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Article

Bacterial and Genetic Features of Raw Retail Pork Meat: Integrative Analysis of Antibiotic Susceptibility, Whole Genome Sequencing and Metagenomics

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Abstract: The global antibiotic resistance crisis, driven by antibiotic overuse and misuse, is multifaceted. This study aimed to assess the microbiological and genetic characteristics of raw retail pork meat, including the isolation, antibiotic susceptibility testing (AST), whole genome sequencing (WGS) of selected indicator bacteria, antibiotic residue testing, and metagenomic sequencing. Samples were purchased from 10 randomly selected retail stores in Gauteng, South Africa. Samples were aseptically separated, with a portion sent to an external laboratory for isolating indicator bacteria and antibiotic residue testing. Identification of the isolated bacteria was reconfirmed using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). AST was performed using the Microscan Walkaway system. WGS and metagenomic sequencing were performed using the Illumina NextSeq 550 instrument. The isolated *E. coli* and enterococci exhibited little phenotypic resistance, with WGS revealing tetracycline resistance genes. Isolated bacteria and meat samples all harboured tetracycline resistance genes, and the antibiotic residue concentrations were within acceptable limits for human consumption. In the metagenomic context, most identified bacteria were of food/meat spoilage and environmental origin, with the resistome analysis primarily indicating beta-lactam, tetracycline and multidrug resistance genes. Further research is needed to understand the broader implications of these findings on environmental health and antibiotic resistance.

Keywords: whole genome sequencing; metagenomics; antibiotic residue testing; retail pork meat; South Africa

1. Introduction

The global crisis of antibiotic resistance is multifaceted and has been linked to antibiotic overuse and misuse in humans and animals, improper prescribing practices and the widespread use of antibiotics as growth promoters in livestock production [1,2]. Pork production is widespread in South Africa, with the largest contributions coming from Limpopo and North West provinces, which account for 24% and 20% of the total production, respectively [3].

In animals, antibiotics such as sulphonamides, tetracyclines, and fluoroquinolones are widely used for therapeutic, metaphylactic and prophylactic purposes or as growth promotors in animal feed [4-6]. The Food and Agriculture Organization and the European Union have established tolerance or maximum residue limits (MRL) for antibiotic residues in animal-derived food products

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[7]. In conjunction, the MRL for foodstuffs in South Africa are regulated in South Africa by the Foodstuffs, Cosmetics and Disinfectants Act No. 54 of 1972 [8].

Previous studies have found that food products not only serve as a reservoir for antibiotic resistant bacteria and antibiotic resistance genes (ARG) but also act as a mediator, facilitating the transfer of antibiotic resistant bacteria and ARGs between the environment and humans through indirect contact through the consumption of contaminated foods [2,5,9,10]. Understanding the diversity and abundance of ARGs, virulence factor (VF) genes and antibiotic residues in food, especially retail pork meat, is important for controlling antibiotic resistance [2,4]. This involves implementing effective antibiotic stewardship programs, regulating the use of antibiotics in food production, improving hygiene and sanitation practices, and promoting responsible antibiotic usage in both human and veterinary medicine [5]. The environment harbours a high prevalence of multidrug-resistant (MDR) strains, which affect both humans and animals, and facilitates the easy transmission of ARGs through horizontal gene transfer among different bacterial species (spp.) [11]. These spp. include both commensal flora and pathogenic foodborne pathogens like *Campylobacter* spp., *Enterococcus* spp., *Escherichia coli* and non-typhoidal *Salmonella* spp. (NTS) that can cause disease in humans and animals [11]. The aim of this study was to isolate and characterise four common indicator bacteria that overlap between humans and animals, *Campylobacter* spp., *Enterococcus* spp., *E. coli* and NTS, to test for antibiotic residues and to assess the microbial community as well as the resistome present in purchased raw retail pork meat.

2. Materials and Methods

2.1. Ethical Clearance and Study Definitions

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Human Research Ethics Committee of the University of the Witwatersrand (M190244; 10/05/2019).

Supermarkets were defined as stores that sold raw meat and various other grocery items, while butcheries were defined as stores selling primarily raw meat commodities. Raw meat samples were defined as pork chops (alternative names included loin, rib, sirloin, top loin and blade chops). Indicator bacteria in this study were defined as *Campylobacter* spp., *Enterococcus* spp., *E. coli* and NTS spp.

2.2. Study Setting and Sampling

Raw meat samples (i.e. pork chops) were purchased once off from 10 randomly selected retail outlet stores in Johannesburg and Pretoria, Gauteng on the 4th of January 2022. The stores selected for sample collection included both supermarkets (n=5) and butcheries (n=5). Pork chops were chosen as it is the most popular cut among consumers [12]. Raw meat samples (containing at least two pork chops in the same container/packet) were randomly selected from the store shelves. All the raw meat samples were collected within the recommended dates for human consumption. Store demographics were captured by the study investigators on the day of sample collection (Supplementary Table S1). A unique number was assigned to each raw meat sample as well as the sampled stores to maintain anonymity and to ensure that the results cannot be linked back to a specific store.

The purchased raw meat samples were transported on ice to the Centre for Healthcare-associated infections, Antimicrobial Resistance and Mycoses (CHARM), National Institute for Communicable Diseases (NICD), a division of the National Health Laboratory Service (NHLS). The outside of all the meat containers/packets was wiped with 70% ethanol before segregation and processing to avoid cross-contamination. The raw meat samples were separated aseptically. One raw meat sample from each store was sent on ice within 24 hours after collection to a subcontracted laboratory in Gauteng, South Africa for 1) the isolation of four selected indicator bacteria and 2) antibiotic residue testing using liquid chromatography tandem-mass spectrometry (LC-MS/MS). The remaining raw meat samples underwent metagenomics sequencing at the Sequencing Core Facility (SCF), NICD. All the raw meat samples were processed within 24 hours to 48 hours after sample collection.

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2.3. Antibiotic Susceptibility Testing (AST) and Whole Genome Sequencing (WGS) of Isolated Indicator Bacteria from Raw Meat Samples

The bacteria isolated from the raw meat samples were transported at room temperature (20°C to 25°C) from the subcontracted laboratory to CHARM within 24 hours after isolation. Organism identification was reconfirmed at CHARM, NICD with the matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) (Microflex, Bruker Daltonics, Germany). AST was performed using the Microscan Walkaway System with the Gram-negative NM44 (Beckman Coulter, USA) and Gram-positive PM33 (Beckman Coulter, USA) cards. The AST results were interpreted using the 2023 European Committee on antimicrobial susceptibility testing (EUCAST) guidelines [13].

The genomic DNA (gDNA) of all isolated organisms from the raw meat samples were extracted with the QIAamp mini kit (Qiagen, Germany) with the inclusion of lysozyme (10 mg/mL; Sigma-Aldrich, USA) to ensure sufficient lysis. The quantity and quality of the extracted gDNA were determined on Qubit 4.0 (Invitrogen, USA) with the high sensitivity assay kit (Invitrogen, USA). Multiplexed paired-end libraries were prepared using the Nextera DNA Prep kit, followed by sequencing (2×150 bp) on a NextSeq 550 instrument (Illumina Inc., USA) with 100x coverage at the SCF, NICD. Raw paired-end reads were analysed using the Jekesa pipeline (v1.0) [14]. Briefly, Trim Galore! (v0.6.7) was used to filter the paired-end reads (Q >30 and length >50 bp) [15]. *De novo* assembly was performed using SKESA (v2.3.0; https://github.com/ncbi/SKESA) and the assembled contigs were polished using Shovill (v1.1.0; https://github.com/tseemann/shovill) [16]. Assembly metrics were calculated using QUAST (v5.0.2) [17]. The multilocus sequence typing (MLST) profiles were determined using the MLST tool (version 2.16.4; https://pubmlst.org/; https://github.com/tseemann/mlst) [18]. VF genes and ARGs search was performed using ABRicate (version 1.0.1; https://github.com/tseemann/abricate), against the Comprehensive Antibiotic Resistance Database (CARD), CARD-prevalence, Virulence Factor Database (VFDB) and ResFinder - Center for Genomic Epidemiology (CGE) database; with the gene alignment coverage cut-off of ≥95% and blastn sequence similarity of ≥95% [19-24]. The assembled genome files were deposited in the National Center for Biotechnology Information GenBank under BioProject number PRJNA1006163.

2.4. Metagenomics

Total gDNA was extracted using the QIAamp Fast DNA stool mini kit (Qiagen, Germany) and host depletion was done using the NEBNext Microbiome DNA enrichment kit (New England Biolabs, USA) with the inclusion of negative and positive controls (ZymoBIOMICS Gut Microbiome Standard). Sequencing was done on the NextSeq 550 (2×150 bp and 10 M reads) (Illumina, USA).

Initial sequence analysis was performed by the SCF, NICD. This included de-multiplexing (assigning reads to the respective sample using the barcodes that were assigned during the library preparation stage), quality checking using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and trimming and discarding of reads with a Q-score of less than 20 (TrimGalore, v0.6.2; https://github.com/FelixKrueger/TrimGalore). Subsequent sequence analysis included the removal of host sequences (Suis scrofa v11.1 and Homo sapiens GRCh38.p13; Available at https://www.ensembl.org/index.html) using Bowtie2 v2.5.0 (https://github.com/BenLangmead/bowtie2). Taxonomic assignment was done using the host-depleted metagenomic sequence reads, where the microbial diversity profile analyses were carried out with Kraken (v2.1; https://github.com/DerrickWood/kraken) using the Standard-16 database [25]. This is followed by the estimated related abundance of genera were calculated using Bracken (v2.5) and the proportion of related abundance were visualised using R (v4.2.1; https://www.r-project.org/).

2.5. Resistome Gene Abundance Estimates

With the host-depleted metagenomic sequence reads, standard *de novo* assembly of the metagenomic data were done using MEGAHIT (v1.2.9) [26]. Using the assembled contigs, the ARG, toxin genes and VF genes were predicted using PathoFact (v1.0) pipeline [27]. The predicted ARG, as

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well as the toxin genes and VF genes with high confident prediction (1: secreted toxin or 1: secreted VF) were selected for relative abundance analysis, using ShortBRED (v0.9.4) [28] against the CARD database (v2023-06) and VFDB (database update: 06 August 2023). The predicted resistome' relative abundance was quantified by ShortBRED-Quantify calls USEARCH, where reads with 95% identity to the resistome were counted and normalized by reads per kilobase of reference sequence per million sample reads (RPKMs). The antimicrobial resistance (AMR) category "multidrug" was defined as bacterial strains that have become resistant to multiple classes of antibacterial drugs or other agents (https://card.mcmaster.ca/ontology/41472).

3. Results

3.1. Isolation of Indicator Bacteria

All 10 raw meat samples were tested for the selected four indicator bacteria (Table 1). *E. coli* was detected and isolated from one butchery raw meat sample (PC9-B4). Enterococci were detected and isolated from two supermarket raw meat samples (PC3-S3 and PC4-S4) and one butchery raw meat sample (PC10-B5). *Campylobacter* spp. and *Salmonella* spp. were not detected in any of the collected raw meat samples (Supplementary Table S2).

Table 1. Indicator organisms isolated from raw meat samples.

Sample ID	*E. coli* (CFU)	*Salmonella* spp. (CFU)	Enterococci spp. (CFU)	*Campylobacter* spp. (CFU)
PC1-S1	Absent	Absent	Absent	Absent
PC2-S2	Absent	Absent	Absent	Absent
PC3-S3	Absent	Absent	16*	Absent
PC4-S4	Absent	Absent	1*	Absent
PC5-S5	Absent	Absent	Absent	Absent
PC6-B1	Absent	Absent	Absent	Absent
PC7-B2	Absent	Absent	Absent	Absent
PC8-B3	Absent	Absent	Absent	Absent
PC9-B4	20*	Absent	Absent	Absent
PC10-B5	Absent	Absent	3*	Absent

PC = Pork chop; S = Supermarket; B = Butchery; CFU = Colony forming units; * = Isolated bacteria from raw meat samples were subsequently processed (AST and WGS).

3.2. AST and WGS of Isolated Indicator

A single *E. coli* isolate was obtained from sample PC9-B4, while one *E. faecalis* isolate each was obtained from samples PC3-S3, PC4-S4 and PC10-B5. The IDs of all the isolates were reconfirmed and AST was performed. All three *E. faecalis* isolates had the same antibiotic susceptibility profile (Table 2). There are no AST guidelines for isolates obtained from raw meat samples; thus, the AST results were interpreted using the 2023 EUCAST guidelines for human isolates [13].

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Table 2. Antibiotic susceptibility testing of one *Escherichia coli* isolate and three *Enterococcus faecalis* isolates isolated from four raw meat samples.

Antibiotic class	**Antibiotic**	***E. coli* PC9-B4 (µg/mL)**	***MIC interpretation#***	***E. faecalis* PC3-S3 (µg/mL)**	***E. faecalis* PC4-S4 (µg/mL)**	***E. faecalis* PC10-B5 (µg/mL)**	***MIC interpretation#***
Aminoglycoside	Amikacin	≤8	***S***	32	32	32	***NI****
	Gentamicin	≤2	***S***	4	4	4	***NI****
	Gentamicin synergy	NT	-	≤500	≤500	≤500	***NI***
	Streptomycin synergy	NT	-	≤1000	≤1000	≤1000	***NI***
	Tobramycin	≤2	***S***	≤2/38	≤2/38	***≤2/38***	***NI****
Beta-lactam (penicillins)	Ampicillin	≤8	***S***	4	4	4	***S***
	Ampicillin/Sulbactam	≤8/4	***S***	NT	NT	NT	-
	Amoxicillin/Clavulanic acid	≤8/4	***S***	≤4/2	≤4/2	≤4/2	***S***
	Oxacillin	NT	-	>2	>2	>2	***NI***
	Penicillin	NT	-	8	8	8	***NI***
	Piperacillin	≤8	***S***	NT	NT	NT	-
	Piperacillin/Tazobactam	≤8	***S***	NT	NT	NT	-
Beta-lactam (cephalosporins)	Cefepime	≤1	***S***	NT	NT	NT	-
	Cefotaxime	≤1	***S***	NT	NT	NT	-
	Cefotaxime/ Clavulanic acid	≤0.5	***NI***	NT	NT	NT	-
	Cefoxitin	≤8	***S***	NT	NT	NT	-
	Cefuroxime	≤4	***S***	NT	NT	NT	-
	Ceftazidime	≤1	***S***	NT	NT	NT	-
	Ceftazidime/Clavulanic acid	≤0.25	***NI***	NT	NT	NT	-
	Cephalothin	≤8	***NI***	NT	NT	NT	-
Beta-lactam (carbapenems)	Doripenem	≤1	***S***	NT	NT	NT	-
	Ertapenem	≤0.5	***S***	NT	NT	NT	-
	Imipenem	≤1	***S***	≤4	≤4	≤4	***S***
	Meropenem	≤1	***S***	NT	NT	NT	-
Beta-lactam (monobactams)	Aztreonam	≤1	***S***	NT	NT	NT	-
Amphenicol	Chloramphenicol	≤8	***S***	≤8	≤8	≤8	***NI***
Cyclic lipopeptide	Daptomycin	NT	-	≤1	≤1	≤1	***NI***
Fluoroquinolone	Ciprofloxacin	≤0.5	***S***	≤1	≤1	≤1	***S***
	Levofloxacin	≤1	***S***	≤1	≤1	≤1	***S***
	Moxifloxacin	NT	-	≤256	≤256	≤256	***R***
	Norfloxacin	≤0.5	***S***	≤4	≤4	≤4	***NI***
Fusidane	Fusidic acid	NT	-	≤2	≤2	≤2	***NI***
Lincosamides	Clindamycin	NT	-	>2	>2	>2	***NI***
	Pristinamycin	NT	-	2	2	2	***NI***
Macrolide	Erythromycin	NT	-	1	1	1	***NI***
Protein synthesis inhibitor	Mupirocin	>16	***NI***	NT	NT	NT	-
Nitrofuran	Nitrofurantoin	≤32	***S***	≤32	≤32	≤32	***S***
Phosphonic acid	Fosfomycin	≤32	***S***	≤32	≤32	≤32	***NI***
Polymyxin	Colistin	≤2	***S***	NT	NT	NT	-
Rifamycin	Rifampin	NT	-	≤0.5	≤0.5	≤0.5	***NI***

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Tetracycline	Minocycline	>8	***NI***	≤1	≤1	≤1	***NI***
	Tetracycline	>8	***NI***	8	8	8	***NI***
	Tigecycline	≤1	***R***	NT	NT	NT	**-**
Glycopeptide and lipoglycopeptide	Teicoplanin	NT	**-**	≤1	≤1	≤1	***S***
	Vancomycin	NT	**-**	2	2	2	***S***
Oxazolidinone	Linezolid	NT	**-**	2	2	2	***S***
Sulfonamide	Trimethoprim/Sulfamethoxazole	>4/76	***R***	NT	NT	NT	**-**

PC = Pork chop; S = Supermarket; B = Butchery; NT = Not tested (antibiotic not included in the antibiotic panel); NI = No EUCAST MIC interpretation; * = All enterococci are intrinsically resistant to aminoglycosides; # = [13].

The isolated *E. coli* (PC9-B4) and *E. faecalis* (PC3-S3, PC4-S4 and PC10-B5) isolates were subjected to WGS (Table 3). The *E. coli* isolate exhibited ARGs conferring resistance to aminoglycoside (*aad*A1), fluoroquinolone (*gyr*A), tetracycline (*tet*B), trimethoprim (*dfr*A1) and sulphonamide (*sul*2). All three *E. faecalis* isolates exhibited ARGs conferring resistance to tetracycline (*tet*M) and lincosamides (*isa*A).

A total of eight VF genes were identified in *E. coli*, and nine VF genes were identified in *E. faecalis*. VF genes associated with *E. coli* included colicins (*cba, cea, cia and cma*), as well as the toxin gene (*astA*). VF genes associated with *E. faecalis* isolates included adhesins (*ace* and *efaAfs*), toxins (*cyl-A, -L* and *-M*), and genes associated with biofilm formation (*ebp-A* and *-B*) and pheromone production (*cad, camE, cCF10 and cOB1)*.

The *E. coli* isolate harboured the IncB/O/K/Z and IncFII (pCoo) plasmids, while the *E. faecalis* isolates harboured the repUS43, repUS11 and rep9a plasmids.

Table 3. Antibiotic resistance genes, virulence factors and plasmids detected in one *Escherichia coli* isolate and three *Enterococcus faecalis* isolates isolated from four raw meat samples.

Sample ID			**PC9-B4***	**PC3-S3***	**PC4-S4***	**PC10-B5***
Organism			*E. coli*	*E. faecalis*	*E. faecalis*	*E. faecalis*
CH Type			11-54	-	-	-
O type			O69	-	-	-
H type			H32	-	-	-
MLST			10^	30#	30#	30#
ARGs	Aminoglycoside	*aadA1*	Y	-	-	-
	Fluoroquinolone	*gyrA*	Y	-	-	-
	Lincosamide	*isaA*	-	Y	Y	Y
	Sulphonamide	*sul2*	Y	-	-	-
	Tetracycline	*tetB*	Y	-	-	-
		tetM	-	Y	Y	Y
	Trimethoprim	*dfrA1*	Y	-	-	-
VF genes	Adhesin	*ace*	-	Y	Y	Y
		efaAfs	-	Y	Y	Y
	Colicin	*cba*	Y	-	-	-
		cea	Y	-	-	-
		cia	Y	-	-	-
		cma	Y	-	-	-
	Cytolysin toxin	*cylA*	-	Y	Y	Y
		cylL	-	Y	Y	Y
		cylM	-	Y	Y	Y
	Endocarditis and biofilm-associated pili genes	*ebpA*	-	Y	Y	Y
		ebpB	-	Y	Y	Y
	Enterococcal leucine rich protein A	*elrA*	-	Y	Y	Y
	Glutamate decarboxylase	*gad*	Y	-	-	-
		gelE	-	Y	Y	Y
	Heat stable toxin	*astA*	Y	-	-	-
	Hyaluronidase	*hylA*	-	Y	Y	Y
	Increased serum survival	*iss*	Y	-	-	-

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	Outer membrane protease	*ompT*	Y	-	-	-
	Plasmid-encoded catalase peroxidase	*katP*	Y	-	-	-
	Sex pheromone	*cad*	-	Y	Y	Y
		camE	-	Y	Y	Y
		cCF10	-	Y	Y	Y
		cOB1	-	Y	Y	Y
	Tellurium ion resistance	*terC*	Y	-	-	-
	Thiol peroxidase	*tpx*	-	Y	Y	Y
	Outer membrane protein complement resistance	*traT*	Y	-	-	-
	Sortase	*SrtA*	-	Y	Y	Y
Plasmids		IncB/O/K/Z	Y	-	-	-
		IncFII(pCoo)	Y	-	-	-
		repUS43	-	Y	Y	Y
		repUS11	-	Y	Y	Y
		rep9a	-	Y	Y	Y

PC = Pork chop; S = Supermarket; B = Butchery; ARG = Antibiotic resistance genes; VF = Virulence factor; MLST = Multilocus sequence typing (https://github.com/tseemann/mlst); Y = Present/Detected; - = Absent/Not detected; ^ = adk 10-fumC 11-gyrB 4-icd 8-mdh 8- purA 8- recA 2; # = aroE 10-ghd 7-gki 1-gyd 1-pstS 11-xpt 2-yqiL 1; * = The corresponding raw meat sample numbers were assigned to each isolate for traceability.

3.3. Antibiotic Residue Testing

The average antibiotic residue concentration detected in all the raw meat samples were <50 µg/kg for all the majority of all the tested antibiotics (Table 4). Sample PC5-S5 had an antibiotic residue concentration of 71,5 µg/kg for chlortetracycline. All tested residue levels were below the Codex/SA MRL acceptable limit [7].

Table 4. Antibiotic residue testing in 10 raw meat samples using liquid chromatography tandem-mass spectrometry (LC-MS/MS).

Antibiotic class (µg/kg)	Antibiotic	Sample ID										Acceptable maximum residue level
		PC1-S1*	PC2-S2*	PC3-S3*	PC4-S4*	PC5-S5*	PC6-B1*	PC7-B2*	PC8-B3*	PC9-B4*	PC10-B5*	
Fluoroquinolones	Ciprofloxacin	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	100#
	Enrofloxacin	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	100#
	Norfloxacin	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	-
Lincosamides	Lincomycin	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	200^
Macrolides	Tylosin	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	100^
Sulfonamides	Sulfadiazine	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	-
	Sulfadimidine	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	100^
	Sulfamethoxazole	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	-
Tetracyclines	Chlortetracycline	<50	<50	<50	<50	71,5	<50	<50	<50	<50	<50	200^
	Doxycycline	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	-
	Oxytetracycline	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	200^
	Tetracycline	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	200^ / 600#
Pleuromutilin	Tiamulin	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	-
Diaminopyrimidines	Trimethoprim	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	-
Quindoxin	Olaquindox metabolite	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	-

PC = Pork chop; S = Supermarket; B = Butchery; * = Muscle; - = No acceptable maximum residue level listed; ^ = [7]; # = [4].

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3.4. Metagenomics

3.4.1. Read Statistics

Approximately 7 million hosts reads (ranging from 5,797,550 – 8,900,728 sequencing reads) were removed from each sample prior to the metagenomic analysis (Table 5). The remaining reads were subject to further bacterial community profiling and ARG prediction analyses.

Table 5. Metagenomics read statistics of 10 raw meat samples.

Sample ID	Raw paired-end reads (n = NGS reads)	Paired-end reads after host removal (n = NGS reads)	Paired-end reads mapped to bacteria (n = NGS reads)	Predicted ARG (n = Annotated ORF)	Predicted secreted VF genes (n = Annotated ORF)	Predicted secreted toxin genes (n = Annotated ORF)
PC1-S1	6559325	761775	249495	47	86	24
PC2-S2	7276334	372205	4242	6	2	5
PC3-S3	9008555	407827	59159	5	11	2
PC4-S4	8346930	353759	25353	1	0	0
PC5-S5	7706014	423019	48284	8	10	3
PC6-B1	6948972	260638	2460	0	1	0
PC7-B2	6318244	388517	47285	7	12	3
PC8-B3	6811831	400456	71941	11	15	3
PC9-B4	8083308	336636	2049	2	0	0
PC10-B5	6727542	295466	2043	2	1	0

PC = Pork chop; S = Supermarket; B = Butchery; NGS = Next-generation sequencing; ARG = Antibiotic resistance genes; ORF = Open reading frame/s; VF = Virulence factor.

3.4.2. Estimated Relative Abundance

Distribution of the bacterial community structures in the raw meat samples reveals functional diversity among bacteria (Figure 1). The relative abundance was similar between raw meat samples from supermarkets and butcheries, but PC9 was different.

Food and meat spoilage organisms such as *Pseudomonas* (90%; 9/10), *Acinetobacter* (80%; 8/10), *Brochothrix* (80%; 8/10), *Psychrobacter* (70%; 7/10), *Photobacterium* (60; 6/10) and *Clostridium* (50%; 5/10) were detected in the majority of raw meat samples [29-33].

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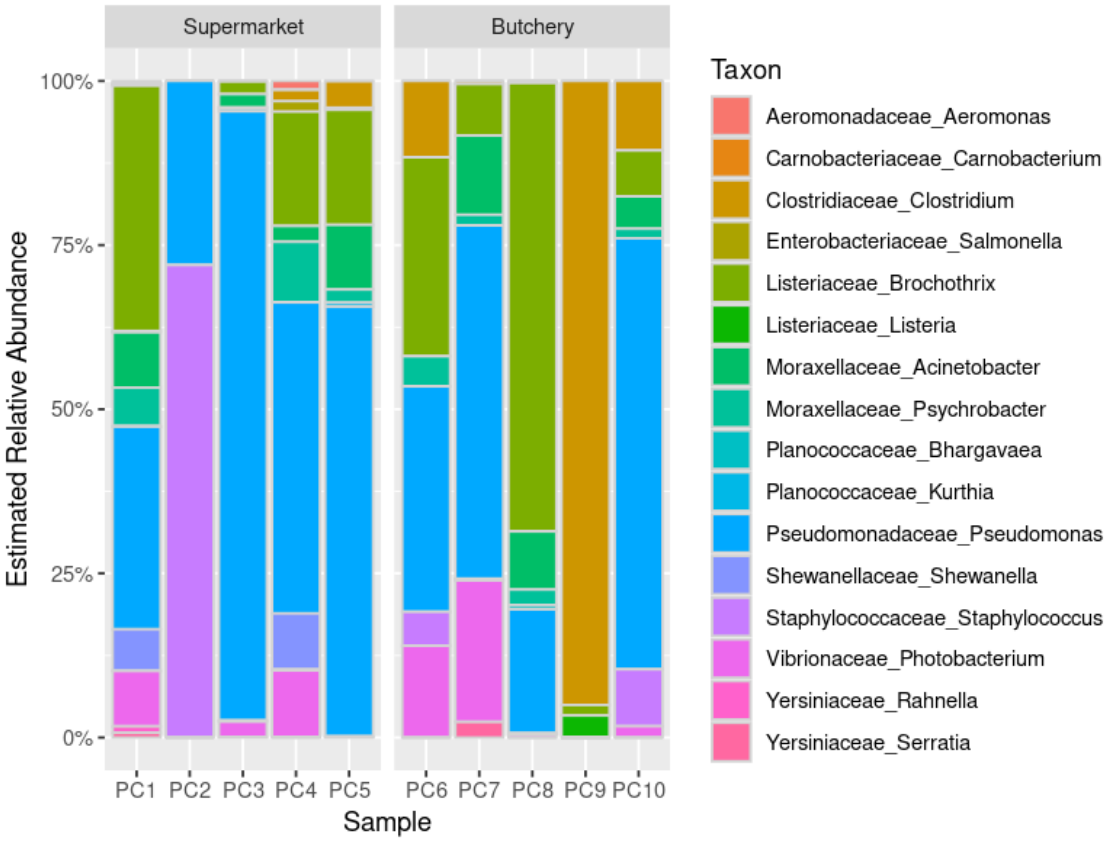

Figure 1. Estimated relative abundance of the bacterial taxonomic profiling (aggregated by family and genus) in 10 raw meat samples. PC = Pork chop.

3.4.3. Resistome prediction

Using the assembled contigs generated from MEGAHIT from the 10 raw meat samples, 61665 ORFs were predicted by PathoFact pipeline, of which, 89, 138 and 40 ORFs are predicted to be involve with AMR, VF secretion and toxin secretion, respectively.

3.4.3.1. Antibiotic Resistance Gene Prediction

A total of 89 ORFs were predicted as ARGs (Table 5; Supplementary Table S3). Raw meat samples from supermarkets were dominated by multidrug (26.8%; 18/67), beta-lactam (20.9%; 14/67) and tetracycline (14.9%; 10/67) resistance genes. Raw meat samples from butcheries were dominated by tetracycline (36.4%; 8/22) and multidrug (13.6%; 3/22) (Figures 2, 3A and 3C) resistance genes.

The majority of the ARGs were involved with resistance mechanisms such as antibiotic efflux (48.3%; 43/89), antibiotic inactivation (23.6%; 21/89), and antibiotic target alteration (12.36%; 11/89) (Supplementary Table S4). No ARG were predicted in sample PC6 (butchery - B1) (Figures 3B and 3D; Supplementary Figure S1).

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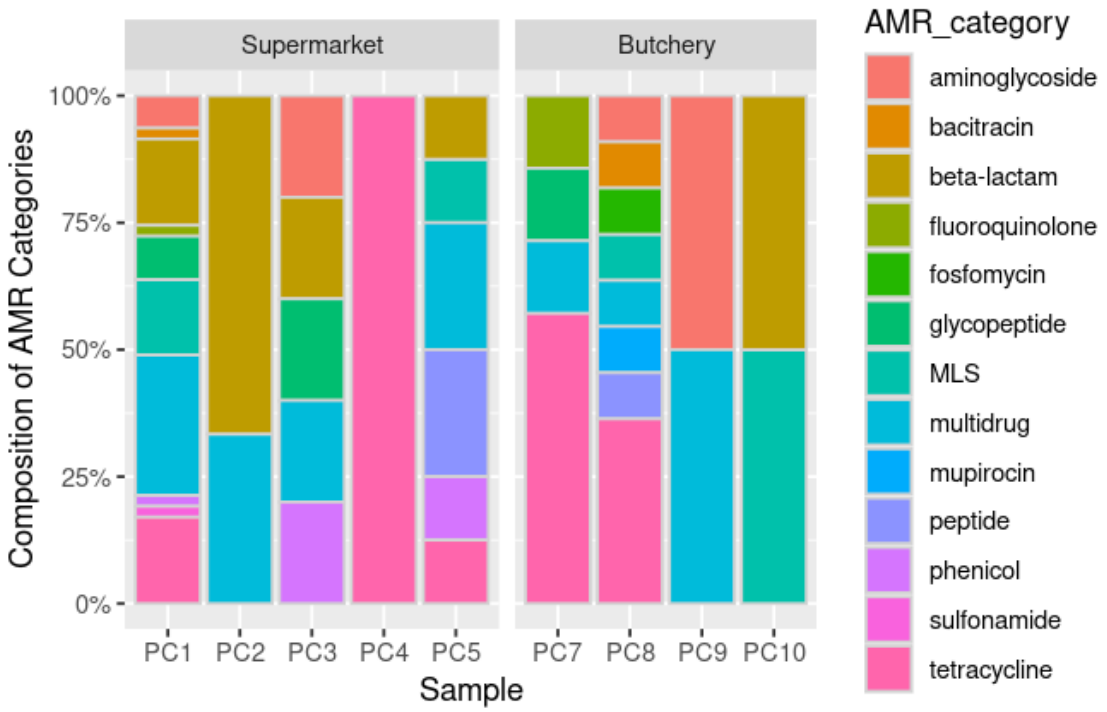

Figure 2. Composition of predicted AMR categories in 10 raw meat samples as predicated by PathoFact database. MLS = Macrolides, lincosamides and streptogramins; AMR category = Default assignment by the PathoFact pipeline; PC6 = No AMR was detected.

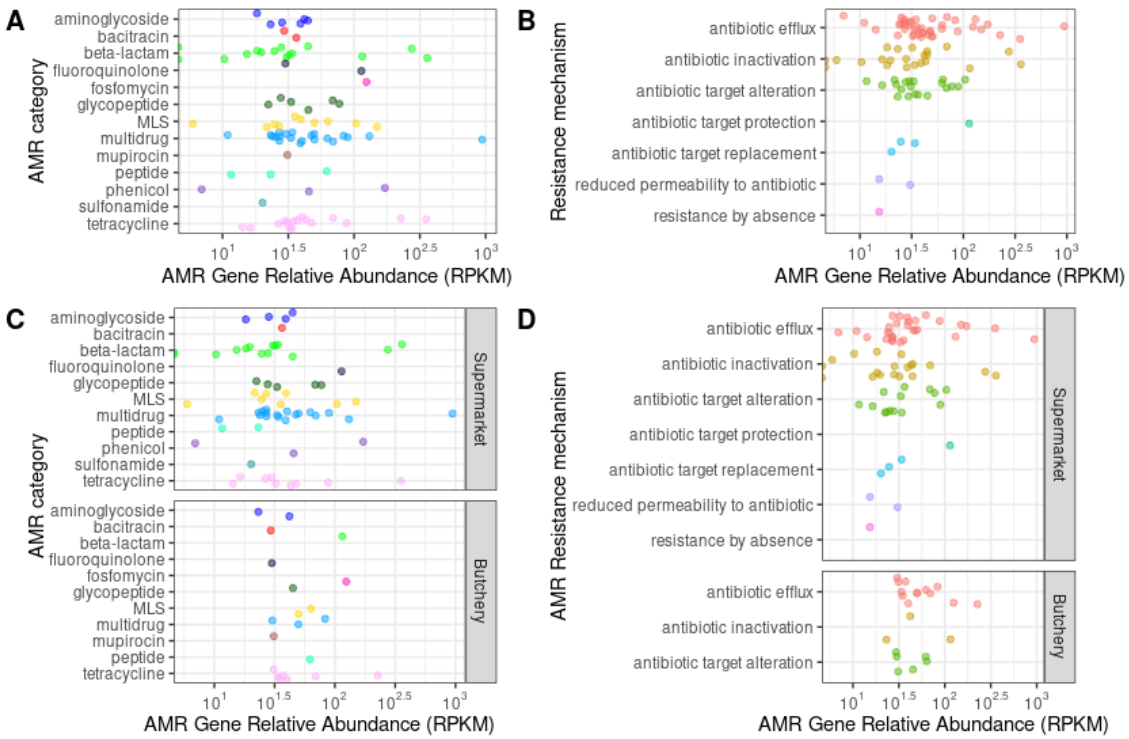

Figure 3. Relative abundance of individual ARGs of 10 raw meat samples (normalized as reads per kilobase per million mapped reads (RPKM), grouped by the AMR category (A and C), and by AMR resistance mechanisms (B and D), stratified by supermarket and butchery (C and D). Each circle indicating the relative abundance of a ARG gene in a single sample and genes within the same AMR category or AMR resistance mechanism are represented with the same colour.

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3.4.3.2 Virulence Factor and Toxin Gene Prediction

A total of 138 ORFs were predicted as secreted VF, and they are categorised into adherence (36.9%; 51/138), antimicrobial activity/competitive advantage (2.9%; 4/138), biofilm (5.1%; 7/138), effector delivery system (13.8%; 19/138), exoenzyme (0.7%; 1/138), exotoxin (0.7%; 1/138), immune modulation (8%; 11/138), invasion (5.1%; 7/138), motility (9.4%; 13/138), nutritional/metabolic factor (10.9%; 15/138), post-translational modification (1.4%; 2/138), regulation (1.4%; 2/138) and stress survival (3%; 4/138) VF categories (Table 5; Figure 4A; Supplementary Table S5; Supplementary Figure S1).

VF genes from all categories were present in the raw meat samples collected from supermarkets (Supplementary Table S6). No exotoxin, invasion, post-translational modification and stress survival VF genes were detected in the raw meat samples collected from butcheries. Fifteen types of toxin genes were detected in the raw meat samples collected from supermarkets and butcheries (Figure 4B).

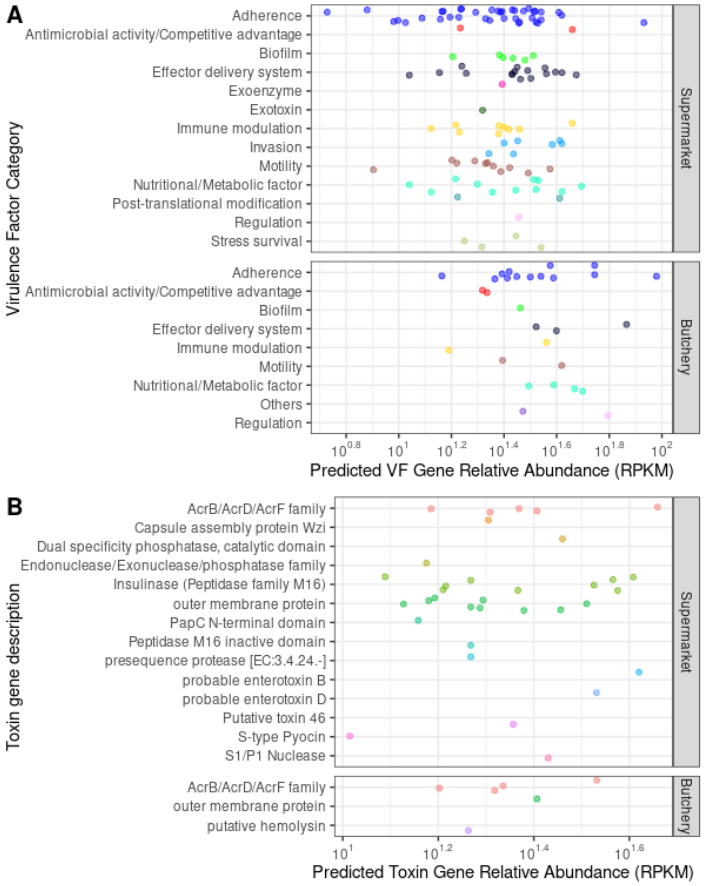

Figure 4. Relative abundance of the individual predicted virulence factor (VF) and toxin genes in 10 raw meat samples (normalized as reads per kilobase per million mapped reads (RPKM), grouped by the VF category according to VFDB (A), and by toxin gene description according to the PathoFact database (B). Each circle indicating the relative abundance of a VF/toxin gene in a single sample. Genes within the same VF category or toxin gene description are represented with the same colour.

4. Discussion

The global crisis of antibiotic resistance is multifaceted and linked to factors such as antibiotic overuse and misuse [1,2]. In this study, four selected indicator bacteria (*Campylobacter* spp., *Enterococcus* spp., *E. coli* and NTS spp.) that overlap between human and animals, the diversity and abundance of bacterial communities, ARGs and antibiotic residues in raw pork meat samples were investigated.

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The majority of the four indicator organisms were absent from the collected raw meat samples. A single *E. coli* isolate was obtained from sample PC9-B4, while one *E. faecalis* isolate each was obtained from raw pork meat samples PC3-S3, PC4-S4 and PC10-B5.

The isolated *E. coli* and *E. faecalis* isolates showed minimal phenotypic resistance. Specifically, the *E. coli* isolate showed resistance to tigecycline and trimethoprim/sulfamethoxazole, while all three *E. faecalis* isolates showed resistance to moxifloxacin only. WGS data for the *E. coli* (PC9-B4) and *E. faecalis* isolates (PC3-S3, PC4-S4 and PC10-B5) showed mainly the presence of tetracycline resistance genes. International studies have shown that *E. faecalis* was the most dominant *Enterococcus* spp. isolated (>80%) from pork samples [34,35]. High tetracycline resistance and associated resistance genes were reported in these studies, corresponding with the findings of this study [34,35]. In contrast, Aslam *et al.* (2012) in Canada found additional ARGs in enterococci isolated from retail meats, including aminoglycosides (*aac*, *aph*A3, *aad*-A and -E and sat4), macrolides (*erm*-B and -A), streptogramin (*vat*E), bacitracin (*bcr*R) and lincosamide (*lin*B) [34]. Hart *et al.* (2004) *in* Australia also reported widespread resistance to tetracycline in *E. coli* isolated from pigs [36]. Tetracycline, a broad-spectrum antibiotic used to treat various infections and as a growth promoter in animals, has contributed to the high tetracycline resistance rates reported in this study from one *E. coli* and three *E. faecalis* isolates [4,5,37-39]. The WGS data further revealed eight and nine different VF gene categories associated with the sequenced *E. coli* (PC9-B4) and *E. faecalis* PC3-S3, PC4-S4 and PC10-B5) isolates, respectively. VF genes enable pathogenic bacteria to colonise host niches and establish infections, contributing both directly and indirectly to disease processes [27]. Aslam *et al*. (2012) reported that cytolysin (*cyl-A, -B, -L* and *-M*), specifically *cyl-A, -L and -M* found in this study, as well as aggregation substances (*agg*), which were not detected in this study, were unconditionally associated the ARGs *tetM, linB* and *bcrR* in retail meats [34]. Another study conducted in Iran has also reported *ace, ebp* and *efaA* VF genes in *E. faecalis* isolates isolated from hospitalised patients [41]. The *E. coli* ST10 detected in this study has been identified as a reservoir for ARG and mobile genetic elements, such as class 1 integrons and plasmids [39,42,43]. The IncB/O/K/Z and *Inc*FII (pCoo) plasmids detected in the *E. coli* isolates have also been reported in *E. coli* from hospitalised patients in South Africa and in carbapenem-resistant Enterobacterales from hospitalised patients in Thailand [44,45]. Therefore, sequencing, especially plasmid monitoring, is essential for future surveillance studies to track the spread and evolution of these genetic elements in humans, animals and food products.

The antibiotic residue concentrations in the 10 tested raw meat samples were below the acceptable MRL (µg/kg) detection rate according to the published guidelines [7]. Ramatla *et al.* (2017) reported higher antibiotic residue concentrations in raw chicken, pork and beef samples (muscle, liver, or kidney) collected in North West, South Africa [4]. Possible explanation for the difference in findings between their study and the current one could be attributed to the sampling frequency (monthly sample collection over a period of five months *vs* one sampling event) and sample types (muscle, liver, and kidney samples *vs* solely muscle samples).

In the context of metagenomics, the majority of the identified bacteria were of food/meat spoilage and environmental origin [29-33]. However, their pathogenicity or ability to cause disease has not been confirmed in this study. Additionally, the resistome analysis revealed the presence of beta-lactam, tetracycline and multidrug resistance genes in the 10 raw meat samples sampled from supermarkets and butcheries. The resistance genes found in this study corresponds to previous local and internal studies [38,46,47].

Further research and analysis are needed to fully understand the potential implications of these findings in terms of environmental health and antibiotic resistance.

This study has several limitations. Firstly, a high number of host reads (i.e. pig DNA) were detected in the metagenomic data, indicating an insufficient number of reads available for the recovery of bacterial metagenome-assembled genomes (MAGs) within the raw meat samples. Consequently, this limited our ability to validate the predications of antibiotic resistance, VF and toxin genes. Using the MAGs approach could have provided more comprehensive information about the bacterial species in the meat samples, potentially identifying novel microorganisms carrying

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ARGs and uncovering new putative, unculturable pathogens. Secondly, it is recommended to consider increasing the sequencing depth or using alternative microbiome enrichment methods. Thirdly, the study's limited sample size could affect the generalisability of the findings to other supermarkets and butcheries across South Africa.

5. Conclusions

In conclusion, this study highlights the importance of evidence-based investigation and laboratory testing for significant bacterial organisms, as well as antibiotic susceptibility testing in food production systems. While none of the indicator organisms were detected in significant colony-forming units in the collected raw meat samples, isolated *E. coli* and *E. faecalis* strains exhibited minimal phenotypic resistance, with notable resistance to tigecycline, trimethoprim/sulfamethoxazole, and moxifloxacin. WGS data highlighted prevalent tetracycline resistance genes, aligning with international studies on tetracycline use in pork production. The presence of various ARG and VF genes in supermarket and butchery meat samples highlights the need for effective antimicrobial use in pork production. Although antibiotic residue concentrations are within acceptable limits, further research is needed to understand the broader implications of these findings on environmental health and antibiotic resistance. This study emphasises the need for vigilance and comprehensive strategies to combat antibiotic resistance in food production systems.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Table S1: Demographic data collected from the sampled stores (five supermarkets and five butcheries); Table S2: Captured demographic data collected from the sampled stores five supermarkets and five butcheries); Table S3: Summary of antimicrobial resistance categories by number of open reading frame genes from 10 raw retail meat samples; Table S4: Summary of antimicrobial resistance mechanisms by number of ORF genes from 10 raw retail meat samples; Table S5: Summary of virulence factor category by number of ORF genes from 10 raw retail meat samples; Table S6: Summary of toxin gene description by number of ORF genes from 10 raw retail meat samples; Figure S1: Mobile genetic element prediction of ARGs of all raw meat samples from both supermarket and butchery groups; Figure S2: Mobile genetic element prediction of VF and toxin genes of all raw meat samples from both supermarket and butchery groups.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Human Research Ethics Committee of the University of the Witwatersrand (Protocol No: M190244).

Informed Consent Statement: Not applicable.

Data Availability Statement: The assembled genome files were submitted deposited in the National Center for Biotechnology Information GenBank and are available under the following BioProject numbers: PRJNA1006163. The supplementary materials contain additional data.

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Conflicts of Interest: The authors declare no conflict of interest.

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