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# Genomic Characterization of *Listeria innocua* Isolates Recovered from Cattle Farms, Beef Abattoirs, and Retail Outlets in Gauteng Province, South Africa

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**Abstract:** Whole-genome sequencing (WGS) was used for genomic characterization of 110 strains of *Listeria innocua* isolated from 23 cattle farms, 8 beef abattoirs, and 48 retail outlets in Gauteng province, South Africa. In silico multilocus sequence typing (MLST) was used to identify the isolates' sequence types (STs). BLAST-based analyses were used to identify antimicrobial and virulence genes. The study also linked the detection of the genes to the origin (sources and types of samples) of the *L. innocua* isolates. The study detected 12 STs, 13 resistance genes, and 23 virulence genes. Of the 12 STs detected, ST637 (26.4%), ST448 (20%), 537 (13.6%), and 1085 (12.7%) were predominant, and the frequency varied significantly ($P<0.05$). All 110 isolates of *L. innocua* were carriers of one or more antimicrobial resistance genes, with resistance genes *lin* (100%), *fosX* (100%), and *tet(M)* (30%) being the most frequently detected ($P<0.05$). Of the 23 virulence genes recognized, 13 (*clpC, clpE, clpP, hbp1.svpA, hbp2, iap/cwhA, lap*, lpeA, *lplA1, lspA, oatA, pdgA*, and *prsA2*) were found in all 110 isolates of *L. innocua*. Overall, diversity and significant differences were detected in the frequencies of STs, resistance, and virulence genes according to the origins (source and sample type) of the *L. innocua* isolates. This, being the first genomic characterization of *L. innocua* recovered from the three levels (farm, abattoir, and retail outlets) of the beef production system in South Africa, provides data on the organism's distribution and potential food safety implications.

**Keywords:** beef production chain; *Listeria innocua*; whole-genome sequencing; sequence type; antimicrobial resistance genes; virulence gene; South Africa

## 1. Introduction

*Listeria* species consist of a group of non-spore-forming Gram-positive facultative anaerobic coccobacilli [1]. There are 21 species of *Listeria* documented since 2020, but few are known to be pathogenic to animals and/or humans [2]. *Listeria monocytogenes* is the only recognized human pathogen and is also pathogenic to animals [3,4]. After con- sumption, several contaminated food types, such as milk and milk products, vegetables, meat, and meat products, have been implicated in listeriosis sporadic cases and/or out- breaks [5–7]. Some clinical manifestations of listeriosis in

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humans include fever, muscle aches, nausea, vomiting, stomach cramps, diarrhoea, abortion, preterm birth, stillbirth in pregnant women, meningitis or encephalitis, and death [8,9].

Currently, *Listeria ivanovii* is known to cause listeriosis in animals [10]. Although rare cases of human and animal infections were reported [11–13], *L. innocua* is not recognized as a human pathogen. *Listeria innocua* co-exists in the same food and environmental niches as *L. monocytogenes*, thus serving as indicator organisms for *L. monocytogenes* [14,15].

*L. innocua* has been documented to share some virulence factors with *L. monocyte- genes* [16], including some *Listeria* pathogenic islands (*Listeria* pathogenic island (LIPI)-1, LIPI-3, and LIPI-4) [17,18]. Virulence genes contribute to the virulence and pathogenicity of *L. monocytogenes.* Several virulence genes have been reported in strains of *L. innocua,* but the prevalence and roles of these virulence genes have not been elucidated [19].

Classical multi-locus sequence typing (MLST) targeting six to eight housekeeping/virulence genes and core genome-based (cg) MLST genotyping have been essential in epidemiological investigation and surveillance of *L. monocytogenes* isolates [20]. Numerous STs have been documented, mainly for *L. monocytogenes*. Since these housekeeping genes are also present in other *Listeria* spp., STs have also been assigned to *L. innocua* and other *Listeria* spp. [21,22]. Furthermore, some STs of *L. monocytogenes* have been associated with human listeriosis, and their occurrence and distribution are affected by geographical locations and food types [23,24].

Strain typing techniques such as traditional serotyping, multi-virulence-locus sequence typing (MVLST), MLST, multilocus variable number tandem repeat analysis (MLVA), and pulse-field gel electrophoresis (PFGE), among others, have been used to detect and characterize *Listeria* spp. [25]. However, whole-genome sequencing (WGS) and various in silico analyses based on WGS are currently the methods of choice for molecular sub-typing for *Listeria* spp. as they provide a higher resolution of strains over the other methods [26–28].

The use and abuse of antimicrobial agents in humans and animals have resulted in the expansion of bacterial antimicrobial resistance, and some bacteria are resistant to multiple antimicrobials [29]. The genes that encode antimicrobial resistance (AMR) in *Listeria* spp. are well documented [22,30]. Still, it is also known that the resistance genes carried by bacteria may not be expressed, limiting their importance in assessing their clinical or therapeutic significance [31]. Variable frequencies of AMR genes have been reported in strains of *L. monocytogenes*, *L. innocua,* and other *Listeria* spp. [32,33]. Hosain *et al.* [34] have highlighted the potential negative impact of AMR on therapy in feed ani- mals and humans. To date, there has not been any information on the genomic carriage of resistance genes by *L. innocua* in South Africa.

Between 2017 and 2018, South Africa experienced the world's largest outbreak of human listeriosis, caused by 'polony,' a ready-to-eat (RTE) beef product [35], and *L. mon- ocytogenes* ST6 was determined to be the aetiological agent [24]. A few studies using poly- merase chain reaction (PCR) has characterized *L. monocytogenes* strains recovered from meat and meat products across the country [36] and MLVA genotypes of *Listeria monoc- ytogenes* and *L. innocua* isolated from farms, abattoirs, and retail in Gauteng province [37], Mpumalanga and North West provinces [38] were reported.

To the best of our knowledge, there is a dearth of information on the genomic char- acteristics of *L. innocua* strains in the country. Mafuna *et al.* [39] used WGS to character- ize 38 isolates of *L. innocua* recovered from the country's meat and food processing facili- ties, while ElZowalaty *et al.* [40] reported the genomic sequence of one strain of *L. innocua* isolated from a healthy goat. The genomic characteristic of *L. innocua* at the levels of the beef production chain is currently unknown.

Therefore, the specific objectives of this study were to use WGS to characterize strains of *L. innocua* isolates from samples collected from cattle farms, beef abattoirs, and retail outlets in Gauteng, South Africa, to elucidate the diversity in the profiles of their sequence types, resistance genes, and virulence genes. The study also investigated the relationships of the profiles with the sources and sample types from which the isolates originated, and the profiles.

## 2. Materials and Methods

### *2.1. Study design and sources of samples*

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Three cross-sectional studies were conducted at three levels of the beef production system in Gauteng province by collecting samples from 23 cattle farms (March-October, 2021), eight beef abattoirs (May-September, 2022), and 48 retail outlets (October 2019 – April 2021). The sample size was determined as recommended by Thrusfield [41]. De- tails on the types and number of samples collected from the sources mentioned earlier and types of samples in the current study have been documented [42].

*2.2. Isolation, identification of L. innocua, and determination of AMR*

All 110 isolates were previously identified (bacteriological and multiplex PCR) as *L. innocua* as described [43–45]. The confirmed isolates of *L. innocua* were inoculated in 50% brain heart infusion (BHI)/50% glycerol and stored at -20°C until subjected to whole genome sequencing (WGS) analyses. The number of isolates of *L. innocua* used in this study was 110 (11.1%) from 990 samples. The prevalence of *L. innocua* at the three levels of beef production was 10.4% (34/328), 5.7% (15/262), and 15.3% (61/400) for cattle farms, beef abattoirs, and retail outlets, respectively. The current study assessed all the isolates recovered from these sources and the types of samples.

In an earlier study [42] on the 110 isolates of *L. innocua* used in the current study, their phenotypic AMR to 16 antimicrobial agents was determined using the disc diffusion method. The antimicrobial agents used comprised penicillin (10 units), amoxicillin clavu- lanic acid (30 µg), ampicillin (10 µg), cephalothin (30 µl), cefotaxime (30 µg), streptomycin (25 µg), gentamicin (10 µg), kanamycin (30 µg), tetracycline (30 µg), doxycycline (30 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), enrofloxacin (5 µg), azithromycin (15 µg), clindamycin (10 µg), and sulfamethoxazole-trimethoprim (23.75/1.25 µg).

*2.3. Whole-genome sequencing, genomic analysis, assembly, and annotation*

DNA extraction was done using the Qiagen DNAEasy Blood & Tissue kit, manual, Gram-positive protocol as per the manufacturer's instructions. All isolates were sequenced either on an Illumina NextSeq platform (150-bp paired-end reads; Illumina or on an Illumina MiSeq platform (250-bp paired-end reads; Illumina, Inc., San Diego, CA) using the Nextera XT library preparation kit per the manufacturer's instructions.

Quality control, including adapter removal of the raw data, was done using BBDuk (v.38.91; https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbduk- uide/; sourceforge.net/projects/bbmap/). SPAdes v.3.15.3 [46] created a *de novo* assembly of each isolate. Only contigs longer than 500 bp were retained for further analysis. Completeness and contamination of the *de novo* assemblies were assessed with CheckM v.1.1.3 [47], and taxonomic classification was done using GTDB-Tk v.1.7.0 [48].

*2.4. In silico MLST*

MLST STs were determined using the MLST tool (http://github.com/tseemann/mlst) [49] which makes use of the PubMLST website (https://pubmlst.org/) developed by Keith Jolley (Jolley & Maiden 2010, BMC Bioinformatics, 11:595) and sited at the University of Oxford.

*2.5. Resistance, and virulence profiles*

ABRicate (https://github.com/tseemann/abricate) [50] was used to detect antimicrobial resistance genes, and virulence factors in species of interest. Abricate was run with default parameters, and the NCBI database was selected for AMR detection. This database was locally updated on 2 November 2022 and, at the time of usage, including 6,334 sequences (doi: 10.1128/AAC.00483-19). For virulence factors, the "vfdb" database was used, updated on 2 November 2022, and containing 4,332 sequences (doi:10.1093/nar/gkv1239).

*2.6. Construction of the phylogenetic tree for L. innocua isolates and correlation with source and type of samples*

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A multiple protein sequence alignment was constructed using GTDB-Tk v.1.7.0 [50] and is based on 120 GTDB core bacterial marker genes. FastTree v.2.1.11 was used to infer a phylogenetic tree and visualized in R with ggtree [51].

*2.7. Data analysis*

All data analyses were done using R v.4.1.2 [52] implemented In RStudio v.2022.2.3.492 [53]. Distance matrices were calculated using the "daisy" function with the "gower" parameter specified to determine Gower distances with the R package "cluster" [54]. Minimum spanning trees were calculated using the "ape" package [55], with the "mst" function, and visualized using "igraph" [56] and "ggnetwork" [57]. R packages ggstatsplot [58], ggsci [59], and ggpubr [60] were further used for data analyses and visualization. Balloon plots were constructed using the *ggballoonplot* function available from ggpubr. The ggstatsplot function, *ggscatterstats,* was implemented to perform correlation analyses based on Pearson's correlation coefficient. Pearson's Chi-squared Test for Count Data, implemented by the *chisq.test*, was used to test for associations. Pearson's correlation coefficients were calculated using the *cor* function. Bar charts were produced using the ggstatplot function *ggbarstats* and Pearson's chi-squared test used to test for significant differences.

## 3. Results

*3.1. Frequency of STs of L. innocua*

For samples collected from the three levels of beef production, 12 STs were detected in the 110 isolates of *L. innocua,* and no ST could be assigned in two isolates (1.8%) (1 each from the farm and retail outlet sources). The frequency of STs found was as follows: ST637 (29 isolates, 26.4%), ST448 (22, 20%), ST537 (15, 13.6%), ST1085 (14, 12.7%), ST1489 (8, 7.3%), ST1482 (7, 6.4%), ST1008 (5, 4.5%), ST1610 (4, 3.6%), and ST1619, ST602, ST1087 and ST43 (1 isolate each, 0.9%). The differences in the frequencies of STs observed across all the samples were statistically significant (P<2.2e-16).

3.1.1. Distribution of STs by the source of samples

The proportion of STs in *L. innocua* isolates within each of the nine sources varied significantly (P<0.05), except for those recovered from the small retail outlets (P=0.77), as shown in Figure 1a.

The associations of STs with the nine sources were statistically significantly higher for ST1087 (communal farm), ST1482 (feedlot), ST1610 (feedlot), ST43 (feedlot), ST537 (low throughput abattoir), ST537 (high throughput abattoir), ST1619 (small retail outlet), ST602 (small retail outlet), ST448 (medium retail outlet), ST537 and ST637 (cow-calf farm).

3.1.2. Distribution of STs by the type of sample

The distribution of STs among *L. innocua* isolates according to the types of samples from which they originated is shown in Figure 1b. Within each of the 8 sample types, the proportion of STs of *L. innocua* was highly statistically significantly different (P<0.05) except for Offal and Organs.

Across the eight food types, the association of STs with certain sample types was statistically significant (P<0.05) for ST637 (feed), ST537 (carcass), ST448 (milled beef), ST1619 (raw beef), and ST1008 (offal and organs) (Supplementary data: Table S1).

3.1.3. Minimum spanning tree (MST) based on ST profiles

The MST of the STs of *L. innocua* isolates is displayed in Figure 2. Clear clustering of the samples based on the ST profiles was evident. The origin of the samples based on "Sample Type" and "Source" further highlighted the composition of the clusters. Overall, seven distinct clusters with two larger clusters appear to contain isolates from different sample types and sources, while the smaller clusters were homogenous. Three of the clusters were predominantly filled with samples from retail outlets. This indicates a propensity of certain ST profiles in this environment. This is the case with clusters biased toward farming or production environments.

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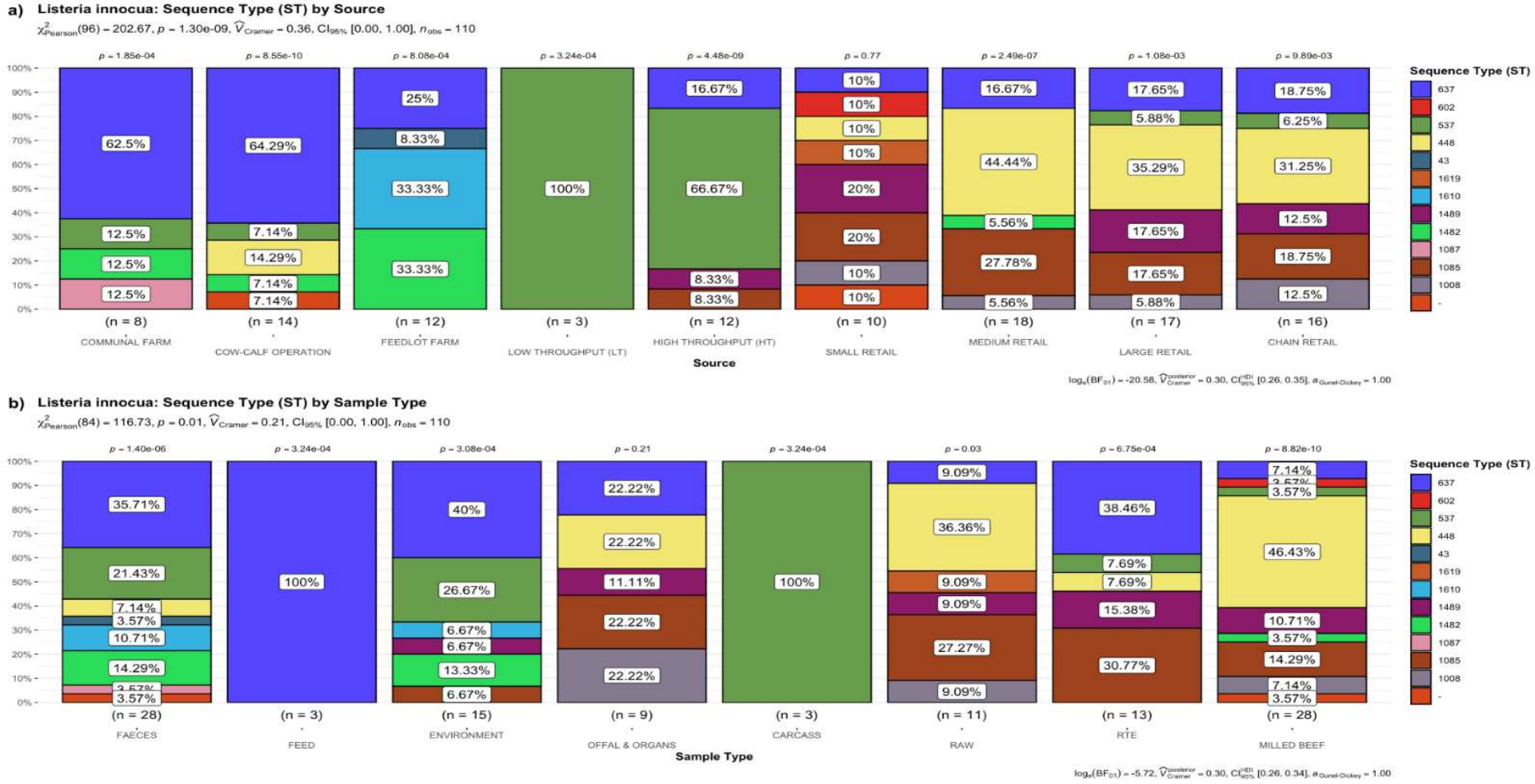


**Figure 1.** Frequency of *L. innocua* sequence types by source and sample type. *L. innocua STs grouped by source*: The STs displayed a significant deviation from the ex- pected distribution across Sources. All Sources, except for Small Retail, were found to be significantly associated with specific STs (Figure 1a). *L. innocua STs grouped by sample type:* A significant deviation from the expected ST distribution was also found for the ST by Sample type distribution. All sample types, except Offal & Organs, were found to be significantly associated with certain STs (Figure 1b).

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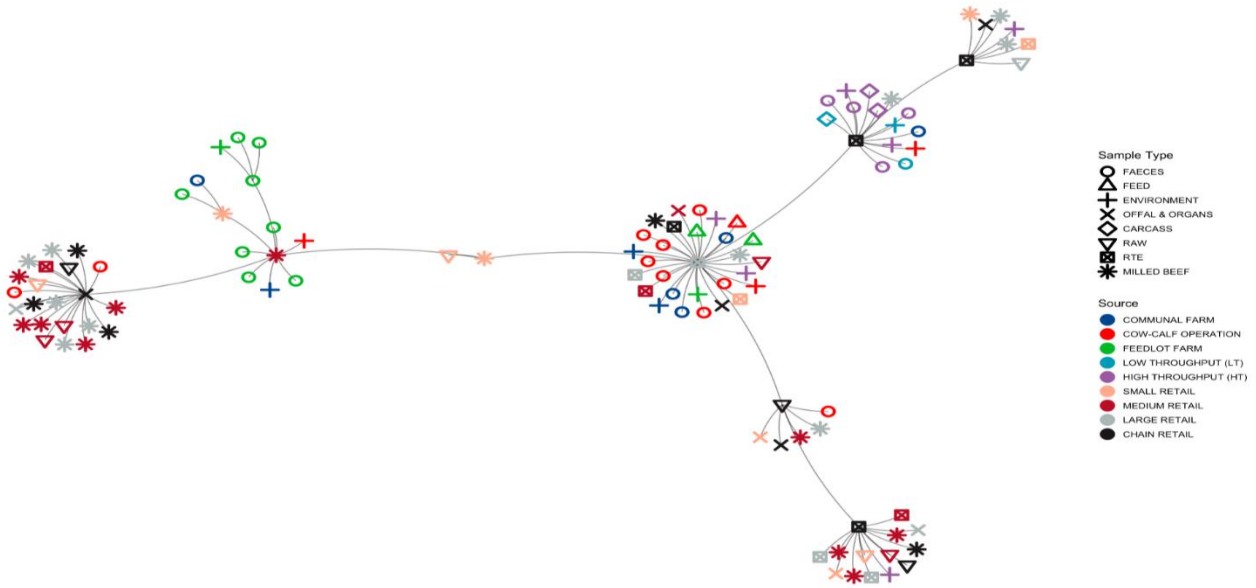


**Figure 2.** Minimum spanning tree for the sequence types of *L. innocua* isolates according to the source and type of sample.

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The clustering of samples based on ST is evident. The shape and colour of each sample indicate the Sample Type and Source, respectively. The smaller clusters are relat- ively homogenous regarding Sample Type and Source. In particular, three smaller clusters are associated with the sample from Retail environments.

*3.2. Detection of antimicrobial resistance genes in L. innocua*

Thirteen resistance genes were detected in the 110 *L. innocua* isolates from the sources tested. They were as follows: *fosX*, 110 (100%), *lin*, 110 (100%), *tet(M)*, 33 (30%), d*frG*, 9 (8.2%), *ImuD*, 6 (5.5%), *mphB*, 5 (4.5%), *mefA*, 4 (3.6%), *msrD*, 4 (3.6%), *tet(S)*, 4 (3.6%), *ant.6.1a*, 1 (0.9%), *InuG*, 1 (0.9%), *vatB*, 1 (0.9%), and *vga*, 1 (0.9%). The differences were statistically significant ($p<0.05$). The details of the sources, sample types, sequence types, and resistance genes identified in the 110 *L. innocua* isolates are shown in Supple- mentary data: Table S1.

3.2.1. Distribution frequency of antimicrobial resistance genes by source

Balloon plots of the 13 resistance genes distribution by source are in Figure 3. Within each of the nine sources investigated, high diversity of the resistance genes was detected in feedlots, 61.5% (8/13), and small retail outlets, 53.8% (7/13), compared with the low diversity of 33.3% (3/9) found in communal farms and LT abattoirs. The differences were statistically significant (P=0.0302). Across the nine sources, the frequency of resistance genes (*fosX* and *lin*) was high, 100% (9/9) but low, 11.1% (1/9) for six genes (*vatB*, *vgaB*, *msrD*, *InuD*, *mefA*, and *ant6.1a*). The difference was statistically significant (P=0.0004).

3.2.2. Distribution frequency of antimicrobial resistance genes by type of sample

Figure 4 shows the frequency distribution of resistance genes by the type of samples. Faeces, environmental samples, and offal and organ samples yielded *L. innocua* with high carriage of resistance genes at 69.2% (9/13), 69.2% (9/13), and 61.5% (8/13), respectively, compared with that for feeds and carcasses, 23.1% (3/13). The difference was statistically significant (P=0.018). Across the 8 sample types, resistance genes *fosX*, *lin*, and *tet(M)* were each detected in 8 (100%), indicating co-occurrence, while only 1 (12.5%) sample type was positive for genes *vatB*, *vgaB*, *InuG*, and *ant.6.1a*. The difference was statistically significant (P=0.0014).

3.2.3. Patterns of multiple antimicrobial resistance genes

For the 110 isolates of *L. innocua* recovered from the farms, abattoirs, and retail outlets, nine antimicrobial resistance gene patterns with a range from 2-7 resistance genes per pattern were as follows, *foxX-lin*, 65 (59.1%), *fosX-lin-tetM*, 27 (24.5%), dfrG- fosX-lin, 8 (7.3%), *fosX-lin-Inu.G-tet.M*, 2 (1.8%), *dfrG-fosX-lin-tetM*, 1 (0.9%), *fosX-lin-InuD-tet.M*, 1 (0.9%), *fosX-lin-tetM-vat.B-vga.B*, 1 (0.9%), *ant.6.Ia-fosX-lin-tet.M-mph.B*, 1 (0.9%), and *fosX-lin-InuD-mefA-mph.B-msrD-tet.S*, 4 (3.6%) ($p<0.05$).

3.2.4. MST for AMR

The MST for the AMR profiles of *L. innocua* isolates according to the sources and sample types is shown in Figure 5. This analysis grouped the isolates into two major clusters with more than 25 isolates each and four minor clusters. The largest cluster contained isolates from all sample types except carcass and all sample sources, while the second largest cluster contained isolates from all sample types and sources. The two smaller clusters are predominantly associated with farming and retail environments. The clustering observed indicates the sharing of AMR profiles across all sectors. Each minor cluster comprised 8, 4, 1, and 1 isolates from limited sample types and sources. The smaller clusters contained samples from similar environments, highlighting some unique AMR profiles in isolates associated with these environments.

3.2.5. Putative resistance phenotypes

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The frequency of putative resistance phenotypes detected in the 110 isolates of *L. innocua* consisted of trimethoprim, 8.2% (9/110), tetracycline, 33.6% (37/110), streptomycin, 0.9% (1/110), streptogramin, 0.9% (1/110), macrolide, 4.5% (5/110), lincosamide, 100% (110/110), fosfomycin, 100% (110/110), and erythromycin, 3.6% (4/110). The differences in the frequencies of putative resistance phenotypes were statistically significant ($p<0.05$).

Putativce phenotypes by the source of isolates

The frequency of putative resistance phenotypes detected by the source of the isolates is shown in Figure 6a. For each of the nine sources studied, the frequencies of the putative resistance phenotypes were significantly associated with each particular source ($p<0.05$).

Putative resistance phenotypes by the sample type

The frequency of putative resistance phenotypes detected is shown in Figure 6b. All eight sample types showed significant under- or over-representation of resistance pheno- type ($p<0.05$).

*3.3. Detection of putative virulence genes*

Twenty-three putative virulence factors (clpC, clpE, clpP, fbpA, gtcA, hbp1.svpA, hbp2, iap. cwhA, lap, llsA, llsB, llsD, llsG llsH, llsP, llsX, llsY, lpeA, lplA1, lspA, oatA, pdgA, and prsA2) previously identified in L. monocytogenes were detected across the 110 isolates of L. innocua from farms, abattoirs, and retail outlets.

Among the 34 isolates from cattle farms, 19 (82.6%) of 23 genes were detected, except for *llsB, llsD, llsP, and llsY*. The frequency of detection of virulence factors was from 0% (0/34) to 100% (34/34) in 14 genes.

For the 15 abattoir isolates, all 23 (100%) virulence genes were detected, with a range from 6.7% (1/15) for *llsB, llsD, llsP, and llsY* to 100% (15/15) for 15 genes.

For the 61 isolates of *L. innocua* from retail outlets assessed for virulence genes, all 23 (100%) genes were detected, and the frequency range was from 8.2% (1/61) for *llsP* to 100% (61/61) in 14 genes ($p<0.05$).

Overall, the frequency of virulence genes detected across the three levels of beef production ranged from 82.6% - 100%, but the differences were not statistically signifi- cant (P=0.109); however, the frequency of the types of virulence genes varied significantly- in 14 genes (P<0.001).

Supplementary data: Table S2 shows the details of the sources, sample types, sequence types, and virulence factor genes identified in the 110 *L. innocua* isolates.

3.3.1 Occurrence of virulence genes by source

Within each of the nine sources, all (100%) the isolates of *L. innocua* that originated from HT abattoirs, small retail outlets, and large supermarkets were positive for the 23 virulence genes detected, while 19 (82.6%) of the 23 virulence genes were detected in five sources (communal farms, cow-calf farms, feedlots, LT abattoirs, and medium retail outlets) (P=0.1085) (Figure 7).

3.3.2. Virulence genes by sample type

Within the food types, all the samples that originated from sample types, 5 (55.6%) samples (environment, offal and organs, raw beef, RTE, and milled beef) were each positive for all (100%) 23 virulence genes. (Figure 8). However, only 15 (65.2%) of the 23 virulence genes were found in feed samples (P=0.038).

3.3.3. MST based on virulence gene profiles

The MST based on the virulence gene profile of each isolate is shown in Figure 9. As with the previous trees, certain profiles are unique to similar environments. A similar clustering pattern was observed for the virulence factor profile here; two large clusters are evident. Four distinct clusters,

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with two larger clusters each, made up of isolates from all the different sample sources and types, while the two smaller clusters, each con- tained five (from 4 different sample types and three sources) and seven (from 3 sample types and 3 sources) isolates, respectively. The large clusters indicate the sharing of viru- lence gene profiles across various environments, with smaller clusters alluding to unique profiles for certain environments.

*3.4. Distribution of virulence genes by sequence type*

For the 108 isolates for which the STs were determined, five patterns of carriage of virulence genes by ST were detected (Figure 10).

***Pattern 1*** comprised 44 (40.7%) isolates recovered from the farms, abattoirs, and outlets distributed across three STs. The four STs were 637 (n=29, 26.9%), 1085 (n=14, 13%) and 602 (n=1, 0.9%). Fifteen (65.2%) of the 23 virulence genes were detected: *clpC, clpE, clpP, fbpA, gtcA, hbp1.svp, hbp2, iap.cwhA, lap, IpeA, IpIA1, IspA, oatA, pdgA,* and *prsA2.*

***Pattern 2*:** consists of 51 (47.2%) isolates recovered from farms, abattoirs, and retail outl- ets, spread across six STs. These are STs 448 (n=22, 20%), 537 (n=15, 13.6%), 1482 (n=7, 6.4%), 1008 (n=5, 4.5%), 1619 (n=1, 0.9%), and 43 (n=1, 0.9%). A total of 19 (82.6%) of the 23 virulence genes were detected in this pattern: *clpC, clpE, clpP, fbpA, gtcA, hbp1.svp, hbp2, iap.cwhA, lap, llsA, llsG, llsH, llsX, IpeA, IpIA1, IspA, oatA, pdgA,* and *prsA2*

***Pattern 3:*** has only 8 (7.4%) isolates recovered from abattoirs and retail outlets. All (100%) of the 23 STs were detected.

***Pattern 4****:* consists of 4 (3.7%) isolates from farms and detected ST 1610 only. Eighteen (78.3%) virulence genes were found: *clpC,* clpE, *clpP, fbpA, hbp1.svpA, hbp2, iap.cwhA, lap, llsA, llsG, llsH, llsX, IpeA, IpIA1, IspA, oatA, pdgA,* and *prsA2.*

**Pattern 5:** composed of 1 (0.9%) isolate from 18 (78.3%) virulence genes detected were clpC, clpE, clpP, fbpA, gtcA, hbp1.svp, hbp2, iap.cwhA, lap, llsA, llsG, llsH, IpeA, IpIA1, IspA, oatA, pdgA, and prsA2.

*3.5. Phylogenies of L. innocua isolates according to the STs*

The genetic relationships of the *L. innocua* isolates recovered from three beef production levels and several types of samples are shown in Figure 11. The tree indicated grouping based on phylogenetic relatedness with distributions across various environments for the groups. The isolates formed five distinct clusters, which appear to be a mix of the different sample types and sources. Some smaller clusters indicated a propensity for specific environments, with the larger clusters containing a diverse spread of sample origins. This could indicate an inclination toward certain *L. innocua* iso- lates to environments with others distributed across disjoint origins. Overall, the phylo- genetic tree suggests that the positioning of the isolates of *L. innocua* isolates in the phylo- genetic tree did not depend on the sample type or source.

Similar to the MST trees, there seems to be a subset of isolates unique to particular origins, with the larger isolate groupings, found distributed across many sample origins.

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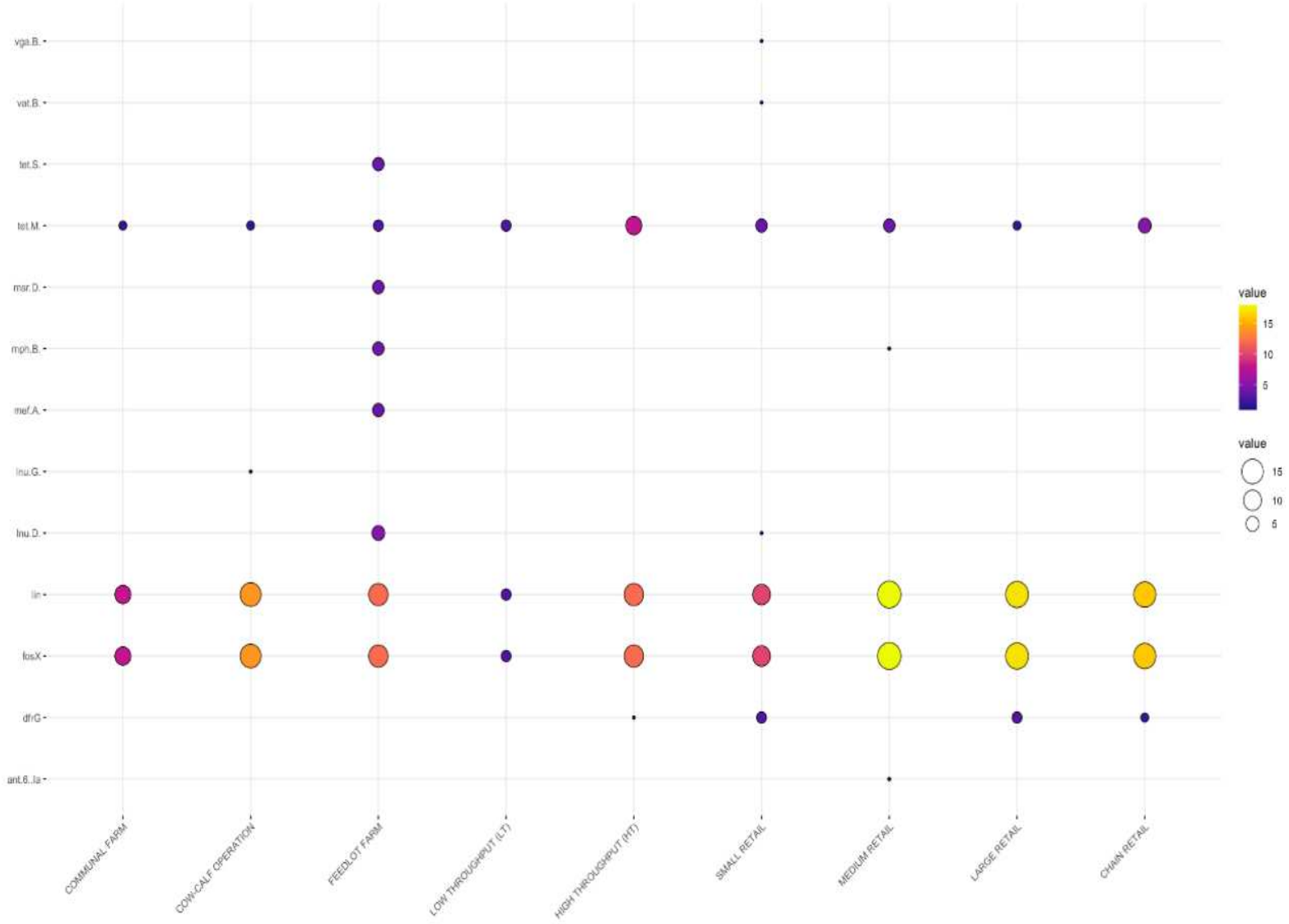


**Figure 3.** Balloon plots showing the distribution of resistance genes by source.

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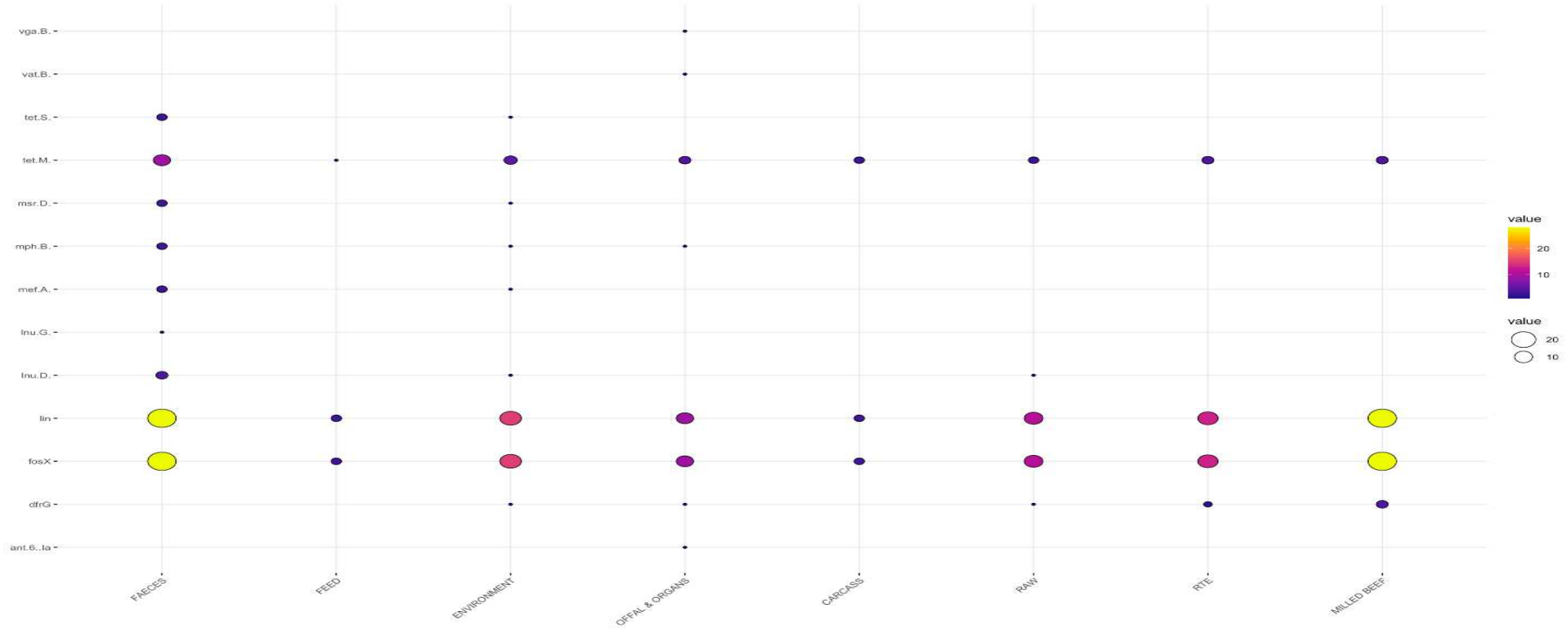


**Figure 4.** Balloon plots showing the distribution of resistance genes by sample type.

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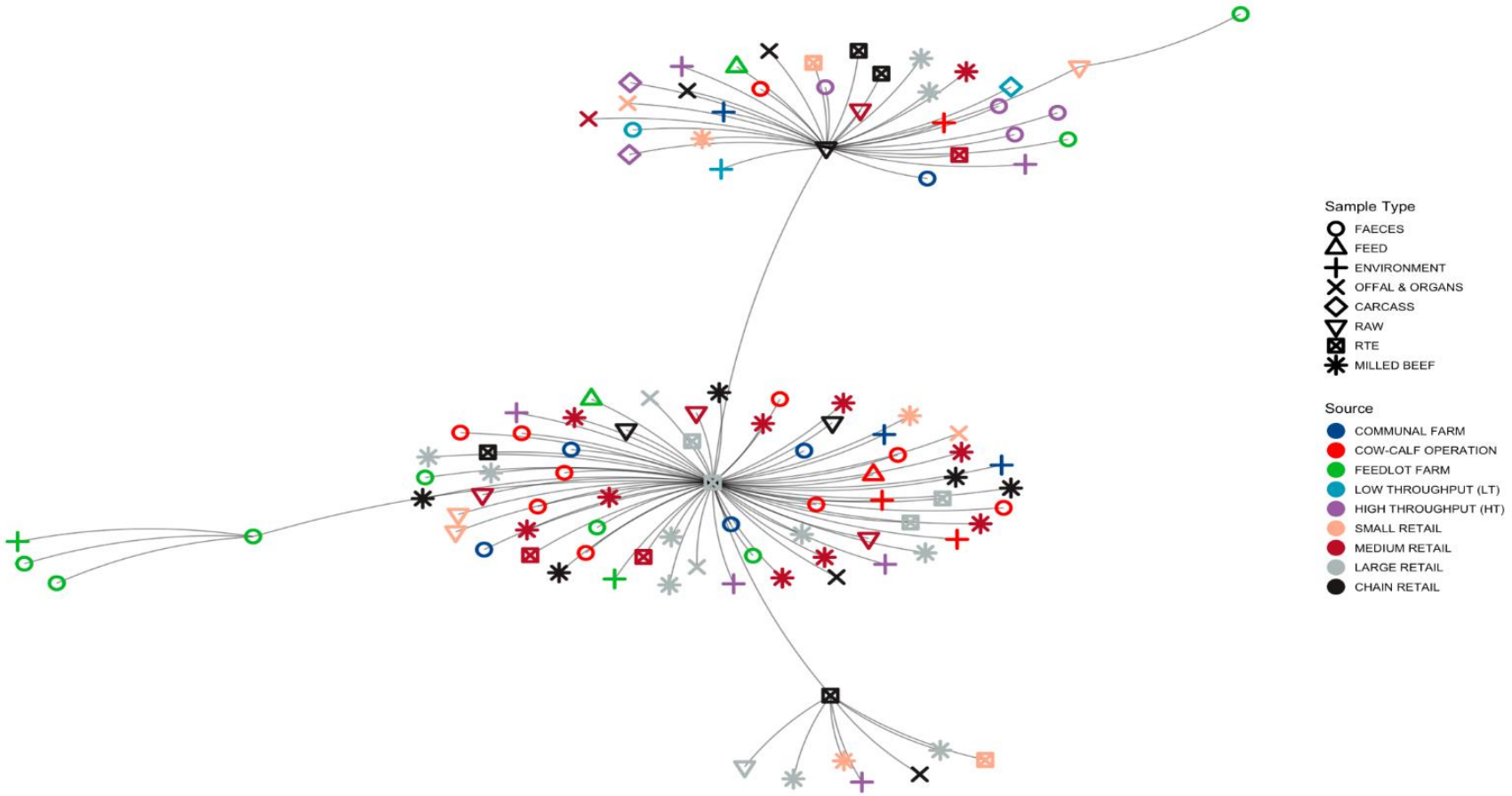


**Figure 5.** Minimum spanning tree for AMR of *L. innocua* isolates.

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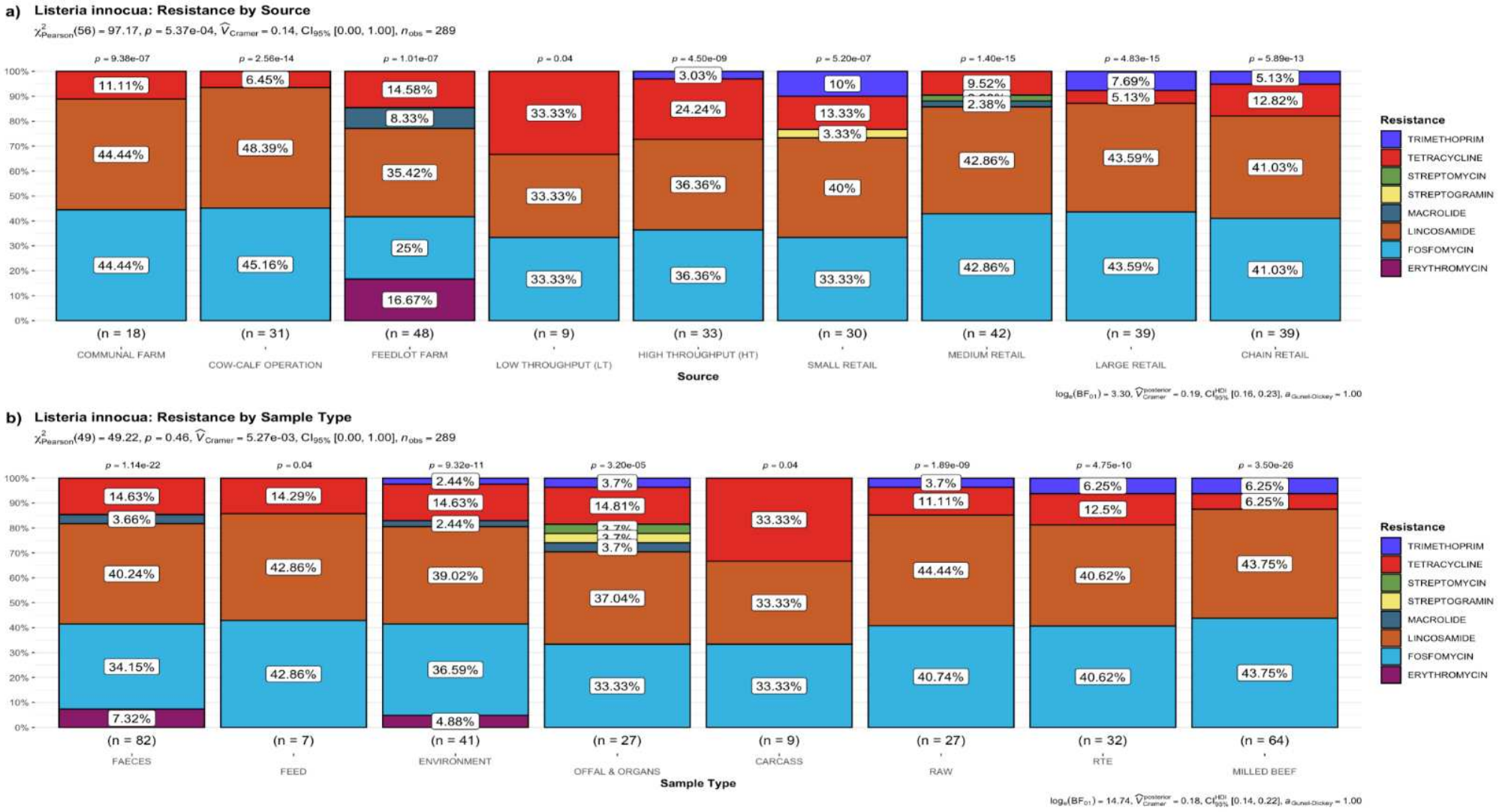


**Figure 6.** Putative resistance of *L. innocua* by source and type of samples.

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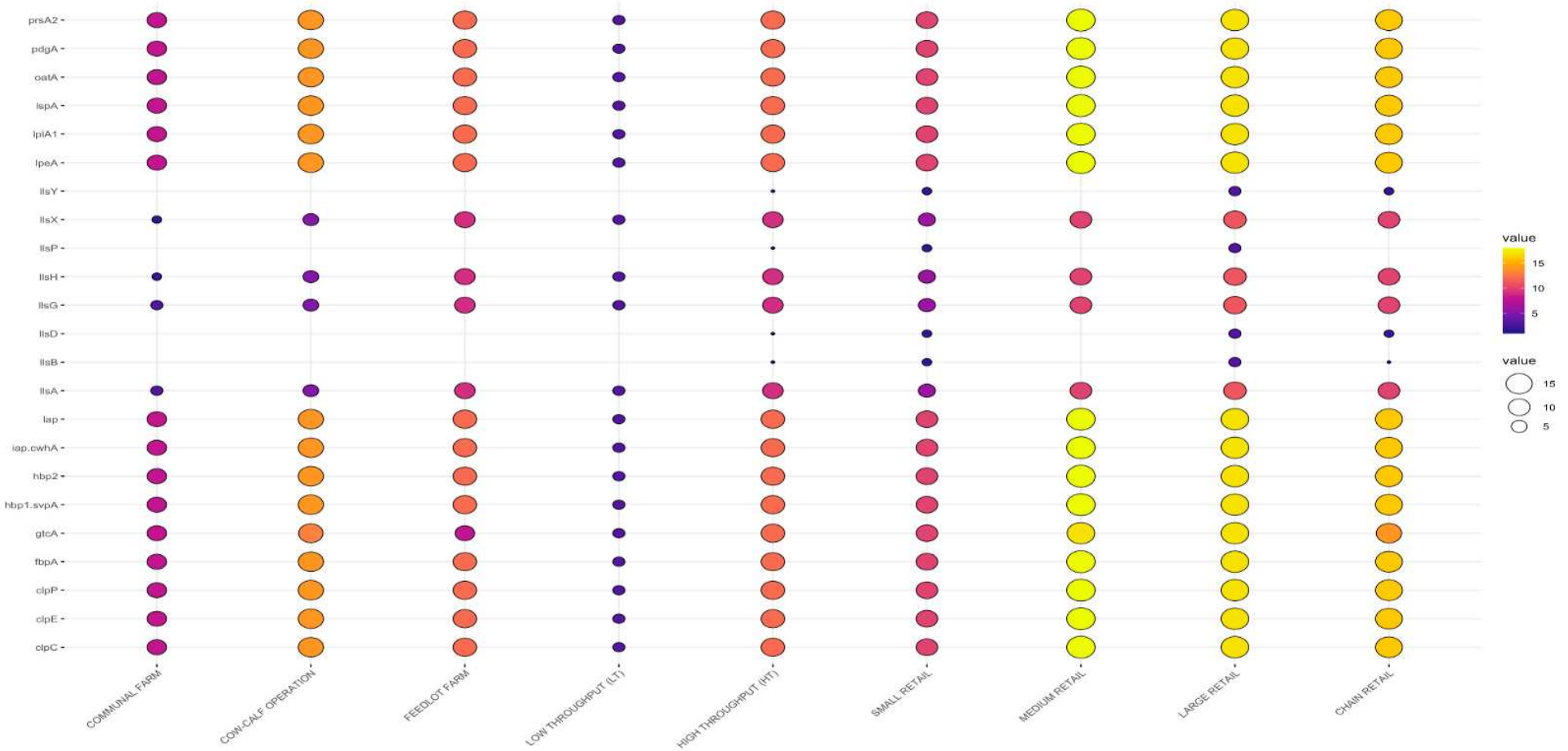


**Figure 7.** Balloon plot of virulence factors by source.

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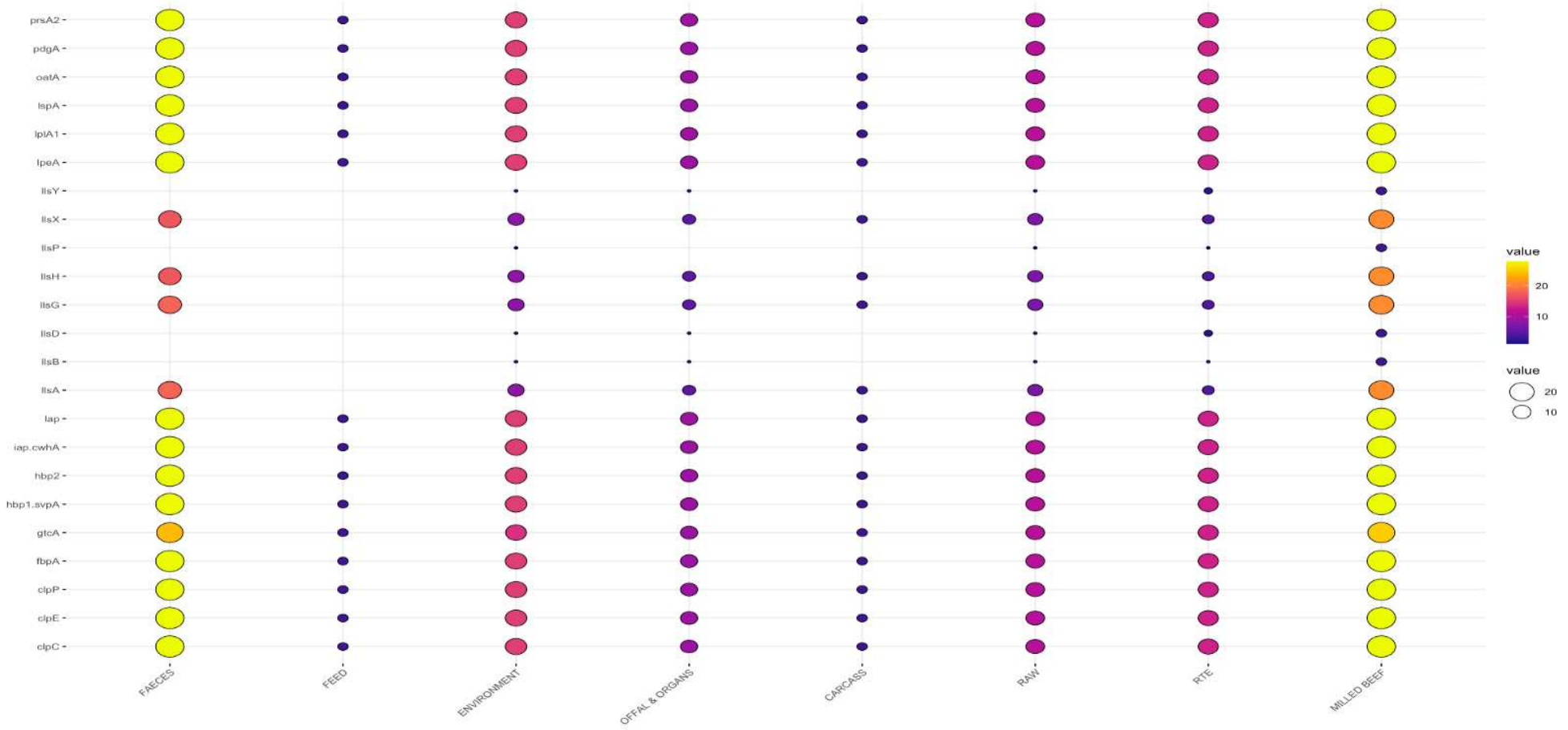


**Figure 8.** Balloon plot of virulence factors by sample type.

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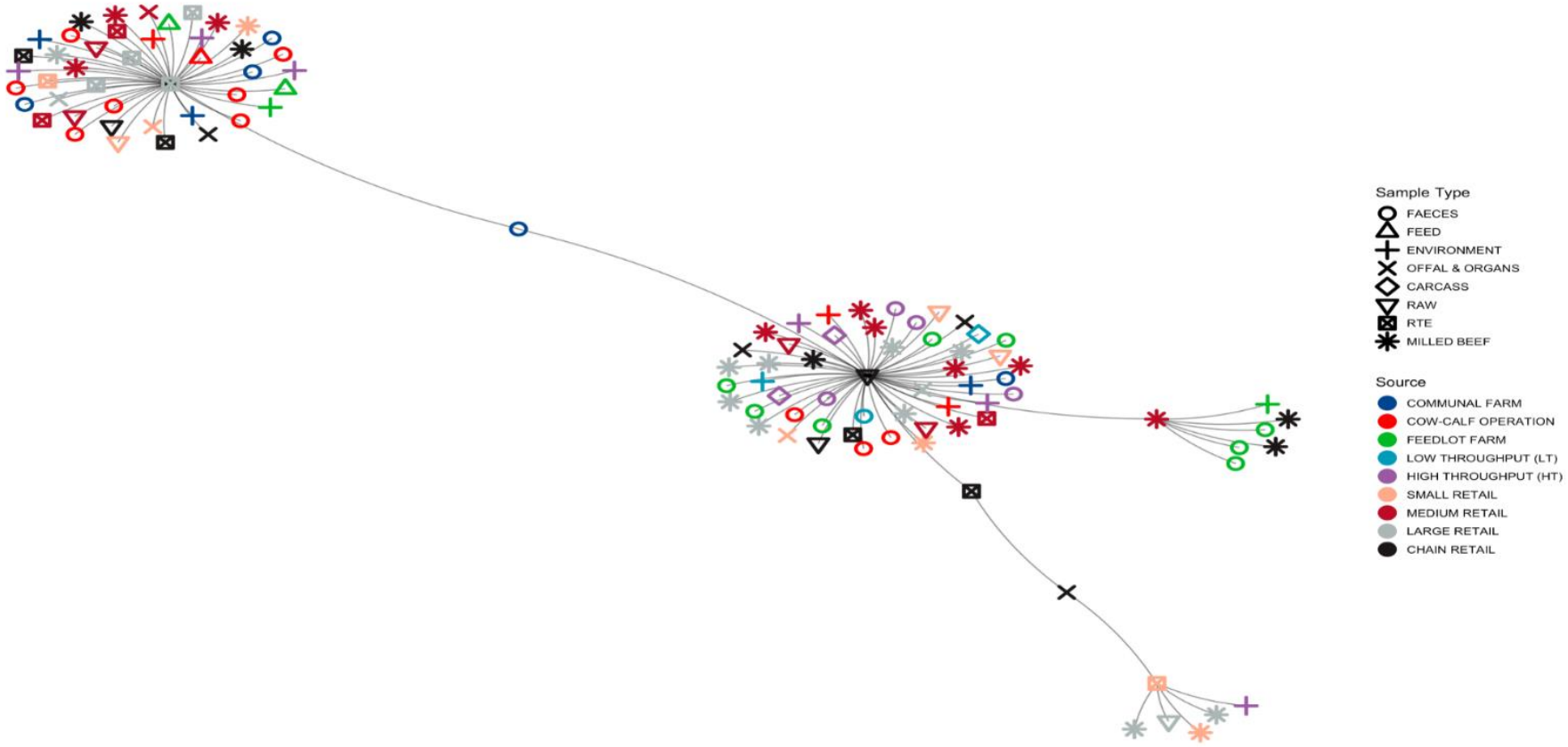


**Figure 9.** Minimum spanning tree of the virulence gene profiles of *L. innocua* isolates.

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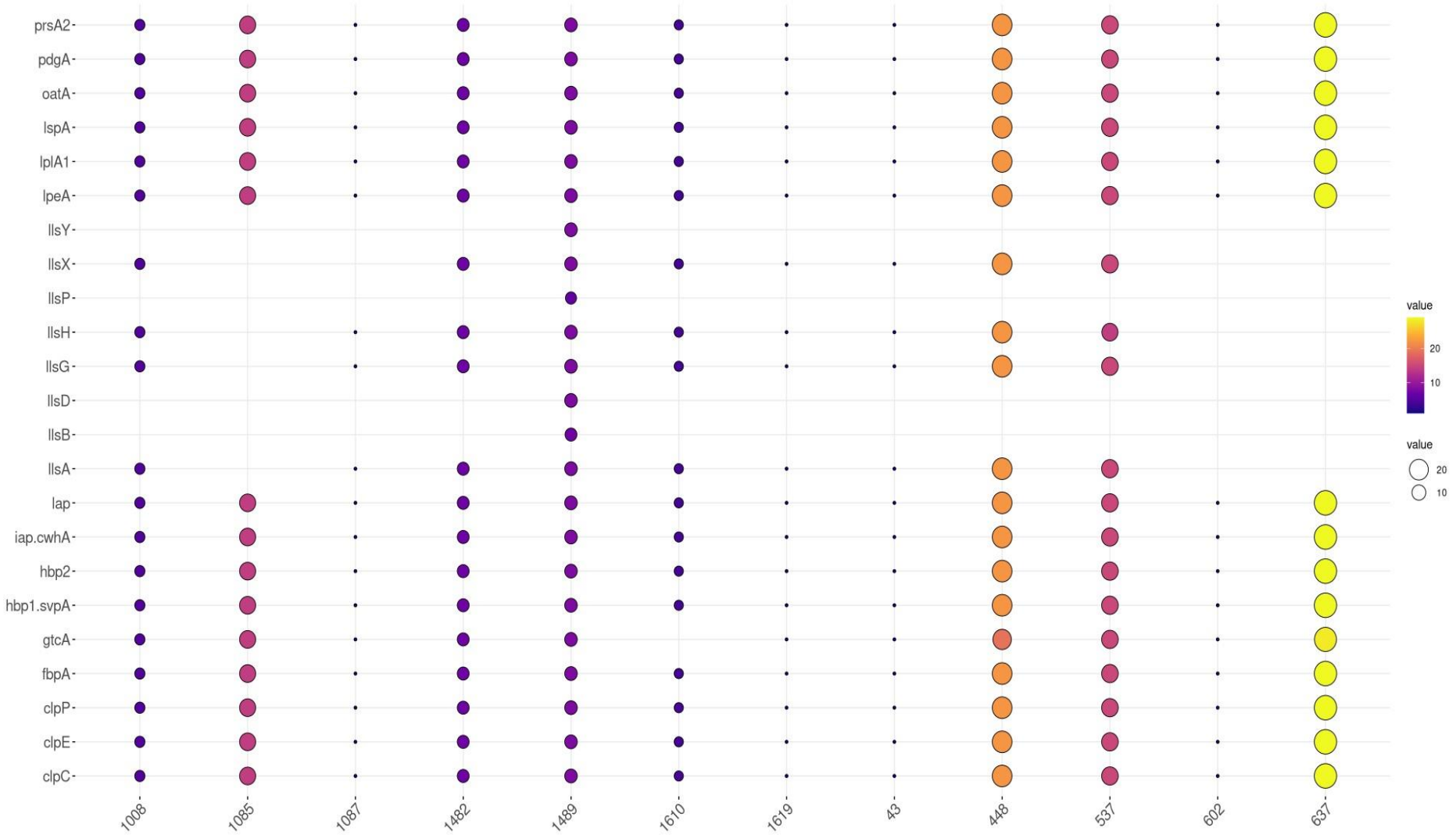


**Figure 10.** Balloon plots of virulence genes and ST of *L. innocua* isolates.

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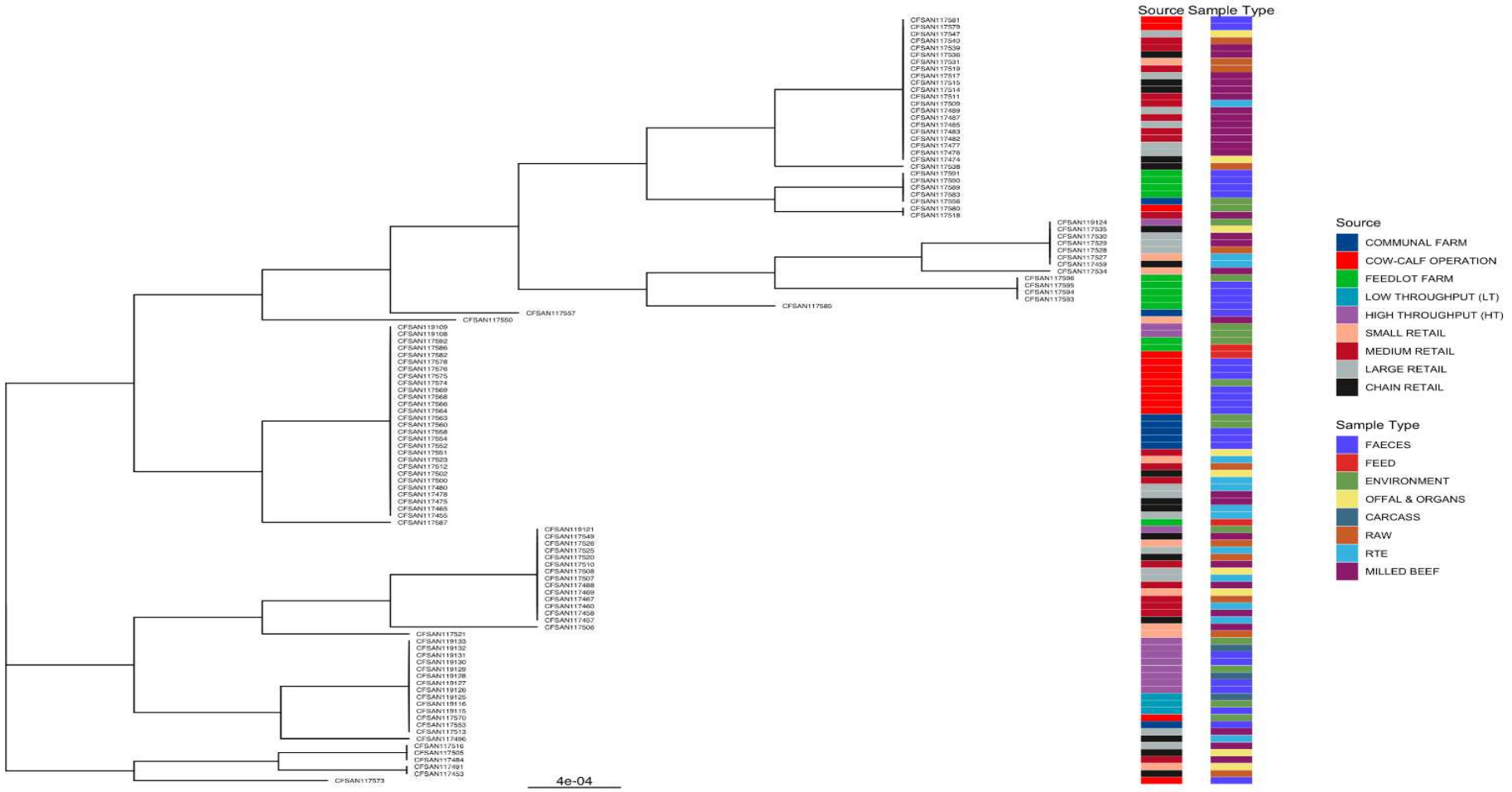


**Figure 11.** Phylogenic tree of 110 isolates of *L. innocua* by source and type of samples.

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## 4. Discussion

The current study is the first comprehensive study undertaken in South Africa on *L. innocua* recovered from three levels of cattle production (cattle farms), beef abattoirs (cattle slaughter), and beef/beef products retailing (retail outlets), concerning the genomic characterization of sequence types, resistance genes, and virulence genes. Both *L. monocytogenes* and *L. innocua* occupy the same niche in foods [14,15]; the detection of *L. innocua*, indicates the possible presence of *L. monocytogenes* in foods. Unlike the present study, the few published genomic characterization of *Listeria* species were studies cond- ucted on *L. monocytogenes* strains recovered from the 2017-2018 large outbreak of human listeriosis [35,61], the report by Mafuna *et al.* [39] on the strains of *L. innocua* and *L. welsh- imeri* isolated from meat and food processing facilities in the country and the sequencing of one isolate of *L. innocua* from a healthy goat [40]. The current study provides important data on *L. innocua* in the country's farm-abattoir-retail association. In other countries, *L. innocua* isolates recovered from meat are characterized using molecular methods [22,62,63]. It is interesting that in our study, the predominant STs of *L. innocua* detected differed significantly as to the source of isolates, ST637 (cattle farms), ST537 (abattoirs), and

ST448 (retail outlets). This is in comparison to the *L. innocua* isolates obtained from retail outlets in Gauteng province, where nine STs were identified, of which ST448 (33.3%), ST1085 (23.3%), and ST637 (15%) were prevalent. Mafuna *et al.* [39] also identified nine STs, of which the most common were ST537 (56%) and ST1085. Also, only four STs (ST537, ST637, 448, and 1085) were common in both studies. The differences in the STs detected between both studies may be explained partly by the types of samples collected (beef versus meats), the source (retail outlets versus food processing facilities), and the number of locations (one province versus nine provinces). Reports by others have documented diversity in the STs, and their frequencies are affected by the geographical location, source, and types of samples from where the isolates originate, among other factors [23,36]. In our study, resistance genes *fosX* (100%), *lin* (100%), and *tet(M)* (30%) were predominantly detected. Similarly, Hanes and Huang [64] reported that in the USA, from 2010 through 2021, data analysis identified *fosX, lin, abc-f,* and *tet(M)* as the four most AMR genes found in *L. monocytogenes*. Compared with published reports on resistance genes in *L. innocua*, the distribution of the resistance genes varied considerably [32,33].

In our study, it is important that there was a high diversity of resistance genes detected in the isolates of *L. innocua* obtained from feedlots (61.5%) compared to the low diversity of resistance genes found in the isolates from communal farms. This is no surprise because animals at intensively managed feedlots receive cattle from diverse sources (farms and auctions) mostly experience antibiotic pressure to control infections and disease. On the other hand, the communal farms in South Africa rear fewer cattle (<10 per herd) in extensive or semi-intensive management systems with minimal antimicrobial agents use, often dictated by financial limitations posed to farmers by the cost of treatment.

The significantly higher diversity of AMR genes detected in *L. innocua* recovered from faecal and environmental samples may be explained partly by the fact that some of the faecal samples were pooled from around the feeding areas and environmental water and effluent samples; thus, a sample may have originated from several animals. Reports by others support our findings, where the frequency and distribution of resistance genes of *L. innocua* varied considerably by the types of samples from where the isolates origin- ated [34,65].

Our study also revealed that the frequency of resistance genes was significantly affected by the STs of the *L. innocua* isolates in five STs 637, 1482, 537, 1008, and 1489. It is also interesting to have detected ST-specific AMR genes as demonstrated by the presence of gene *dfrG* only in *L. innocua* ST1489 and the four isolates that belonged to ST1610 were each carrier of multi-drug resistance (MDR) genes (*fosX, lin, inuD, metA, mph, msrD*, and *tetS)* in all four ST 1610 isolates. The association of resistance genes with STs has been documented by others [66]. Regardless of the STs, it is of potential therapeutic significance that nine MDR genes were detected in our study ranging from two to seven genes per isolate. Palaiodimou *et al.* [66] have also reported the occurrence of MDR genes

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*bcrABC*, *emrC,* and *qacH* and emphasized the risk of the AMR and MDR transfer to other bacteria, including *L. monocytogenes* [62,67].

Of the three predominant resistance genes (*fosX*, *lin*, and *tet(M)*), putative resistance to fosfomycin and tetracycline appears to be pertinent to South Africa because these antimicrobial agents are inexpensive, readily available, and used by farmers on livestock in the country [68,69]. However, tetracycline is the country's most frequently used antimicrobial gene on livestock. Therefore, the detection of 30% of the *L. innocua* isolates recovered from the three levels of sampling (farm, abattoirs, and retail outlets), and the putative resistance encoded by *tet(M)* and *tet(S)* genes based on WGS was 33.6%. Therefore, there is a potential for tetracycline-resistant *L. innocua* strains to enter the human food chain. It is relevant to mention that the prevalence of phenotypic tetracycline resistance exhibited by the same isolates of *L. innocua* using the disc diffusion method was 36.8% [42]. Interestingly, this phenotypic resistance correlates well with the putative resistance to tetracycline due to both *tet(M)* and *tet(S*) genes, suggesting that the genes may have been responsible partly for the resistance detected. These findings suggest that tetracycline resistance may have been acquired with the potential for these antimicrobial genes to be transferred to commensal and pathogenic bacteria through the food chain, in addition to the fact that antimicrobial resistance in *L. monocytogenes* may have an adverse effect on the effective treatment of listeriosis in humans, as mentioned by Escolar et al. [62]. Studies have been reported on the resistance of bacterial pathogens, such as *E. coli*, *Salmonella*, and *Listeria* species, to tetracyclines in the livestock industry in South Africa [36,69,70]. The resistance of bacteria to tetracycline in South Africa has been attributed to the unregulated use of veterinary drugs, including tetracycline, in the country. This is attributed to the existing Fertilizers, Farm, and Agricultural and Stock Remedy Act (Act 36, 1947), which legalizes the use of certain antimicrobial agents, such as sulphonamides and trimethoprim, to be purchased over-the-counter, and they are used for treatment and as growth promoters [71]. Interestingly, the phenotypic resistance exhibited to tetracycline (36.8%) determined by Gana [42] correlates well with the putative resistance to tetracycline due to both *tet(M)* and *tet(S*) genes based on WGS on the same isolates, suggesting that the genes may have been responsible partly for the resistance detected. Other studies have similarly reported the correlation between phenotypic resistance and the carriage of corresponding encoding resistance genes [67,72,73]. However, a lack of correlation between these variables has also been reported by others [62]. It has been documented that bacteria may possess resistance genes but not express them, or they may be lost, thus limiting their application to their therapeutic implications and significance [74,75].

*L. innocua* is considered non-pathogenic. Previous analyses have suggested that *L. monocytogenes* and *L. innocua* evolved from a common virulent ancestor. During the evolution, consecutive loss of virulence genes critical to host adaptation were associated with the emergence of *L. innocua* [18]. Rare, atypical *L. innocua* strains that harbor LIPI-1 and *inlA* and are hemolytic and weakly virulent may represent an intermediary evolutionary stage. In addition, rare, atypical *L. monocytogenes* strains resulted from the spontaneous loss of virulence genes and were nonhemolytic [76]. In the present study, 23 virulence factors were detected in the 110 isolates using WGS, thus providing a spectrum of the virulence factors carried by the isolates, unlike PCR which provided information specific only to the primers targeted [77,78]. Unlike *L. innocua*, the ability of *L. monocytogenes* to cause listeriosis is known to be multifaceted and has been attributed to six virulence genes *prfA*, *plcA*, *hly*, *mpl*, *actA*, and *plcB, which are located in the PrfA*-dependent virulent gene cluster known as LIPI-1 [78], other *Listeria* pathogenicity islands, namely LIPI-3 and LIPI-4, Internalins (*inl*) genes, and other virulence genes as reported by Glimour *et al.* [79]. None of our *L. innocua* isolates contained virulence genes and are therefore classified as non-pathogenic [80]. However, it has emerged that some strains of *L. innocua* have been demonstrated to contain virulence genes that have contributed to their weak virulence [18]. Some of the factors documented by others in strains of *L. innocua* include the carriage of virulence factors, such as LGI2, LGI3, LIPI-3, and LIPI-4 [17,39,81]. It was noteworthy to have detected that the grouping of virulence genes by the STs revealed five distinct patterns. For example, pattern 1 (44 isolates) comprised three STs (637, 1085, and 602) consisting of 15 virulence factors, and pattern 2 (51 isolates) consisted of 6 STs (448, 537, 1482, 1008, 1619, and 43), with 15 virulence factors.

According to the STs, this distribution of virulence genes has been documented in *L. monocytogenes*, where some are more associated with listeriosis depending on the virulence genes they carry, as reported in a recent outbreak of human listeriosis was caused by *L. monocytogenes*, ST6 [24,35]. Notwithstanding the high frequency of virulence genes in *L. innocua* isolates recovered from the three levels of beef production in South Africa, it is important to note that the presence/absence of virulence genes in *L. monocytogenes* was not a predictor of the virulence potential of *L. monocytogenes* [82]. Similarly, we should interpret the presence of virulence genes in *L. innocua* with caution. Further assessments, including hemolytic and virulence assays, on our *L. innocua* strains are needed.

## 5. Conclusions

For the first time in South Africa, this study provided a comprehensive genomic characterization of resistance and virulence genes in *L. innocua* isolated from three levels (production, processing, and retailing) of the beef industry using WGS. The MSTs using the profiles of the STs, AMRs, and virulence genes revealed a diversity in their spread and clustering regardless of the sources and sample types from where the *L. innocua* isolates originated. Furthermore, the phylogenies confirmed the genetic relatedness of the *L. innocua* isolates from the three sampling levels. The high frequency of resistance genes *tet(M)* and *fosX* observed in this study suggests that the use of tetracycline and fosfomycin in the livestock industry in the country and its role in the development of bacterial antimicrobial resistance should be reviewed. Finally, caution is needed in extrapolating the data based on the presence and absence of genes to the potential phenotype (i.e., resistance and virulence potential). The study has provided invaluable data on the status of *L. innocua* in the cattle industry food chain in the country.

**Supplementary Materials:** The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table S1. Sources, types of samples, sequence types, and resistance genes detected in 110 L. *innocua* isolates. Table S2. Sources, types of samples, sequence types, and virulence genes detected in *L. innocua* isolates.

**Author Contributions:** Conceptualization, A.A.A., and N.G.; methodology, J.G., Y.C., R.E.P., A.A.A., and NG; software, R.E.P., Y.C., and NG; validation, R.E.P., and Y.C.; formal analysis, R.E.P., Y.C., A.A.A., and JG; resources, A.A.A., N.G., and R.M.; data curation, A.A.A., JG, and R.E.P.; writing—original draft preparation, A.A.A., J.G., N.G., and R.E.P.; writing—review and editing, A.A.A., J.G., N.G., R.E.M., Y.C. and R.M.; visualization, R.E.P., Y. C., N.G., J.G., and A.A.A.; supervision, A.A.A., N.G., and R.M.; project administration, A.A.A., N.G., and J.G.; funding acquisition, A.A.A., N.G., and R.M.. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** All the data are contained within the article and the supplementary materials.

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**Disclaimer**: The views expressed in this article are those of the authors and do not necessarily reflect the official policy of the Department of Health and Human Services, the U.S. Food and Drug Administration, or the U.S. Government.

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