predicted mobility were determined by whole-genome sequencing and the MOB-suite tool.
[e]Inc−, plasmid with no detectable Inc type.
[f]Cip, ciprofloxacin; Gen, gentamicin; Str, streptomycin; Amp, ampicillin; Axo, ceftriaxone; Tmp, trimethoprim; Chl, chloramphenicol; Lin, lincomycin; Sxz, sulfisoxazole; Ery, erythromycin; Azi, azithromycin; Tet, tetracycline; Fos, fosfomycin.
[g]This plasmid had two copies of the *sul1* gene.

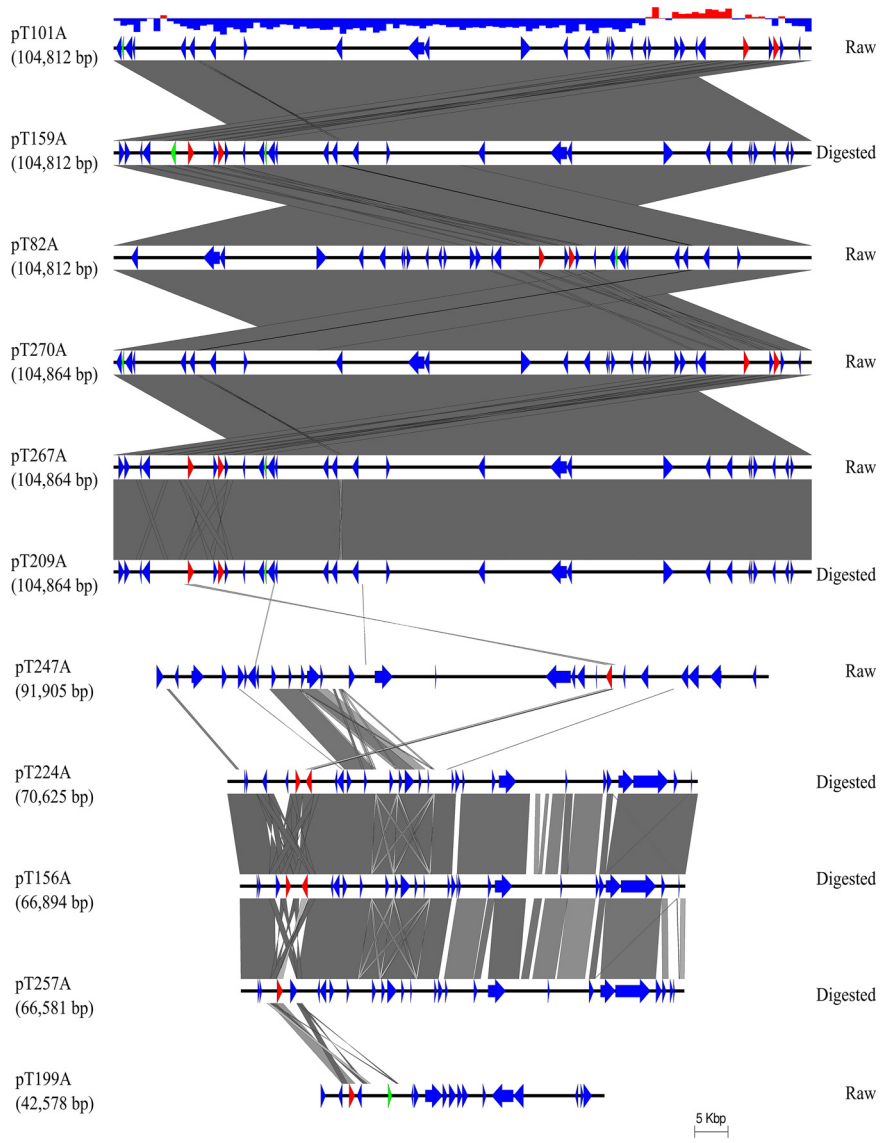


FIG 3 Comparison of 11 closed plasmid sequences obtained from hybrid assembly on short- and long-read sequencing platforms. Coding sequences annotated by PROKKA tool are represented by colored arrows. Red arrows are β-lactamase genes, green arrows are mobile genetic elements, and blue arrows are other functional genes. The top graph showed GC content of the first plasmid sequence: red, GC content greater than 50%; blue, GC content lower than 50%.

both *ermB* and *ermF* gene targets increased in abundance with digestion, and the bla_{OXA20} target did not change in abundance (Fig. 5). The abundance of total bacteria based on the 16S rRNA gene (*rrnS*) did not significantly decrease in digested samples (Fig. 5a). The impact of digestion on the abundance of all gene targets except *incW* was the same whether expressed as the absolute or the relative abundance (Fig. 5a and b). The *incW* plasmid incompatibility group gene target was significantly reduced in absolute abundance with digestion, whereas it was not reduced in relative abundance (Fig. 5a).

DISCUSSION

As revealed by viable plate count, anaerobic digestion significantly reduced the abundance of viable Gram-negative bacteria but not that of Gram-positive bacteria. These results are consistent with a previous study in which most of Gram-negative bacteria significantly decreased with digestion but *Clostridium perfringens* and *Enterococcus* spp. did not (29). In another study, *E. coli* and total coliform counts decreased below the limit of

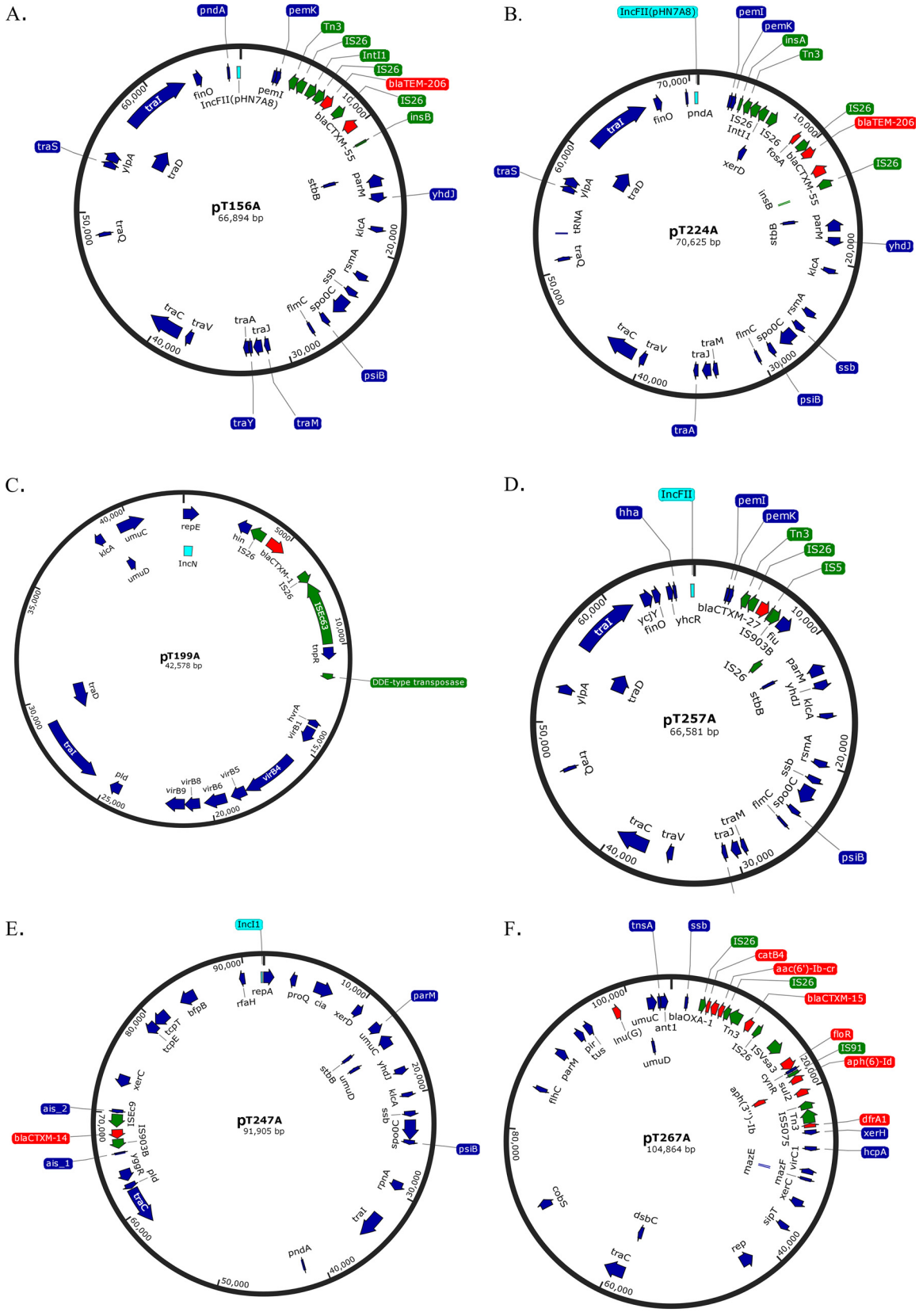


FIG 4 Plasmid maps of six distinct plasmids harboring ESBL genes which were captured in *E. coli* CV601 strain. (A) pT156A, (B) pT224A, (C) pT199A, (D) pT257A, (E) pT247A, (F) pT267A. Red and turquoise arrows are resistance genes and incompatibility plasmid sequence, respectively, detected by starAMR tool. Green arrows are mobile genetic elements detected by RAST and BLAST tools. Dark blue arrows are other functional genes which were annotated by PROKKA tool. Figures were created using SnapGene.

TABLE 5 Antibiotic resistance phenotypes of 25 transconjugants using Gram-negative susceptibility panels (CMV4AGNF and ESB1F) on the Sensititre automated system[a,b]

	Phenotype for transconjugant																								
Antibiotic(s)	T156A	T247A	T277A	T224A	T413A	T308A	T257A	T455A	T199A	T295A	T304A	T306A	T476A	T409A	T478A	T496A	T082A	T270A	T145A	T159A	T101A	T267A	T286A	T221A	T209A
Amoxicillin/clavulanic acid	S	S	S	S	S	S	S	S	I	I	I	I	I	I	I	I	I	**R**	S	**R**	**R**	**R**	**R**	**R**	**R**
Ampicillin	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
Azithromycin	S	S	S	S	**R**	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Cefazolin	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
Cefepime	S	S	S	S	S	S	S	S	**R**	S	**R**	S	S	**R**	**R**	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**R**	**R**	**R**
Cefotaxime	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
Cefoxitin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	I	S	S	S	S	S	S	S
Cefpodoxime	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
Ceftazidime	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
Ceftriaxone	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
Cephalothin	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
Chloramphenicol	S	S	S	S	S	S	S	S	S	S	**R**	**R**	S	**R**	**R**	**R**	**R**	**R**	S	**R**	**R**	**R**	**R**	**R**	**R**
Ciprofloxacin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Gentamicin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Imipenem	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Meropenem	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Nalidixic acid	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Piperacillin/tazobactam	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	I	S	S	S	S	S	S	S
Streptomycin[c]	S	S	S	S	**R**	S	S	S	S	S	**R**	**R**	S	**R**	**R**	**R**	**R**	**R**	S	**R**	**R**	**R**	**R**	**R**	**R**
Sulfisoxazole[c]	S	S	S	S	NS	S	S	S	S	NS	NS	NS	S	NS	NS	NS	NS	NS	S	NS	NS	NS	NS	NS	NS
Tetracycline	S	S	S	S	**R**	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Trimethoprim/ sulfamethoxazole	S	S	S	S	S	S	S	S	S	**R**	**R**	**R**	S	**R**	**R**	**R**	**R**	**R**	S	**R**	**R**	**R**	**R**	**R**	**R**

[a]The results were interpreted by the Sensititre automated system unless otherwise stated.

[b]Streptomycin and sulfisoxazole results were interpreted according to the breakpoint guideline provided by U.S. Food and Drug Administration (FDA) (https://www.fda.gov/media/108180/download).

[c]R, resistant (bolded for clarity); I, intermediate; S, susceptible; NS, not susceptible. NS only applies to sulfisoxazole where MICs were limited by the range of tested panels; however, the values were larger than the susceptible breakpoint in the guideline provided by U.S. Food and Drug Administration (FDA) (https://www.fda.gov/media/108180/download).

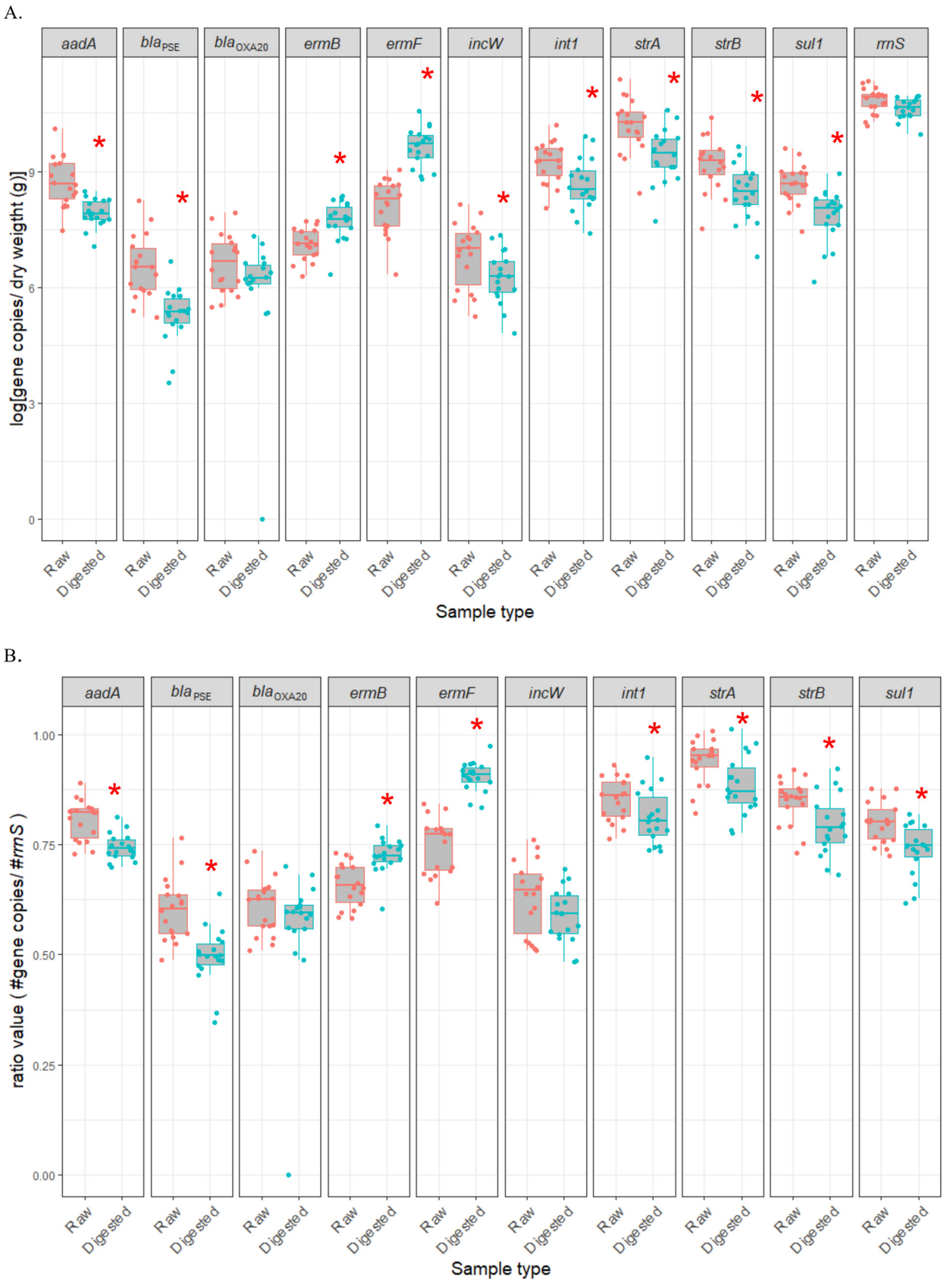


FIG 5 A comparison of antibiotic resistance-associated gene targets in raw and digested samples across six farms. (A) The log of [gene copy number per matter dry weight (g)]. (B) The ratio of the gene copy number to the number of total bacteria. Asterisks indicate a statistically significant difference ($P < 0.05$).

detection within 4 to 7 days of dry mesophilic anaerobic codigestion of food waste and pig manure, whereas it took longer to remove *Enterococcus* spp. (12 days) (30). It is likely due to the fact that these bacteria are more thermotolerant (*Enterococcus* spp., *Staphylococcus* spp.) or that they are spore-forming (*Clostridium perfringens*) (9, 31). In our study, digesters at most farms were operated in the mesophilic temperature range of 38.5 to 40°C.

With *E. coli* CV601 as the recipient, bacteria enriched from raw manure as the donor yielded transconjugants more frequently than did bacteria enriched from digested manure. Presumably, this was due to the significant reduction in the abundance of potential donors, as evidenced by viable plate counts. Manure was preenriched with buffered peptone containing cefotaxime prior to inoculation with the *E. coli* GFP-tagged CV601 recipient. In the absence of this preenrichment step, we were unsuccessful in obtaining any transconjugants in digested samples. In other studies, biparental/triparental mating was used to capture mobilizing plasmids from granules of an anaerobic wastewater treatment plant, cow manure, swine manure slurries, and fresh water; however, none of these studies investigated the fate of these plasmids after anaerobic digestion (32–34). In our study, capturing plasmid-mediated ESBL genes in either preenriched raw or preenriched digested manures was still achievable without the addition of helper plasmids such as pBBR1MCS-derivative plasmids. Although there was a significant decrease in cefotaxime-resistant transconjugants found in digested manures compared to that found in raw manures, both raw and digested samples appeared to share similar RE profile clusters. The prevalent plasmid, pT267A (~100 kb), was present abundantly in both raw and digested manures from all farms participating in this study. We also identified other less frequent plasmids that were only found in either raw or digested manures.

The variance components from our multilevel models also provided us with additional insights concerning our data. Interestingly, farm-level effects appeared to contribute to a substantial proportion of the variance in the outcomes examined, although we should be cautious in this interpretation due to the lack of precision in our estimates (i.e., wide confidence intervals for the variance components). Farm-level factors, which could not be statistically analyzed due to the relatively small number of farms included in our study, that may explain this farm-level variance include retention time, manure input and output flows, pH, and other differences in digestion processes. Future studies, using a larger number of farms, would allow researchers to measure the effect of these manure digestion processing factors.

A variety of ESBL genes were identified in 25 sequenced transconjugants, including $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-27}}$, $bla_{\text{CTX-M-55}}$, and $bla_{\text{PER-1}}$. The $bla_{\text{CTX-M-15}}$ gene was the most prevalent ESBL gene detected. This is not surprising because $bla_{\text{CTX-M}}$ variants are commonly found in both clinical and community settings (35–38). First discovered in the 1980s, they became the most commonly encountered ESBL genes and have become more frequently found than bla_{TEM} and bla_{SHV} (38). The CTX-M lineage was divided into at least five phylogroups: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25 (38). In our study, two phylogroups were identified: CTX-M-1 ($bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-55}}$) and CTX-M-9 ($bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-27}}$). The $bla_{\text{CTX-M-15}}$ gene was the most prevalent gene among them in our study; this agrees with previous reports regarding the global spread of this gene (39–45). The non-CTX-M ESBL $bla_{\text{PER-1}}$ gene, originally from *Pseudomonas aeruginosa*, was also found in other species, including *Salmonella enterica* serovar Typhimurium, *Proteus mirabilis*, and *Providencia stuartii* (41, 46–48).

Other non-ESBL β-lactamase genes which colocated with one $bla_{\text{CTX-M-}}$ gene were also detected, including $bla_{\text{TEM-1b}}$, $bla_{\text{TEM-206}}$, and $bla_{\text{OXA-1.}}$ The $bla_{\text{TEM-1b}}$ gene, colocated with either $bla_{\text{CTX-M-27}}$ or $bla_{\text{CTXM-55}}$, was considered one of the parental penicillinase genes along with $bla_{\text{TEM-1a}}$ and $bla_{\text{TEM-2}}$ genes. Their derivatives, resulting from various combinations of eight amino acid substitutions, including five amino acids which expanded the enzymatic substrate specificity, were classified as TEM-type ESBL genes

(49). More interestingly, there was a rare case where an *E. coli* isolate carrying this bla_{TEM-1b} gene expressed ESBL phenotype. Lagace-Wiens et al. postulated that the strain either hyperproduced non-ESBL β-lactamase or got permeability reduced (50). There is very little available information concerning $bla_{TEM-206,}$ which was classified as a non-ESBL gene in one study (51). Two different bla_{TEM} variants were reported by two different tools ($bla_{TEM-141}$ by ABRicate versus $bla_{TEM-206}$ by starAMR) because our truncated sequence, which lacked 115 bp of the front sequence, shared the same coverage (87%) with both of them (Fig. S1). Therefore, this gene might not be functional at all in our case. The bla_{OXA-1}, another non-ESBL gene in *E. coli*, was found to cooccur with $bla_{CTX-M-15.}$ This cooccurrence was also observed in several previous studies (39, 42, 52). Also, there was an association between the OXA-1 enzyme and reduced susceptibility to penicillin/β-lactamase inhibitor combinations among ESBL-producing *E. coli* (53).

ESBL-producing *E. coli* isolates are more likely to be multidrug resistant, gentamicin resistant, and fluoroquinolone resistant than are AmpC-producing *E. coli* isolates (37, 54). In the present study, the most prevalent ESBL gene ($bla_{CTX-M-15}$) was found to colocate with multiple other resistance genes: *aac(6′)-Ib-cr*, *aph(3′′)-Ib*, *aph(6)-Id*, *catB4*, *dfrA1*, *floR*, *lnu*(G), and *sul2*. Although some plasmids harbored a determinant for gentamicin and ciprofloxacin resistance [*aac(6′)-Ib-cr*], their phenotypic MIC values did not meet the CLSI standard to be interpreted as "resistant." Nevertheless, MICs in some transconjugants were slightly elevated compared to that in the host alone. The *aac(6′)-Ib-cr* gene, a derivative of aminoglycoside acetyltransferase *aac(6′)-Ib*, was first reported in 2003 and has widely disseminated ever since (44, 45, 55). This gene is interesting because it was shown to confer resistance to two distinctly different antibiotic classes (aminoglycoside and fluoroquinolone) (55). Other resistance genes were also detected along with ESBL genes, including *fosA3* that confers resistance to fosfomycin: *mph*(E) and *msr*(E) for macrolide resistance and *tet*(A), *tet*(C), *tet*(E), and *tet*(X) for tetracycline resistance.

Resistance genes found in manures are likely related to the use of drugs that were approved for veterinary medication (prescription drug list; PDL) such as oxytetracycline, penicillin, florfenicol, trimethoprim-sulfadoxine, and ceftiofur (15, 56). These antibiotics were used by the participating farms; a full list of drugs (antibiotics included) is found in Table S5. Penicillins, penicillin combinations, cephalosporins, tetracyclines, trimethoprim-sulfonamide combinations, and lincosamides are the most commonly used antibiotic classes in dairy farms in Canada for dry cow therapy and clinical mastitis treatment (57). Penicillin G procaine (Pen G), ampicillin (Polyflex), and ceftiofur (Excede) might contribute to the dissemination and maintenance of β-lactamase, especially ESBL genes. Other drugs such as florfenicol, oxytetracycline, trimethoprim, and lincomycin could promote ESBL plasmids that were also resistant to other drugs as shown in this study.

Mobile genetic elements such as plasmids, transposons, and insertion sequences have been known to largely contribute to the spread of antibiotic resistance genes (58, 59). Plasmids found in our study fell within the size range of 40 to 180 kb. The most prevalent plasmid had a low GC content backbone which is similar to previous observations (60, 61). Only areas containing resistance genes appeared to have a higher GC content, suggesting that these genes were recruited from foreign DNA in this plasmid. In addition, a variety of insertion sequences and integrases were found near resistance genes. Among them, IS*26* was detected in a majority of our sequenced plasmids. IS*26*, first discovered almost 4 decades ago, is a major contributor to the evolution and/or the dissemination of antibiotic resistance genes (62–65). IS*26* was found to cooccur with several β-lactamase genes, such as bla_{S2A} and bla_{TEM-1} (62, 66). Two mechanisms were presented to explain IS*26* movement: cointegrate forming and cut-and-paste mechanisms (64). The end product is normally an array of two or more directly oriented IS*26*s (64). In another study, IS*26* was shown to use replicative transposition to reorganize clinically isolated plasmids (67). Only one out of four possible recombination outcomes resulted in two inversely oriented IS*26*s after replicative transposition

provided that the donor insertion sequence (IS) and target sites were present in the same replicon (intramolecular transposition) and DNA rearrangement occurred in the *trans* pathway (58, 67). In our study, a sophisticated structure of multiple disoriented IS*26*s along with the presence of other mobile genetic elements were seen in several plasmids, suggesting multiple gene exchange events.

The majority of gene targets significantly decreased in abundance after digestion except for bla_{OXA20}, *erm*(B), and *erm*(F). The abundance of *erm*(B) and *erm*(F) significantly increased in digested samples. The abundance of *erm*(B) and *erm*(F) was also seen to increase in municipal wastewater treatment plants (68). This might be due to the shift of certain bacterial populations, for example from nutrient removal functional bacteria (i.e., *Nitrospira*, *Dechloromonas*, *Dokdonella*, *Comamonas*, *Thauera*, and *Zoogloea*) to fermentative bacteria (i.e., *Smithella*, *Petrimonas*, *Saccharicrinis*, *Syntrophomonas*, and *Ercella*) (68). Lab-scale mesophilic anaerobic digestion also amplified both *erm*(B) and *erm*(F) genes, while thermophilic digestion provided more effective reduction of *erm*(B) and *erm*(F) (69). Both *erm*(B) and *erm*(F) are carried by various Gram-positive bacteria of animal origin, including *Streptococcus* spp., *Staphylococcus* spp., and *Peptostreptococcus* spp. (70).

The present study showed that on-farm anaerobic digestion reduced the horizontal transfer potential of plasmids carrying ESC genes including ESBL genes into *E. coli* CV601, consistent with the abatement of viable coliform bacteria. Anaerobic digestion abated some but not all antibiotic resistance gene targets. This inconsistent response is presumably due to shifts in the population carrying these genes according to those that perish during digestion and those that survive or proliferate. Future studies on the shift of bacterial populations during digestion using amplicon/shotgun sequencing will help unravel the dynamics of populations and the genes that they carry.

MATERIALS AND METHODS

Participating farms, anaerobic digestion process, and manure sampling method. Seven farms were recruited, but in the present study, farm six was not sampled since this farm composted manure rather than processing the manure through a digestion system. The six participating farms in this project were located within 500 km of London, Ontario, Canada. The herd size for each farm is indicated in Fig. S2. Cow breeds at theses farms were Holsteins.

All farm digestion systems run on a continuous flow methodology, through mesophilic/thermophilic digesters (Fig. S2). Two digesters typically run in a range of 38.5 to 40°C with a retention time of roughly 48 days in farms 1, 2, 3, and 4. For farms 1 and 3, the output of the secondary digester continued going through screw presses to separate solids from liquids. Farm 5 had three digesters operated at 38 to 39°C (digesters 1 and 2) and 51 to 52°C (digester 3) with a total retention time of 100 to 110 days depending on feeding rates. Farm 7 had only a primary digester operated at mesophilic temperatures (38 to 39°C). After this digester, the material went through two screw presses to remove more moisture (dewatered) and were fed into two rotating kiln dryers running at 150°C. Digesters had a volume of about 1,000 m^3 each, except for the digester on farm 7 with a size of 3,000 m^3.

Samples were collected on a monthly basis from the six farms during an approximately 1-year period, except for 1 month when samples were collected on a biweekly basis. A brief description of the manure sampling approach from raw manure to final digestate, including sampling points, can be found in Text S1. The drug list in Table S5 was obtained from the dairy farms' record keeping book because dairy farms were required to record drug usage on the farm as part of their animal care program.

Sample preparation and enumeration of total enteric bacteria. A total of 106 raw and 138 digested manure samples of multiple digestion stages as indicated in Fig. S2 were used for bacterial enumeration. Manure samples were prepared for bacterial enumeration as 10-fold serial dilutions (10^{-1} through 10^{-4}) by aseptically adding 5 g of manure into 45 ml of sterile sodium metaphosphate buffer (2 g/liter, pH 7.0). Subsequent serial dilutions were prepared by aseptically transferring 1 ml of the previous dilution to 9 ml of sterile metaphosphate buffer and vortexed for 30 s.

Total coliforms and *Escherichia coli* were enumerated by direct plating of 100 μl of each serial dilution onto Chromocult agar (Millipore-Sigma, Toronto, ON). Plates were incubated at 37°C for 18 to 20 h. *Enterococcus* spp. were enumerated by direct plating as above onto m-Enterococcus agar (Thermo Fisher Scientific, Toronto, ON). Plates were incubated at 37°C for 48 h. *Staphylococcus* spp. were enumerated by direct plating onto Mannitol salt agar (MSA, VWR International, Mississauga, ON) and Chromagar *Staphylococcus* agar (Dalynn Biologicals, Calgary, AB) and incubated for 48 h at 37°C. *Clostridium perfringens* was enumerated by direct plating of serial dilutions onto mCP agar (Accumedia, VWR International, Mississauga, ON) and incubated at 44.5°C for 24 h in anaerobic boxes under anaerobic conditions (Anaeropak system, VWR International).

Conjugation method with enriched manure samples. Conjugation experiments used 174 enriched samples obtained from the six dairy farms as donors. Sixty-three samples were raw and 111 were digested. Manure was enriched by adding 1 g (if solid, raw manure) or 1 ml (if slurry, digested manure)

into 9 ml buffered peptone water (Difco) supplemented with cefotaxime (4 mg/liter) overnight under static condition at 30°C before being used in conjugation experiments.

Overnight enriched manure was centrifuged at 300 × *g* for 5 min to remove particulates, and the supernatant was decanted into a clean sterile conical tube. Subsequently, the supernatant was centrifuged at 8000 × *g*, 15 min to collect bacteria. The pellet was resuspended in 2 ml 1/10× LB, centrifuged at 3,100 × *g* in 5 min, washed once with 2 ml 1/10× LB, and centrifuged again. Finally, the pellet was resuspended in 200 μl saline.

The β-lactam-sensitive GFP-labeled *E. coli* CV601 (*gfp*$^+$kan^{R}rif^R) was used as the recipient strain in conjugation experiments (71–73). The strain carries some chromosomal resistance genes [*aph(3′)-III*, *mdf* (A)], and its antimicrobial resistance phenotype can be found in Table S4. The strain was inoculated into LB supplemented with rifampin (50 mg/liter) and cultured overnight at 37°C on a rotary shaker set at 200 rpm. The next day, cells were harvested by centrifugation at 3,100 × *g* for 5 min, washed twice with 1/10× LB, then resuspended in 100 μl saline and mixed with the above enriched manure in a ratio of 1:1, and spotted onto LB plates supplemented with cycloheximide (100 mg/liter) plates. Manure aliquots and the recipient strain *E. coli* CV601 were also spotted individually on LB plus cycloheximide (100 mg/liter) plates as negative controls.

After overnight incubation at 30°C, mating spots were washed and resuspended in saline, and different dilutions were plated on Chromocult medium containing rifampin (50 mg/liter), kanamycin (50 mg/liter), and cefotaxime (4 mg/liter) to select transconjugants. Negative controls were also plated onto the same selective medium. Transconjugant green-florescent phenotypes were also confirmed using a handheld UV light.

Conjugation frequency was calculated by taking the ratio of the number of colonies counted on transconjugant-selective plates (Chromocult agar supplemented rifampin [50 mg/liter], kanamycin [50 mg/liter], and cefotaxime [4 mg/liter]) over the number of colonies on recipient-selective plates (Chromocult agar supplemented rifampin [50 mg/liter], kanamycin [50 mg/liter]) which supported the growth of both transconjugants and plasmid-free recipients.

ESBL disc diffusion confirmation test. The ESBL confirmation disc set was used to confirm ESBL phenotypes. This set is a combination of four individual discs of cefotaxime/cefotaxime plus clavulanic acid/ceftazidime/ceftazidime plus clavulanic acid, (BD BBL, Thermo Fisher Scientific, Ottawa, ON). Potential ESBL transconjugants were restreaked on transconjugant-selective plates to obtain pure colonies and confirm their cefotaxime-resistant phenotypes. Following this, they were mixed in 10 ml saline solution and swabbed on Mueller-Hinton agar (Oxoid) plates before the four diffusion discs were applied. Plates were then incubated at 37°C overnight. The results were interpreted by comparing the diameters around the discs following the Clinical and Laboratory Standards Institute (CLSI) guidelines (99).

Based on the results, transconjugants were determined to possess either ESBL or non-ESBL phenotype. Samples where phenotypic ESBL transconjugants were obtained were recorded as ESBL phenotypes. Likewise, samples where non-ESBL phenotype transconjugants were obtained were recorded as non-ESBL phenotypes. Samples that had transconjugants of any phenotype (ESBL, non-ESBL, or both) were recorded as extended-spectrum cephalosporinase (ESC). The numbers were used for statistical analysis as described further below.

Susceptibility tests. MICs of various antimicrobial agents were determined using the Sensititre automated system (Trek Diagnostic Systems, Cleveland, OH, USA) using the Gram-negative (CMV4AGNF and ESB1F) susceptibility panels. The MIC data were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) breakpoints (99) and the breakpoint guideline provided by the U.S. Food and Drug Administration (https://www.fda.gov/media/108180/download).

Plasmid miniprep, enzyme digestion, and whole-genome DNA extraction. Plasmids were isolated from 459 transconjugants obtained from the conjugation experiment using either the Qiaprep Spin Miniprep kit (Qiagen, Toronto, ON) or the Plasmid Mini AX kit (A&A Biotechnology, Gdynia, Poland). They were then digested with EcoRI and run electrophoretically on 0.8% agarose gel at 110 V for 50 min.

For whole-genome DNA extraction, strains were inoculated in LB supplemented with cefotaxime (4 mg/liter) and incubated overnight at 37°C. On the next day, 1 ml cell culture was collected and used for genome DNA extraction using Lucigen MasterPure Complete DNA and RNA with additional RNase step (Lucigen, Mandel Scientific, Guelph, ON, Canada) following the manufacturer's manual.

Illumina/MinION sequencing protocol and bioinformatics tools. Illumina paired-end sequencing was performed on the MiSeq platform (Illumina, Inc., San Diego, CA, USA) using the 600-cycle sequencing kit with libraries prepared using Nextera XT at the National Microbiology Laboratory (Guelph, ON, Canada) to a target of 60-fold coverage.

Oxford Nanopore MinION (Oxford Nanopore Technologies, New York, NY) sequencing was performed according to the default manufacturer protocol for rapid barcoding. Samples were prepared using the SQK-RBK004 rapid barcoding kit and subsequently run on a FLO-MIN106 R9.4 flow cell. Each multiplexed run produced between 4,719 and 111,488 reads per sample, with the mean read length ranging between 3,485 and 11,880 bp. Albacore v. 2.1.3 (Oxford Nanopore Technologies) was used to perform demultiplexing, base-calling, and quality filtering of the raw reads.

Illumina only and hybrid *de novo* assemblies of transconjugants' whole genomes were produced using the Unicycler pipeline v. 0.4.4 (74). MOB-suite v. 2.0.0 was used to characterize the plasmid content of the *de novo* assemblies (75). MOB-recon was used to reconstruct the individual plasmids in the draft *de novo* assemblies, and plasmid typing was performed on each plasmid using MOB-typer using the default parameters for both. PROKKA (Galaxy version 1.13+galaxy1) and RAST (https://rast.nmpdr.org) were used to annotate genes on plasmids (76–80). Mobile genetic elements that were detected by RAST were further specified by blasting sequences against the NCBI nonredundant database (81). Snippy tool (Galaxy version 4.4.3+galaxy0) was used to determine SNPs between a reference genome with closed

plasmid sequence and Illumina assemblies of transconjugants carrying potentially identical plasmids (82). The assemblies were also used as input to StarAMR (Galaxy version 0.7.1+galaxy1) and ABRicate (Galaxy version 0.8) to detect antibiotic resistance genes based on the resfinder resistance gene database (83–88). Plasmid maps were constructed using ApE v. 2.0.47 (https://jorgensen.biology.utah.edu/wayned/ape/) and SnapGene v. 5.1.3.1. Easyfig v. 2.2.2 was used to create multialignment figure (89).

Extraction of DNA from manure samples for quantitative PCR. A subset of 18 raw samples and 18 digested samples were randomly chosen for further investigation using quantitative PCR (qPCR). A total of 250 mg of solid manure was used for DNA extraction using the DNeasy PowerSoil kit (Qiagen, Canada) following the manufacturer's instructions. The final elution volume was 100 μl. If manure samples were liquid, 1 ml of each sample was centrifuged at 15,871 $\times$ *g* for 5 min in a tabletop centrifuge, and the pellet was treated with the Qiagen DNeasy PowerSoil kit following the manufacturer's instructions. The final elution volume was 100 μl. DNA concentrations and quality were determined using a NanoDrop ND-1000 microspectrophotometer (NanoDrop Technologies, Wilmington, DE).

Detection and quantification of antibiotic resistance-associated gene targets. The abundance of 10 selected gene targets associated with antibiotic resistance or horizontal gene transfer was determined by qPCR using a Bio-Rad CFX96 real-time PCR instrument with Bio-Rad CFX Manager software, version 3.0, as described previously (90, 91). The primers and hydrolysis probes used in the present study are listed in Table 6. All primers and hydrolysis probes were synthesized by Sigma (Sigma-Aldrich, Toronto, ON). The reaction was performed with the Brilliant II QPCR Master mix (Agilent, Toronto, ON, Canada) for TaqMan PCR and the Brilliant II SYBR green Low ROX qPCR Master mix (Agilent) for SYBR green PCR. A total of 2 μL of template DNA (corresponding to 0.1 to 10 ng DNA) was added and PCR grade water (Sigma-Aldrich, Toronto, ON) was used to reach a final volume of 25 μl. The PCRs were run in Hard-Shell Thin-Wall 96-Well Skirted PCR plates with clear bottom (Bio-Rad Laboratories, Canada). An optically clear and adhesive seal, the Microseal 'B' seal (Bio-Rad Laboratories, Canada) was placed on a plate before the PCR run. Each sample reaction included the no template control reaction and was run with the following cycle conditions: 1 cycle at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and the required annealing temperature as specified in Table 6 for extension time of 35 s. For the SYBR green assay, a melting curve step was added in order to check the purity of the PCR product. This step consisted of a ramp temperature increase from 65 to 95°C, with an increment of 0.5°C and holding for 5 s for each step. The identities of the quantified gene targets were ensured on the basis of hybridization when using TaqMan chemistry or melting behavior when using SYBR green.

The quantity of each experimental sample was calculated using a standard curve as described in a previous study (91). The DNA fragment was cloned in the pSC-A-amp/kan plasmid using the StrataClone PCR cloning kit (Agilent) and transformed into *E. coli*. Plasmids were extracted using the Qiagen plasmid midi kit (Qiagen, Mississauga, ON). The plasmids were then linearized by NotI enzyme (NewEngland Bio Labs, Mississauga, ON) and purified with the Qiagen QIAquick PCR purification kit. Plasmid copy numbers were calculated using the measured DNA concentration from NanoDrop ND-1000 microspectrophotometer (NanoDrop Technologies, Wilmington, DE). The standard curve consisted of 10-fold serial dilution (from 10^6 down to 1 copy per μl) of a known target plasmid in triplicate and was included in each plate for PCR. The limit of quantification (LOQ) was set at the lowest dilution giving three positive results in the linearity range when tested with negative soil samples. If the gene target was detected at a copy number between one and four copies per reaction, it was considered to be detected below the LOQ.

Statistical analysis. Bacteriological enumeration data for the raw and digested samples were evaluated for normality using the Shapiro-Wilk test. Bacteriological data did not show a normal distribution and subsequently were analyzed using the Mann-Whitney U test to determine significance in treatment effect with a *P* value cutoff of <0.05. Bacteriological enumeration data were plotted as box plots in SigmaPlot (v. 13.0, Systat Software, San Jose, CA).

Antibiotic resistance-associated gene targets in raw and digested samples were evaluated for normality using the Shapiro-Wilk test. Gene targets that did not show a normal distribution [*erm*(F), bla_{OXA-20}] subsequently were analyzed using the analysis of variance (ANOVA) on ranks test to determine significance in treatment effect with a *P* value cutoff of <0.05. Other targets that met normality criteria [*sul1*, *str*(A), *str*(B), *erm*(B), *intI1*, *aadA*, *incW*, bla_{PSE}] were analyzed using the one-way ANOVA test with a significant *P* value cutoff of <0.05. The statistical analysis was done using SigmaPlot (v. 13.0, Systat Software, San Jose, CA). The data were then plotted as box plots using RStudio (v. 1.2.1335, RStudio Inc., Boston, MA).

The conjugation results from 63 raw samples and 63 digested samples were used in multilevel logistic and linear regression models. To evaluate the effect of anaerobic digestion on conjugation, these digested samples were taken from the digestate holding pit for farm 7 and from the secondary digesters for other farms (Fig. S2). The prevalence estimates of samples with transconjugants that have ESC, ESBL, and non-ESBL phenotypes were reported with their 95% exact confidence intervals. The mean ($\log_{10}$) and median transformed conjugation frequencies were reported with their 95% confidence intervals and interquartile ranges, respectively. All estimates were reported for all samples and when stratified by processing stage and season.

Multilevel logistic regression models were fitted to examine the associations between manure processing stage (i.e., raw versus digested) and season (i.e., spring [March, April, May], summer [June, July, August], fall [September, October, November], and winter [December, January, February]) and the odds of a sample having the following characteristics: presence of transconjugants resistant to ESC, presence of transconjugants with an ESBL phenotype (ESBL), and presence of transconjugants with a non-ESBL phenotype. A multilevel linear regression model was fitted to examine the associations between the same independent variables and the $\log_{10}$ of the conjugation frequency. The conjugation frequency was log transformed to meet model assumptions concerning the normality and homoscedasticity of

TABLE 6 Primers and probes used for quantitative PCR in detection of antibiotic resistance genes

Primer or probe	Sequence (5′-3′)[a,b]	Concn (nM)	Annealing temp (°C); extension time (s)	Amplicon size (bp)	Target	Reference
Universal bacteria						
BACT1369F	CGGTGAATACGTTCYCGG	300	59; 40	123	*rrn*S gene	94
PROK1492R	GGWTACCTTGTTACGACTT					
TM1389F	HEX-CTTGTACACACCGCCCGTC-BHQ1	300				
int1						
Int1-F2	TCGTGCGTCGCCATCACA	400	62; 60	67	Integrase class 1	95
Int1-R2	GCTTGTTCTACGGCACGTTTGA					
sul1						
sul1-F	GACTGCAGGCTGGTGGTTAT	200	64; 60	105	Sulfamethazine resistance gene 1	91
sul1-R	GAAGAACCGCACAATCTCGT					
str(A)						
strA-F	TATGGTTGTTTGCCATGGTG	400	62; 60	126	Streptomycin phosphotransferase A	96
strA-R	TTCTCTTCGGCGTTAGCAAT					
str(B)						
strB-F	ATCGCTTTGCAGCTTTGTTT	300	61; 30	143	Streptomycin phosphotransferase B	96
strB-R	ATGATGCAGATCGCCATGTA					
*str*B-P	HEX-ATGCCTCGGAACTGCGT-BHQ1	200				
erm(B)						
ermB-F	AAAACTTACCCGCCATACCA	400	65; 60	139	Erythromycin resistance gene locus B	97
ermB-R	TTTGGCGTGTTTCATTGCTT					
erm(F)						
ermF-F	TCGTTTTACGGGTCAGCACTT	300	61; 30	182	Erythromycin resistance gene locus F	97
ermF-R	CAACCAAAGCTGTGTCGTTT					
aad(A)						
aadA-F	CAGCGCAATGACATTCTTGC	200	63; 30	294	Aminoglycoside adenylyltransferase	96
aadA-R	GTCGGCAGCGACA(C/T)CCTCG					
aadA-P	HEX-TGGTAGGTCCAGCGGCGGAG-BHQ1	300				
bla_{OXA-20}						
$bla_{OXA20-F}$	TGATGATTGTCGAAGCCAAA	400	60; 60	101	Oxacillinase gene bla_{OXA20} (group II)	98
$bla_{OXA20-R}$	GCCTGTAGGCCACTCTACCC					
bla_{PSE}						
blaPSE-F	ACCGTATTGAGCCTGATTTA	400	59; 30	101	Beta-lactamase PSE gene	98
blaPSE-R	GCCGGCAATACTGAACGTAG					28
blaPSE-P	HEX-TCTTGGATGGTGAACAATCAAG-BHQ1	300				
IncW *rep*A						
IncW-F	GGCCATCGTATCAACGAGAT	300	61; 30	153	Plasmid incompatibility group W	91
IncW-R	ATTGGTGCGCTCAAAGTAGC					
IncW-P	HEX-AGCTGGCTTAGTCGGCTACA-BHQ1	200				

[a]HEX, 2′,4′,5′,7′-tetrachloro-6-carboxy-4,7-dichlorofluorescein succinimidyl ester.
[b]BHQ-1, black hole quencher-1.

residuals. Where the conjugation frequency was 0, half the lowest value recorded was used for analyses since the $\log_{10}$ of zero is mathematically undefined. For all the multilevel models, random intercepts were included for the samples collected on the same date on a specific farm and for farm. Processing stage was forced into all models, and season was included if it was a statistically significant independent variable or acted as a confounding variable (92). Season was considered a confounding variable if its removal from the model resulted in a 20% or greater change in the model coefficient for processing stage. Interaction effects between processing stage and season were not examined due to small sample sizes for interaction terms. Variance partition coefficients were estimated for each model with the latent variable technique used for the multilevel logistic regression models (93). The standardized residuals (multilevel linear model) and the best linear unbiased predictions (BLUPs for all models) were examined using normal quantile plots and scatterplots of the BLUPs against the predicted outcomes to determine if they met the assumptions of normality and homoscedasticity, respectively. The significance level for all

analyses was 5% (i.e., $\alpha = 0.05$). All multilevel models were fitted using the "melogit" (multilevel logistic regression) and "mixed" (multilevel linear regression) commands using Stata 15 statistical software (StataCorp, College Station, TX).

Data availability. Complete nucleotide sequences of 11 closed plasmids were deposited in GenBank under accession numbers MW298652 to MW298662. Whole-genome sequences can be accessed on the NCBI server under BioProject ID PRJNA681611.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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