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Transmission of Antibiotic-Resistant *Escherichia coli* from Chicken Litter to Agricultural Soil

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A growing concern regarding the use of animal manure as fertilizer is the contamination of soil, plants, and the environment with a variety of antibiotic-resistant and pathogenic bacteria. This study quantified and characterized the antibiotic resistance profiles of *Escherichia coli* in soil before and after chicken litter application to determine the impact of manure on the soil resistome. Litter and soil samples were collected from a sugarcane field before and after litter application. *E. coli* was isolated and quantified using the Colilert®-18/Quanti-tray® 2000 and 10 randomly selected isolates from the positive wells of each Quanti-tray were putatively identified on eosin methylene blue agar. Real-time PCR was used to confirm the isolates by targeting the *uidA* gene. Antibiotic susceptibility test against 18 antibiotics was conducted using the disk diffusion method, and the multiple antibiotic resistance index was calculated. Soil amendment with chicken litter significantly increased the number of antibiotic-resistant *E. coli* in the soil. Among the 126 *E. coli* isolates purified from all the samples, 76% showed resistance to at least one antibiotic, of which 54.2% were multidrug-resistant (MDR). The highest percentage resistance was to tetracycline (78.1%), with the least percentage resistance (3.1%) to imipenem, tigecycline, and gentamicin. The isolates also showed resistance to chloramphenicol (63.5%), ampicillin (58.3%), trimethoprim-sulfamethoxazole (39.6%), cefotaxime (30.2%), ceftriaxone (26.0%), cephalexin (20.8%), cefepime (11.5%), amoxicillin-clavulanic acid (11.5%), cefoxitin (10.4%), Nalidixic acid (9.4%), amikacin (6.3%), and ciprofloxacin (4.2%). Of the 54.2% (52/96) MDR, the highest number was isolated from the litter-amended soil (61.5%) and the least isolates from soil samples collected before litter application (1.9%). The relatively higher mean MAR index of the litter-amended soil (0.14), compared to the soil before the amendment (0.04), suggests soil pollution with antibiotic-resistant *E. coli* from sources of high antibiotic use. *E. coli* could only be detected in the soil up to 42 days following manure application, making it a suitable short-term indicator of antibiotic resistance contamination. Notwithstanding its relatively short detectability/survival, the application of chicken litter appeared to transfer antibiotic-resistant *E. coli* to the soil, enhancing the soil resistome and highlighting the consequences of such agricultural practices on public health.

Keywords: *E coli*, multidrug-resistance, chicken litter, litter-amended soil, unamended soil, environment, transmission, pollution

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INTRODUCTION

The increasing prevalence of antibiotic-resistant bacteria (ARB) in the environment is a growing global threat to public health in the 21st century (Udikovic-Kolic et al., 2014; Wellcome Trust, 2018). The misuse and overuse of antibiotics in food-animal production contributes to the emergence and subsequent spread of antibiotic resistance from animals to the environment (Laxminarayan et al., 2013; WHO 2020). A significant route by which ARB enter the environment and the food chain is through manure from antibiotic-treated animals applied to agricultural soil (Heuer et al., 2011; Marti et al., 2013). However, the impact of this agricultural practice on the soil resistome is not well known, particularly in African countries.

Chicken litter is often applied to agricultural soil as a substitute for inorganic fertilisers to meet the growing demand for crops and improve soil fertility, particularly in organic farming (Jechalke et al., 2013; Marti et al., 2013; Atidégla et al., 2016). Also, the application of chicken litter to agricultural soil as organic fertiliser is the cheapest means of disposing the large volumes of poultry waste generated from the rapidly growing poultry industry worldwide (Kyakuwaire et al., 2019). Furthermore, chicken litter, a mixture of chicken faeces, waste feed, wood shavings, and other small invertebrates, is a major soil amendment that improves and maintains the chemical, physical, and biological soil properties (Brye et al., 2004).

A growing concern about the application of untreated animal manure to agricultural soil is the possibility of contamination with pathogenic ARB and antibiotic resistance genes (ARGs), as animal manure is considered a significant reservoir of both enteric and pathogenic ARB and ARGs (Robins-Browne, 2005; Johnson et al., 2016). Previous studies that investigated the impact of animal manure on soil resistome demonstrated that fertilisation with animal manure resulted in the appearance or increased level and diversity of ARB and ARGs in soils (Jensen et al., 2013; Chen et al., 2016b; Fatoba et al., 2021; Sun et al., 2021). Jensen et al. (2013) reported the appearance of *E. coli* in soil after the application of animal slurry to three Danish agricultural fields. Additionally, Chen and others (2016b) observed a significant increase in the bacterial diversity and the abundance of tetracyclines genes in the soil following manure amendment in China. Fatoba et al. (2021) also observed an increase in MDR *Enterococcus* spp. in agricultural soil following chicken litter application. Another study from the United States showed that manure application significantly increased the diversity of surface soil microbiome and resistome and also introduced tetracycline and sulphonamide resistance genes to the soil (Sun et al., 2021).

The addition of ARB and ARGs of animal manure origin to the soil can also lead to horizontal transfer of ARGs between the manure-borne bacteria and the indigenous soil bacteria through mobile genetic elements (MGEs) (Heuer et al., 2011). Gao et al. (2015) tracked *E. coli* harbouring ESBL genes from pig manure to agricultural soil fertilised with pig manure and showed that MDR ESBL-producing *E. coli* isolates from the manure-amended soil had overlapping phenotypes and over 90% genetic similarity with strains from the pig manure samples. Seventy and fifty-six percent of the isolates from the manured soil and pig manure, respectively, harboured the IncF-type replicon plasmids, which suggest possible horizontal gene transfer in the soil (Gao et al., 2015).

Moreover, most studies have indicated that such increases are temporal because bacteria from manure are less adapted to soil environments (Sengeløv et al., 2003b; Heuer and Smalla, 2007; Binh et al., 2008; Marti et al., 2014; Muurinen et al., 2017) However, other studies have found certain ARB to survive in manure-amended soil for extended periods (Islam et al., 2004; Merchant et al., 2012; Çekiç et al., 2017). These ARB and ARGs from the manure-amended soil can subsequently enter the food chain through contaminated farm produce or spread to community surface water bodies *via* run-offs(Marti et al., 2013; Zhang et al., 2019), posing severe human health risks. Several infection outbreaks have been linked to *E. coli* in food contaminated by animal manure (Atidégla et al., 2016; Yang et al., 2017; Shonhiwa et al., 2019).

There is a paucity of information on the environmental dimensions of AMR in Africa, as most of the AMR surveillance and research focuses on the prevalence of ARB in humans and food animals (farm-to-fork) (Mbelle et al., 2019; McIver et al., 2020; Abdalla et al., 2021). However, a substantial number of ARB can be transferred to the soil via animal manure application, and attempts to identify them may not be financially and technologically feasible. Therefore, identifying an organism suitable to be an indicator of such pollution is necessary. *E. coli* has been used as an indicator of faecal pollution for centuries. Recently, the World Health Organization (WHO) has recommended using *E. coli* to trace AMR because its molecular mechanisms of resistance are well characterised (WHO, 2020). However, several studies have reported on the relatively shorter duration of survival of *E. coli* in the environment compared with other organisms (Sengeløv et al., 2003a; Bolton et al., 2011; Abia et al., 2015b). In addition, a previous study showed that *Enterococcus* could be found in litter amended soils up to 105 days following manure application (Fatoba et al., 2021). Therefore, this study investigated the potential transmission of antibiotic-resistant *E. coli* from chicken litter to agricultural soil and sought to determine how long *E. coli* could be detected in litter amended soil following chicken litter application. We evaluated the prevalence, antibiotic resistance profiles, and the MAR indices of *E. coli* isolated from chicken litter and the soil of a sugarcane field before and after chicken litter application.

MATERIALS AND METHODS

Study Site and Sample Collection

This study was carried out on a sugarcane field located in uMshwathi Local Municipality in uMgungundlovu District, KwaZulu-Natal, South Africa, fertilized with chicken litter. The study site and its surroundings have previously been described (Fatoba et al., 2021). The sample collection was carried out for one hundred and 16 days (October 2018 to February 2019). Soil samples were collected for 5 days before manure application

(i.e., days 1, 2, 3, 5, and 9). Samples were also collected on the day of manure application over 111 days. Soil samples were collected until no microbial counts were recorded in most of the sampling points at three consecutive sampling rounds. The chicken litter was a mixture of raw chicken faeces and wood shavings from a large-scale chicken farm that supplements feed and water with antibiotics in the uMgungudlovu District. The poultry farmer uses zinc bacitracin, olaquindox, and avilamycin, for growth promotion. Doxycycline, macrolide-lincosamides (tylosin, kitasamycin), enrofloxacin, sulfadiazine-trimethoprim, and zinc bacitracin are used for therapeutic purposes. A detailed sampling regime has previously been described (Fatoba et al., 2021).

Quantification and Purification of *E. coli*

E. coli was detected and quantified using the Colilert® -18/Quanti-Tray® 2000 system (IDEXX Laboratories (Pty) Ltd., Johannesburg, South Africa) according to the manufacturer's guidelines. All the samples collected were processed as previously described by Abia et al. (2015a). Briefly, 5 g of homogenised soil or litter samples were resuspended in 5 ml sterile distilled water, and the supernatant was analysed using the IDEXX defined substrate multiple tube technique as recommended by the manufacturer. A detailed analysis of the samples' supernatant has been previously described by Fatoba et al. (2021). The most probable number (MPN) of *E. coli* in 100 ml of sample (MPN/100 ml) poured inside the quanti-Tray® 2000 system was calculated from the number of fluorescent (positive) wells per sample as recommended by IDEXX. Corresponding value of *E. coli* counts per grams (MPN/g), was calculated taking into consideration the dilution factor. Following incubation, pure *E. coli* isolates were obtained by subculturing the content of ten randomly selected fluorescent wells repeatedly on Eosin Methylene Blue agar plates (Oxiod, Hampshire, England) and incubating at 37°C for 24 h as previously described (Abia et al., 2015b). The presumptive pure, distinct colonies obtained from the selective media plates were stored in trypticase soy broth (TSB) with 20% glycerol at −80°C for further analysis.

Molecular Confirmation of the *E. coli* Isolates

The stored isolates were revived on nutrient agar. Overnight grown *E. coli* cultures on nutrient agar plate at 37°C were used for the DNA extraction using the boiling method previously described (Dashti et al., 2009). The extracted DNA was stored at −80°C for subsequent analysis. All the *E. coli* isolates were then confirmed by real-time polymerase chain reaction using specific primers sets that targeted the *uidA* (encoding beta-glucuronidase) gene as described by López-Saucedo et al. (2003) using the forward and reverse primers 5′-AAAACGGCAAGAAAAAGC AG-3′ and 5′-ACGCGTGGTTAACAGTCTTGCG-3′, respectively. The positive control used was *E. coli* ATCC 25922 (American Type Culture Collection, Manassas, VA, United States), and the no-template control was the reaction mixture without template DNA. The PCR protocols were as previously described (Abia et al., 2015b).

Antibiotic Susceptibility Testing

The antibiotic susceptibility test of the *E. coli* isolates was carried out according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2020) using the disk diffusion method on a panel of 18 antibiotics (**Figures 1**,**2**). Zones of inhibition were interpreted according to CLSI breakpoints except for tigecycline (15 μg) and cephalexin (30 μg), where the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints were used. Multidrug resistance is defined as resistance to at least one drug in three or more different classes of antibiotics. *E. coli* ATCC 25922 was used as the control strain. The multiple antibiotic resistance (MAR) index of each isolate was calculated as a/b, where a is the number of antibiotics to which a tested isolate expressed resistance, and b is the number of antibiotics to which the isolate has been evaluated for susceptibility (Krumperman, 1983).

Statistical Analysis

The data on *E. coli* counts were log-transformed and analysed using Microsoft Excel 2016 and Statistical Package for the Social Science SPSS version 26 (IBM Corporation, Armonk, New York, United States). One-way analysis of variance (ANOVA) and Games-Howell Post-hoc test was used to check for any significant differences in the mean counts of *E. coli* and the number of antibiotic-resistant *E. coli* in the soil before and after litter amendment and the chicken litter. All statistical tests were considered significant at $p < 0.05$. For ease of data presentation, the most probable number per Gram (MPN/g) of samples with values < 1 was considered as 1 for log-transformation and average calculations.

RESULTS

Mean Concentrations of *E. coli*

A total of 193 samples (45 chicken litter and 148 soil) were collected. Among the three sample groups, chicken litter had the highest (4.09 E + 07 MPN/g) *E. coli* counts per sample round (**Table 1**). The overall mean count of *E. coli* in the chicken litter (2.11 (±1.29) x 10^7 MPN/g) was significantly higher than the litter-amended soil ($p = 0.020$), and the soil samples collected before the litter amendment ($p = 0.023$) (**Supplementary Table S1**). There was no statistically significant difference ($p = 0.999$) in the overall mean count of *E. coli* in the litter-amended soil (1.51 (±0.99) x 10^7 MPN/g) and the soil samples collected before the litter amendment (1.52 (±0.72) x 10^7 MPN/g) (**Supplementary Table S1**). The *E. coli* counts in the soil and the stored chicken litter fluctuated throughout the sampling period. No *E. coli* was detectable in the soil 49 days after the litter amendment.

Prevalence and Antibiotic Susceptibility Profiles of the *E. coli* Isolates

A total of 126 *E. coli* isolates were recovered from all the positive samples, with 88 from the litter-amended soil, 10 from soil samples before the litter amendment, and 28 from the chicken litter. Seventy-six percent (96/126) of the *E. coli* isolates displayed resistance to at

TABLE 1 | Mean *E. coli* counts ((MPN/g) throughout the sample collection.

Sample collection day	Mean *E. coli* count (MPN/g) per sample point						The geometric mean of *E. coli* count (MPN/g)/Sample group		
	A	B	C	D	E	H	Soil before litter amendment	Litter-amended soil	Chicken litter
D1	1.30 E + 07	1.85 E + 07	2.58 E + 07	2.61 E + 07	—	—	1.99 E + 07	—	—
D2[b]	1.88 E + 07	2.31 E + 07	2.61 E + 07	2.01 E + 07	1.00 E + 00	—	1.84 E + 07	—	—
D3[r]	1.00 E + 00	1.00 E + 00	1.00 E + 00	1.00 E + 00	1.00 E + 00	—	1.00 E + 00	—	—
D5	1.00 E + 00	1.00 E + 00	1.00 E + 00	1.00 E + 00	1.46 E + 07	—	1.07 E + 07	—	—
D9	2.92 E + 07	1.94 E + 06	1.00 E + 00	1.00 E + 00	1.00 E + 00	—	1.38 E + 07	—	—
D0	2.61 E + 06	1.40 E + 07	1.15 E + 07	3.45 E + 06	1.45 E + 07	1.63 E + 07	—	1.72 E + 07	1.56 E + 07
D1	1.00 E + 00	1.77 E + 07	1.89 E + 07	1.00 E + 00	2.03 E + 06	2.15 E + 07	—	1.40 E + 07	2.13 E + 07
D3	1.00 E + 00	2.29 E + 06	2.27 E + 07	1.82 E + 07	2.27 E + 07	1.84 E + 07	—	1.68 E + 07	1.52 E + 07
D7u	1.00 E + 00	1.00 E + 00	1.00 E + 00	1.00 E + 00	1.65 E + 07	1.80 E + 07	—	1.09 E + 06	1.76 E + 07
D14[r]	2.76 E + 07	2.10 E + 07	1.15 E + 07	1.15 E + 07	1.00 E + 00	3.93 E + 07	—	1.45 E + 07	3.76 E + 06
D21	3.60 E + 07	1.40 E + 07	1.00 E + 00	2.80 E + 07	2.99 E + 08	3.41 E + 07	—	1.89 E + 07	4.09 E + 07
D28	1.00 E + 00	1.00 E + 00	1.00 E + 00	1.00 E + 00	1.46 E + 07	1.20 E + 07	—	1.07 E + 07	1.92E + 07
D35	1.40 E + 07	1.00 E + 00	1.00 E + 00	1.00 E + 00	1.00 E + 00	1.60 E + 07	—	1.06 E + 07	1.41 E + 06
D42r	1.00 E + 00	1.00 E + 00	1.00 E + 00	1.00 E + 00	2.84 E + 07	2.83 E + 07	—	1.17 E + 07	2.57 E + 07
D49	1.00 E + 00	1.00 E + 00	1.00 E + 00	1.00 E + 00	1.00 E + 00	3.28 E + 07	—	1.00 E + 00	3.23 E + 07
D56	—	—	—	—	—	1.00 E + 00	—	—	1.00 E + 00
D63	—	—	—	—	—	1.00 E + 00	—	—	1.00 E + 00
D77[r]	—	—	—	—	—	2.29 E + 07	—	—	1.67 E + 07
D91	—	—	—	—	—	1.81 E + 07	—	—	1.51 E + 07
D105	—	—	—	—	—	1.26 E + 07	—	—	1.21 E + 07
Overall Mean count	1.63 E + 07	1.47 E + 07	1.38 E + 07	1.52 E + 07	1.58 E + 07	2.11 E + 07	1.52 E + 07	1.51 E + 07	2.11 E + 07

- Sample not collected. The farm was burnt (b) on day 2 (D2), chicken litter, and urea (u) salt was applied on day 0 (D0) and day 7 (D7) respectively. E. coli *reduced to detection limit in soil on day 49 (D49).*

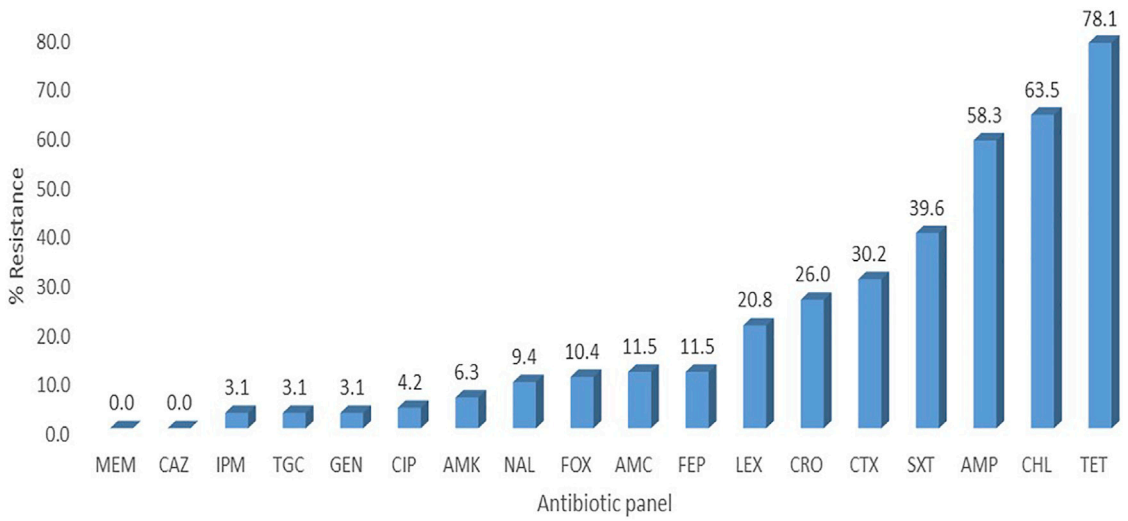

FIGURE 1 | Percentage resistance of the *E. coli* isolates. LEX-Cefalexin, FEP-Cefepime, CTX-Cefotaxime, CAZ-Ceftazidime, AMP-Ampicillin, FOX-Cefoxitin, TGC-Tigecycline, CIP-Ciprofloxacin, TET-Tetracycline, SXT- Trimethoprim-sulfamethoxazole, AMC-Amoxicillin-clavulanic acid, CHL- Chloramphenicol, GEN-Gentamicin, AMK- Amikacin, MEM-Meropenem, CRO-Ceftriaxone, IPM-Imipenem, NAL-Nalidixic acid.

least one of the antibiotics tested. The highest number was recorded in the litter-amended soil (71.9%, 69/96), followed by the chicken litter (27.1%, 26/96) and in soil samples collected before the litter amendment (1%, 1/96). The highest percentage resistance was to tetracycline (78.1%), while the least (3.1%) was to imipenem, tigecycline, and gentamicin (3.1%) (**Figure 1**). In addition, all the isolates were susceptible to meropenem and ceftazidime. Notably, there was an increased detection of *E. coli* isolates resistant to tetracycline, ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole in the soil after the chicken litter application (**Figure 2**). The overall prevalence of antibiotic-resistant *E. coli* in the litter-amended soil was statistically significantly higher than in the soil samples before amendment (p = 0.001) and chicken litter (p = 0.001) (**Supplementary Table S2**).

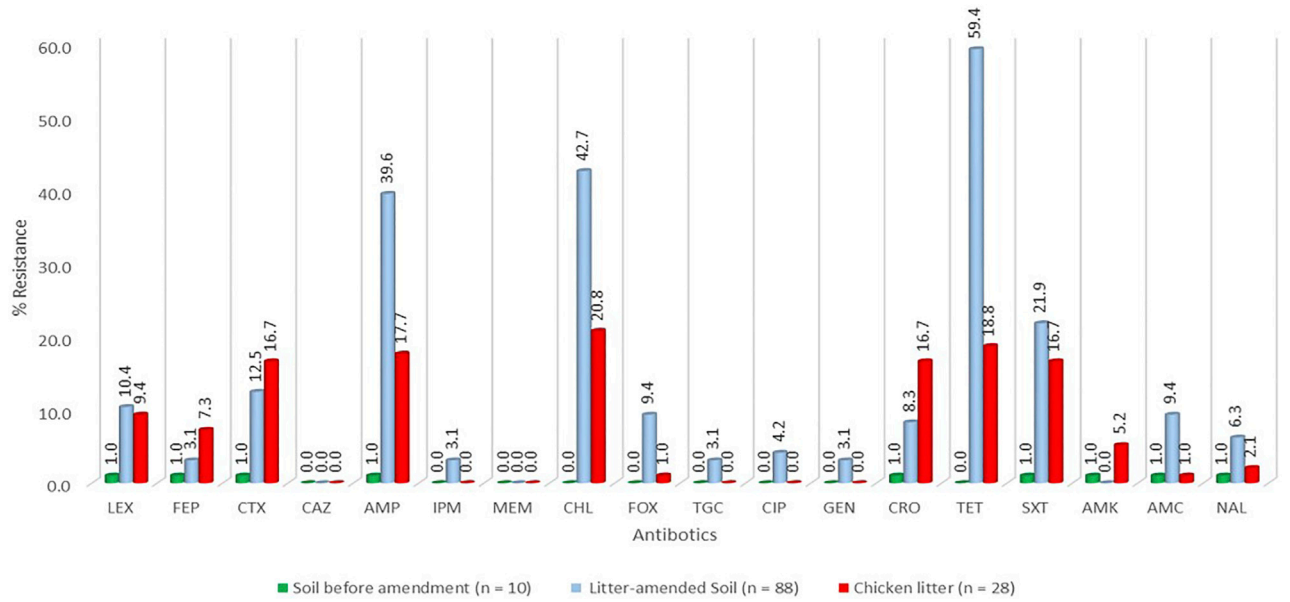

FIGURE 2 | Percentage resistance of the *E. coli* stratified by sample source. LEX-Cefalexin, FEP-Cefepime, CTX-Cefotaxime, CAZ-Ceftazidime, AMP-Ampicillin, FOX-Cefoxitin, TGC-Tigecycline, CIP-Ciprofloxacin, TET-Tetracycline, SXT- Trimethoprim-sulfamethoxazole, AMC-Amoxicillin-clavulanic acid, CHL-Chloramphenicol, GEN- Gentamicin, AMK- Amikacin, MEM-Meropenem, CRO-Ceftriaxone, IPM-Imipenem, NAL-Nalidixic acid.

Multidrug Resistance

Multidrug resistance was evident, and the predominant resistance patterns were ampicillin-chloramphenicol-tetracycline and chloramphenicol-tetracycline-trimethoprim-sulfamethoxazole (**Table 2**). In total, 54.2% (52/96) of the isolates were multidrug-resistant, grouped into 21 different resistance patterns. The highest prevalence of MDR was detected in the litter-amended soil (61.5%) and the least in isolates from soil samples collected before litter application (1.9%) (**Table 2**). Interestingly, two isolates, one from each of the chicken litter and litter-amended soil, displayed resistance to ten antibiotics that belong to six and four classes of antibiotics, respectively.

Multiple Antibiotic Resistance (MAR) Index of the *E. coli* Isolates

The MAR index of all the isolates ranged between 0.11 and 0.56, representing resistance to two and ten antibiotics, respectively (**Table 3**). Overall, 38.5% (37/96) of the resistant isolates had a MARI >0.2 with the highest rate (51.4%) in the litter-amended soil and the least in the soil before litter amendment (2.7%).

DISCUSSION

An anthropogenic activity like the application of manure from food animals exposed to antibiotics to soil can increase the burden of AMR in the soil environment, thereby posing a public health threat, particularly when potential pathogenic ARB like *E. coli* enter the food chain. This study investigated the potential transmission of antibiotic-resistant *E. coli* from chicken litter to agricultural soil in KwaZulu-Natal, South Africa. The chicken litter amendment increased the bacterial count and the number of antibiotic-resistant isolates in the soil. Antibiotic-resistant *E. coli* was detected in all the sample points and the three sample groups with the highest prevalence in the litter-amended soil. The isolates displayed high percentage resistance to tetracycline, ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole commonly used in poultry farms. Also, 54.2% of all the isolates were MDR. The relatively high percentage (51.4%) of isolates with MAR index >0.2 in the litter-amended soil compared to the soil before the litter amendment (2.7%) indicated that the litter amendment resulted in soil contamination with *E. coli* from sources with high use of antibiotics.

Mean Concentrations of *E. coli*

E. coli is an established indicator of faecal contamination because of its ubiquitous presence in the intestines of animals and its prominence in faecal-contaminated environments (Aarestrup et al., 2008). This explains the highest *E. coli* count recorded in the chicken litter, making it a potential source of *E. coli* contamination to the receiving environment. There was an increase in *E. coli* count in the soil at the time (day 0) the chicken litter was applied and days (D3, D14, and D21) after the soil amendment (**Table 1**). The litter amendment could have contributed to the observed increase, as previous studies have indicated that the addition of animal manure to soil increases the number of viable bacteria in soil by the enrichment of indigenous soil bacteria or addition of manure-borne bacteria (Zhu et al., 2013).

TABLE 2 | Multidrug-resistance patterns of the *E. coli* isolates.

Antibiogram	Soil before litter (*n* = 10)	Litter-amended soil (*n* = 88)	Chicken litter (*n* = 28)	Total
Farm burning day (D2)				
AMP-AMC-LEX-CTX-CRO-FEP-AMK-SXT	1	0	0	1
Day 0 of litter application				
AMP-CHL-TET	0	8	0	8
AMP-CTX-CRO-NAL-CHL-TET	0	1	0	1
AMP-CTX-CRO-CHL-TET	0	2	5	7
AMP-CHL-TET-SXT	0	3	0	3
AMP-CTX-CHL-TET	0	1	0	1
AMP-CHL-CIP-GEN-TET-SXT	0	1	0	1
AMP-NAL-CIP-GEN- CHL-TET-SXT	0	1	0	1
AMP-TET-SXT	0	7	0	7
Day 3 after litter application				
AMP-AMC-LEX-FOX-CTX-CRO-FEP-TET-SXT	0	1	0	1
AMP-AMC-LEX-FOX-CTX-CRO-TET-SXT	0	1	0	1
Day 7 after litter application				
AMP-AMC-LEX-FOX-CTX-CRO-FEP-TET-TGC-SXT	0	1	0	1
Day 14 after litter application				
AMP-LEX-NAL-CIP-GEN-CHL-TET	0	1	0	1
AMP-LEX-CTX-CRO-FEP-NAL-CIP-TET-SXT	0	1	0	1
Day 21 after litter application				
AMP-AMC-TET-SXT	0	1	0	1
CHL-TET-SXT	0	1	7	8
AMP-AMC-FOX-TET-SXT	0	1	0	1
Day 49 after litter application				
AMP-LEX-CTX-CRO-FEP-NAL-AMK-CHL-TET-SXT	0	0	1	1
AMP-LEX-CTX-CRO-FEP-CHL-TET-SXT	0	0	4	4
AMP-LEX-CTX-CRO-FEP-NAL-AMK-SXT	0	0	1	1
Day 77 after litter application				
AMK-CHL-TET-SXT	0	0	1	1
Total MDR Isolates (*n* = 52, 54.2%)	1 (1.9%)	32 (61.5%)	19 (36.5%)	52

LEX-Cefalexin, FEP- cefepime, CTX-Cefotaxime, CAZ-Ceftazidime, AMP-Ampicillin, FOX-Cefoxitin, TGC-Tigecycline, CIP-Ciprofloxacin, TET-Tetracycline, SXT- Trimethoprim-sulfamethoxazole, AMC-Amoxicillin-clavulanic acid, CHL- Chloramphenicol, GEN- Gentamicin, AMK- Amikacin, MEM-Meropenem, CRO-Ceftriaxone, IPM-Imipenem, NAL-Nalidixic acid.

TABLE 3 | Multiple antibiotic resistance (MAR) index of the isolates.

MAR index	No. of isolates	Percentage (%)
0.11	26	27.1
0.17	25	26.0
0.22	10	10.4
0.28	9	9.4
0.33	4	4.2
0.39	2	2.1
0.44	8	8.3
0.50	2	2.1
0.56	2	2.1

In this study, we observed short-term (42 days) persistence of *E. coli* in the soil following the amendment suggesting that *E. coli* can serve as a suitable indicator of short-term faecal pollution in agricultural soil. This finding is consistent with previous reports (Sengeløv et al., 2003a; Binh et al., 2008; Bolton et al., 2011), that indicated that some bacteria from animal manure are less adapted to the soil environment and only survive for a short time (9 days–11 weeks). Several indicator organisms like enterococci, *E. coli*, faecal coliforms, and *Clostridium perfringens* are the commonly tested faecal pollution indicators. However, the limitations and strength of each of these indicators suggest that none of these indicator organisms should be used in isolation for predicting the impact of faecal pollution in any environment (Tyagi and Chopra, 2006). *E. coli* hardly survives under environmental stress such as limited moisture, low organic matter, high and low temperatures (Berry and Miller 2005; Williams et al., 2005). Contrary to *E. coli,* high densities of enterococci in soils has been attributed, in part, to the more excellent survival abilities of Gram-positive bacteria (e.g., enterococci and staphylococci) than of Gram-negative bacteria (e.g., *E. coli*, and *Pseudomonas* spp.) under environmental stresses, particularly desiccation and cellular injury (Bale et al., 1993; Byappanahalli et al., 2012). Fatoba et al., 2021 showed that resistant enterococci were still detectable in the litter-amended soil even at 105 days after chicken litter application. However, *E. coli* was no longer detectable after day 42 in the current study, suggesting that the long-term impact and accurate monitoring of the soil environment for bacterial contamination from manure-based fertilizers requires a more persistent indicator organism alongside *E. coli*. Supporting this finding, a previous study of faecal pollution in riverbed sediments in South Africa recommended using *Clostridium perfringens* alongside *E. coli* as indicators of faecal pollution in riverbed soil due to its persistence in the environment(Abia et al., 2015b). Although

E. coli was not recorded in the litter-amended soils after 42 days, some isolates were still recovered from the unapplied chicken litter heap. The survival could be due to the rich nutrient content in the chicken litter, while the disappearance in the litter-amended soil could have also been influenced by other farm practices like urea application.

Antibiotic Susceptibility Profiles of the *E. coli* Isolates

The use of antibiotics in food animal production has been beneficial for economic and animal health reasons. Thus, different antibiotic classes are used in food-animal production, depending on the purpose (prophylaxis, metaphylaxis, treatment, or growth promotion), the kind of animal, and the country's policies. However, their overuse and misuse have led to increased detection of ARB in manure, which in most cases is released into the environment (Looft et al., 2012; Johnson et al., 2016). Additionally, antibiotic administration patterns and quantities used in food animal production vary considerably from country to country, region to region, and farm to farm, resulting in substantial differences in the rate of resistance recorded in many studies (Van Boeckel et al., 2015). Studies conducted within South Africa and other countries have reported varying levels of *E. coli* resistance to the antibiotics included in the current study.

Overall, the *E. coli* isolates examined in this study expressed the highest percentage of resistance to tetracycline (78.1%), followed by chloramphenicol (63.5%), ampicillin (58.3%), and trimethoprim-sulfamethoxazole (39.6%), correlating with the frequent use of doxycycline and trimethoprim-sulphadiazine in poultry farms in KwaZulu-Natal Province, South Africa. Furthermore, two surveys carried out in South Africa on antimicrobial use in food animals showed that the highly consumed antibiotics in food animal production in South Africa include the macrolides, tetracyclines, sulphonamides, and the penicillins (Henton et al., 2011; Eagar et al., 2012). Therefore, the increased detection of *E. coli* resistant to these four antibiotics (tetracycline, ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole) in the soil after the litter amendment suggests emergence on the farm followed by transmission of antibiotic-resistant *E. coli* and/or ARGs from the chicken litter to the soil.

The high prevalence of chloramphenicol resistance was not expected. This antibiotic is not permitted for use in food-animal production in South Africa (Eagar et al., 2012), neither is it among the antibiotics used on the poultry farms in KwaZulu-Natal (personal communication). Therefore, the high chloramphenicol resistance in the absence of chloramphenicol selection pressure may be due to co-selection and/or co-transmission of chloramphenicol resistance due to genetic linkage to genes conferring resistance to antibiotics that are commonly used in poultry farms. For example, the co-selection of chloramphenicol resistance with resistance to sulfamethoxazole, tetracycline, and kanamycin due to frequent use of sulphonamides, aminoglycosides, and tetracyclines in food animals has been reported in the United States (Bischoff et al., 2005). The study demonstrated the conjugative transfer of the chloramphenicol resistance gene *CmlA* with both sulphonamide (*sul*) and aminoglycoside (*aadA*) resistance genes on class 1 integrons from swine-borne *E. coli* donors to the recipient *E. coli* strains (Bischoff et al., 2005). Since aminoglycosides are not used in food-animal production in South Africa, the common use of sulphonamides may be responsible for the spread of chloramphenicol resistance among the isolates.

Although the resistance of *E. coli* to the third-generation cephalosporins (cefotaxime and ceftriaxone) was relatively low compared to tetracycline, chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole, the high frequency observed in chicken litter isolates needs urgent monitoring as cephalosporins are important front-line antibiotics widely used to treat infections caused by Gram-negative bacteria in humans. Thus, to curb the spread of AMR in food animal production and the environment, there is a need to implement policies that will ensure strict and proper use of available antibiotics.

In this study, the chicken litter application significantly increased the detection of antibiotic-resistant *E. coli* in the soil. This could be ascribed to the combined proliferation of indigenous soil *E. coli* and exogenous manure-borne ones, both of which were enhanced by the added nutrients from the chicken litter. These findings are consistent with previous studies that have indicated that land application of animal manure can result in the introduction of new ARB and ARGs of animal manure origin to the soil or increase the quantity of already existing soil ARB and ARGs (Sengeløv et al., 2003a; Udikovic-Kolic et al., 2014). Udikovic-Kolic et al. (2014), in a field experiment conducted in the United States, showed that cow manure amendment increased β-lactam-resistant bacteria in the manured soil. The increase was attributed to the enrichment of the ARB initially present in the soil. Another field experiment conducted on four farms in Denmark indicated that the temporary increase in tetracycline-resistant Gram-negative bacteria in the soil after the spread of pig manure slurry was due to resistant intestinal bacteria of manure origin (Sengeløv et al., 2003a).

E. coli isolates were only detectable until 42 days following manure application. This short-term detection suggests that *E. coli* is only suitable as a good indicator of recent or short-term AMR pollution in agricultural soil environments. Although *E. coli* was not found in the soil after 42 days following manure amendments, it is not certain if its resistance genes were still present in the litter-amended soil. Thus, studies to determine if *E. coli*-associated resistance genes would survive long in the environment should be conducted, as these could be transferred horizontally to closely related bacteria (Poole et al., 2017).

Multidrug Resistance and Multiple Antibiotic Resistance (MAR) Index of the *E. coli* Isolates

In this study, 54.2% of the *E. coli* isolates were MDR, with the highest rate (61.5%) in the litter-amended soil. The highest number and most diverse resistance patterns in soil were recorded on the day of

litter application (day 0), suggesting a major influx of MDR *E. coli* from the litter into the soil environment. Furthermore, similar resistance patterns in the litter-amended soil and the chicken litter indicates possible transmission and mobility of ARGs between the litter-borne *E. coli* and the *E. coli* present in soil throughout sample collection. Therefore, the presence of MDR *E. coli* up to 77 days in the stored chicken litter heap on the sugarcane field is of great concern, as it can be a source of continuous MDR *E. coli* contamination to the soil environment, plants, the drainage channel on the field and surrounding water bodies through run-off.

The MAR indexing method is a simple and cost-effective indicator of ABR trends (Osundiya et al., 2013; Sandhu et al., 2016). This study showed that 60%, 22%, and 10% of the isolates from the chicken litter, litter-amended soil, and soil before litter amendment had a MARI >0.2, indicating that they originated from environments of high antibiotic exposure (Krumperman, 1983). The average MAR index of 0.25 observed in the chicken litter isolates in this study further attests to the high usage of antibiotics in the poultry farm where the chicken litter was obtained. Furthermore, the relatively high percentage (51.4%) of isolates with MAR index >0.2 in the litter-amended soil compared to the soil before the litter amendment (2.7%) shows that the application of chicken litter resulted in soil contamination with *E. coli* from sources with high use of antibiotics. The MAR indices intimate that the litter-amended soil and the chicken litter should be considered significant reservoirs of MDR *E. coli* and chicken litter should undergo pre-treatment (e.g., composting) before it is used as fertiliser.

CONCLUSION

The present study show that chicken litter is a major reservoir of antibiotic-resistant *E. coli* that can be transferred to soil. The increase in the number of antibiotic-resistant *E. coli* following litter application suggests a significant influx of resistant *E. coli* from the chicken litter to litter-amended soil. The higher number of isolates with MAR index >0.2 in the litter-amended soil compared to the soil samples collected before the litter amendment indicates soil contamination with *E. coli* from sources with high use of antibiotics such as the chicken litter. Finally, relying on *E. coli* alone to predict the effect of chicken litter application on AMR in the environment would only provide short-term evidence; other persistent organisms like enterococci should be included in monitoring schemes to understand the long-term effects. The presence of MDR *E. coli* with resistance to antibiotics of clinical importance in agricultural environment constitutes a serious danger to public health including the local communities, consumers of farm produces and occupationally exposed individuals. Thus, to reduce the prevalence and dissemination of ARB in agricultural environment, biosecurity measures that will ensure prudent use of antibiotics in food animal production and pre-treatment of animal manure (composting or anaerobic digestion) which reduces AMR in manure should be put in place in South Africa. Further studies on other sources of pollutants contributing to the load of clinically relevant antibiotics and ARB in agricultural environment are highly needed.

ETHICS STATEMENT

Ethical approval was obtained from the Animal Research Ethics Committee (reference AREC 073/016PD) and the Biomedical Research Ethics Committee (reference BCA444/16) of the University of KwaZulu-Natal (UKZN). The study is registered with the South African National Department of Agriculture, Forestry, and Fisheries [reference 12/11/1/5 (879)].

AUTHOR CONTRIBUTIONS

DF, AA, and SE: co-conceptualized the study. DF: undertook sample collection, performed the laboratory experiments and wrote the manuscript. DF, AA, and DA: analysed the data. AA and SE: supervised the project. SE: facilitated ethical approval and vetted the data analysis. All authors undertook a critical revision of the manuscripts.

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