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Phenotypic and Genotypic Analysis of Antimicrobial Resistance of Commensal *Escherichia coli* from Dairy Cows' Feces

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Abstract: Commensal *Escherichia coli* has the potential to easily acquire resistance to a broad range of antimicrobials, making it a reservoir for its transfer to other microorganisms, including pathogens. The aim of this study was to determine the prevalence of resistant commensal *Escherichia coli* isolated from dairy cows' feces. Phenotypic resistance profiles and categorization were determined by minimum inhibitory concentration (MIC) testing with the broth microdilution method, while the PCR method was used to determine the presence of resistant genes. Out of 159 commensal *E. coli* isolates, 39 (24.5%) were confirmed to have resistance. According to the MIC values, 37 (97.3%) and 1 (2.7%) isolate were phenotypically categorized as ESBL and ESBL/AmpC, respectively. All isolates showed resistance to ampicillin, while 97.4%, 56.4%, and 36% showed resistance to cefotaxime, ciprofloxacine, and azitromycine, respectively. Not all isolates that showed phenotypic resistance were found to be carrying the corresponding gene. The most prevalent resistant genes were *gyrA*, *tetA*, *sul2*, and *tetB*, which were present in 61.5%, 64%, 54%, and 49% of the isolates, respectively. The results clearly indicate that, besides their resistance to multiple antimicrobials, the commensal *E. coli* isolates did not necessarily carry any genes conferring resistance to that particular antimicrobial.

Keywords: ESBL; AmpC; commensal *E. coli*; MIC; resistance; dairy cow; feces

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Citation: Kerluku, M.; Ratkova Manovska, M.; Prodanov, M.; Stojanovska-Dimzoska, B.; Hajrulai-Musliu, Z.; Jankuloski, D.; Blagoevska, K. Phenotypic and Genotypic Analysis of Antimicrobial Resistance of Commensal *Escherichia coli* from Dairy Cows' Feces. *Processes* **2023**, *11*, 1929. https://doi.org/10.3390/pr11071929

Academic Editors: Elisa Gamalero and Luigi Piga

Received: 31 January 2023
Revised: 19 June 2023
Accepted: 23 June 2023
Published: 26 June 2023

1. Introduction

Antibiotics are imperative for human and animal life, care, and health management due to their widespread use to treat various infectious diseases. Concerning their use in food-producing animals, despite the EC Regulation 1831/2003 [1] that bans the use of antibiotics as growth promotors, in some of the developing countries in Europe, like R.N. Macedonia, antibiotic misuse is still present. Exposure to antibiotics could spontaneously lead to mutations in bacteria, which might occur in genes that are directly involved in antibiotic resistance or in genes that regulate the expression of resistance genes. These mutations can result in the acquisition, amplification, or alteration of existing resistance genes, or they can lead to the development of entirely new resistance genes. The ability to evade the effects of antibiotic therapy by pathogenic bacteria is very important in the medical field, but in general, even non-pathogenic bacteria can develop resistance in different environments. [2]. The development and spread of AMR, facilitated by mobile genetic elements like plasmids, pose a significant global problem for both human and animal health [3]. This creates the potential for antimicrobial-resistant bacteria or their ARGs to be transmitted between animals and humans through direct contact or environmental contamination.

Therefore, due to the frequent and extensive administration of antimicrobials, livestock and their surrounding environment have emerged as significant reservoirs of ARGs [4]. It is noteworthy that commensal and environmental microorganisms, which would typically be susceptible to antimicrobials, can acquire ARGs from resistant bacteria, thus contributing to AMR dissemination throughout the food chain [5].

Commensal *E. coli* is the most frequently used indicator bacteria for addressing the spread of antibiotic resistance in various habitats and host species and is a frequent carrier of various antibiotic resistance genes [6]. The emergence of antibacterial resistance in *E. coli* and other bacteria is multifactorial, and data show that *E. coli* presents the highest rates of resistance against different antibiotics that have been in use for the longest time, as is evidenced by the high worldwide resistance rate against sulfonamides [7]. Additionally, the extended-spectrum β-lactamase (ESBL) strains appear to be most prevalent in the food animal industry and in animal feed, soil, drinking water, vegetables, and crops, as well as in health care settings, particularly intensive care units—all of which pose a serious threat to public health [8].

The dairy cattle microbiome is rich with commensal bacteria, many of which are potential carriers of ARGs, acting as an antimicrobial-resistant gene pool. Studies on the molecular characterization of antibiotic-resistant genes in commensal *E. coli* isolated from healthy dairy cows or clinical isolates reveal a high incidence of resistant determinants, which reflects the inappropriate use of clinically important antibiotics. Penicillin, cephalosporins, tetracyclines, sulfonamides, aminoglycosides, phenicol, and macrolides are examples of antimicrobials included in this group. The most common resistant genes in commensal *E. coli* found in livestock include those for ampicillin (*bla*SHV, *bla*CMY, and *bla*TEM-1B), tetracyclines (*tetA* and *tetB*), co-trimoxazole (sulfamethoxazole (*sul1*, *sul2*, and *sul3*) + trimethoprim (*dfrA1* and *dfrA17*)), aminoglycosides (*aph(3″)-Ia*, *aph(6)-Id*, and *aac(3)-IV*), and fuoroquinolones (*qnrA* and *aac(6′)-Ib-cr*) [9,10]. A highly drug-resistant *E. coli* strain obtained from veal calves primarily possesses the genes *bla*CMY-2, *blaCTX-M*, *mph*(A), *erm*(B), *aac (6′) Ib-cr*, and *qnrS1*, which confer resistance to AmpC, macrolides, aminoglycosides, and quinolones [11].

Antimicrobial-resistant bacteria (ARBs) carrying antimicrobial resistance genes (ARGs) can be introduced into the environment through animal feces. Feces, either directly or indirectly, contribute to the dissemination of ARGs in the environment, thereby posing a potential risk of transmission to humans. Animal fecal bacteria communities serve as extensive reservoirs of ARGs, which can be found in both commensal and pathogenic bacteria affecting humans [5]. Investigations into the diversity and prevalence of drug-resistant genes in the intestinal bacterial communities of animals reveal that if ARGs are transferred and become widespread in bacteria, including those capable of causing human infections, it becomes exceedingly challenging to prevent and control bacterial diseases in animals.

Therefore, it is crucial to conduct research on antibiotic resistance in animal feces to effectively prevent and control bacterial diseases, develop strategies to impede the transfer of drug resistance in bacteria, and guide appropriate clinical drug usage. These efforts hold significant importance for public health and food safety. For that purpose, the focus of this paper is to screen the prevalence of commensal *E. coli*, isolated from dairy cows' feces, that exhibits phenotypic and genotypic antibiotic resistance.

2. Materials and Methods

2.1. Farm Selection and Sample Collection

In the period between June 2019 and February 2020, 159 fecal samples were collected from 34 farms located in the Municipality of Debar, R. North Macedonia. The farms were selected randomly from a list of dairy cattle herders provided by the local authorities. These were small-scale dairy farms, each consisting of a maximum of 20 animals. Prior to the sample collection, the farmers were informed in detail about the study's purpose and the protocol for collecting samples, and verbal consent was obtained from them. The animals

were not subjected to any treatment that would affect their welfare, and thus, the study did not require approval from The Ethics Committee for Animal Experimentation.

Freshly voided feces samples were collected from each animal using clean and sterile spoons and stored in sterile cups covered with lids. The samples were maintained in a portable fridge to preserve the cold chain (storage temperature of 3 °C to 5 °C) during transportation to the Laboratory for Microbiology at the Faculty of Veterinary Medicine in Skopje.

2.2. Identification of Commensal E. coli

In the initial stage, 1 g of fecal sample was mixed with Buffered Peptone Water (BPW) (Merck, Darmstadt, Germany) in a 1:10 ratio and incubated at 37 ± 1 °C for 18 to 22 h. Then, a loop full (10 µL loop) of the enriched sample was cultured on TBX agar (Merck, Darmstadt, Germany). After 24 h of incubation at 44 °C, a typical *E. coli* green colony from the TBX plate was subcultured on Nutrient agar (Oxoid, Hampshire, UK) and incubated for 24 h at 37 °C. After incubation, colonies were used to confirm the presence of commensal *E. coli* with biochemical assays such as indole and oxidase tests (Oxoid, Hampshire, UK) and the VITEK 2 Compact System (BioMérieux, Craponne, France).

2.3. Isolation and Identification of Presumptive ESBL/AmpC- and Carbapenemase-Producing Commensal E. coli

Presumptive ESBL, AmpC, and carbapenemase-producing *Escherichia coli* were detected and isolated (EURL-AMR) according to the EU Reference Laboratory for Antimicrobial Resistance's protocol [12]. For that purpose, each isolate of commensal *E. coli* from TBX agar and a loop full of the enriched samples were subcultured onto on MacConkey agar (Oxoid, Hampshire, UK) with 1 mg/L of cefotaxime (CTX) and then incubated at 44 ± 0.5 °C for 18–22 h. Based on the color and morphology of the colony, presumptive ESBL/AmpC-producing *E. coli* MacConkey plates appeared as red/purple colonies. For detection of carbapenemase-producing commensal *E. coli*, including strains producing OXA48 and OXA-48-like carbapenemases, commercial bi-plate selective chromogenic medium (Chromid Carba Smart; BioMérieux, Craponne, France) was used, following the same method as previously mentioned [12].

2.4. Antimicrobial Susceptibility Test (AST) Determination by Broth Microdilution

MICs were determined using broth microdilution, as recommended by the EU Reference Laboratory for Antimicrobial Resistance's protocol and Commission Decision 2013/652/EU) [13]. For the phenotypic categorization of presumptive ESBL, AmpC, and carbapenemase producers, isolates were tested using two Sensititre susceptibility panels (Thermo Fisher Scientific, USA). The first panel, EUVSEC1, contained 14 antimicrobial agents belonging to 10 classes [Ampicillin (AMP), Azithromycin (AZI), Cefotaxime (FOT), Chloramphenicol (CHL), Ciprofloxacin (CIP), Colistin (COL), Gentamicin (GEN), Meropenem (MERO), Nalidixic acid (NAL), Sulphametoxasole (SMX), Ceftazidime (TAZ), Tetracycline (TET), Tigecycline (TGC), Trimethoprim (TMP)]. The second panel, EUVSEC2, contained 10 antimicrobial substances: Cefoxitin (FOX), Cefotaxime (FOT), and Ceftazidime (TAZ) with and without Clavulonic acid (CLV) each, Imipenem (IMP), Meropenem (MERO), Ertapenem (ETP) and Temocillin (TRM). To define resistance to the tested antibiotic, MIC findings were interpreted using epidemiological cutoff values (ECOFFs) published by the European Committee for Antimicrobial Susceptibility Testing (EUCAST). Clavulanic acid was used to test the synergy, essential for phenotypic categorization of ESBL and/or AmpC production.

A few colonies from pure overnight cultures from every strain, after incubation of 24 h at 37 °C on nonselective nutrient agar, were suspended in 4 mL of sterile saline, and the suspension was adjusted to 0.5 McFarland. A total of 10 µL of the suspension was transferred into 10 mL cation-adjusted Mueller–Hinton broth (Oxoid, UK) to obtain an inoculum of 1×10^5 CFU/mL, and then 50 µL of inoculated broth was transferred into each

well of the plates. The plates had to be inoculated within 30 min of standardization of the inoculum suspension to maintain a viable cell number concentration. EUVSEC plates were sealed with a commercially available top and finally incubated under aerobic conditions at 37 ± 1 °C for 18 h. MICs for every substance were defined based on the first well that has no visible pallets or growth and then interpreted according to the ECOFF by EUCAST.

A MIC of 16 mg/L was considered aa a reference for azithromycin resistance in wild-type isolates (no cutoff was set by EUCAST), as proposed for *Salmonella* spp. [14,15]. In this context, the phrases "susceptible" and "resistant" refer to isolates that lack (wild type) and have phenotypically expressed resistance mechanisms, respectively. In conclusion, an ESBL phenotype was determined if isolates were resistant to FOT (>1 mg/L) or TAZ (>1 mg/L) but susceptible to FOX (8 mg/L) and demonstrated clavulanic acid synergy with FOT and/or TAZ (more than a 2-fold reduction in the MIC combined with 4 mg/L CLV compared to the MIC of the cephalosporin alone). If there was no clavulanic acid synergy and the isolates were resistant to FOT or TAZ (>1 mg/L) and FOX (>8 mg/L), they were categorized as having the AmpC phenotype. If isolates were resistant to FOT (1 mg/L) or TAZ (>1 mg/L), resistant to FOX (>8 mg/L) and demonstrated clavulanic acid synergy with FOT and/or TAZ, an ESBL/AmpC phenotype was assigned. Meropenem resistance (>0.12 mg/L) was used to infer a carbapenemase-producing phenotype.

2.5. Detection of Antibiotic Resistance Genes of Commensal E. coli Isolates

Isolates that showed phenotypic resistance were tested for the presence of antimicrobial-resistant genes. For that purpose, a conventional PCR followed by gel electrophoresis was used to determine the presence of genes encoding resistance to β-lactams (*blaCTX-M*, *bla*TEM, *bla*SHV), tetracycline (*tetA*, *tetB*, *tetC*), trimethoprim (*dhfr1*, *dhfr5*, *dhfr12*, and *dhfr13*), ciprofloxacin (*gyrA*), azitromycin (*mphA*), sulfisoxazole (*sul1*, *sul2*), nalidixic acid (*qnrA*), chloramphenicol (*cmlA*), gentamicin (*aac(3)-IV*), colistin (*mcr-1*), and tigecyclin (*tet (X3)*) (Table 1).

Table 1. List of primers used in this study to detect antibiotic-resistant genes of *E. coli* isolated from fecal materials of dairy cattle.

Antimicrobial(s) /Integron	Target Gene	F Primer Sequence (5′ to 3′)	R Primer Sequence (5′ to 3′)	Amplicon Size (bp)	Ref.
β-lactam	*blaCTX-M*	CACACGTGGAATTTAGGGACT	GAATGAGTTTCCCCATTCCGT	970	[16]
	blaTEM	TTCTTGAAGACGAAAGGGC	ACGCTCAGTGGAACGAAAAC	1150	
	blaSHV	TTATCTCCCTGTTAGCCACC	GATTTGCTGATTTCGCTCGG	796	
	blaOXA1	TGAAAAACACAATACATATCAACTTCGC	GTGTGTTTAAATGGTGATCGCATT	820	
	blaOXA2	ACGAT AGTGGTGAGTATCCGACAG	ATCTGTTTGGCGTATCRATATTC	601	
	blaVIM1	AGTGGTGAGTATCCGACAG	ATGAAAGTGCGTGGAGAC	261	
tetracycline	*tetA*	GCGCCTTTCCTTTGGGTTCT	CCACCCGTTCCACGTTGTTA	831	
	tetB	CCCAGTGCTGTTGTTGTCAT	CCACCACCAGCCAATAAAAT	723	[16]
	tetC	TTGCGGGATATCGTCCATTC	CATGCCAACCCGTTCCATGT	1019	
trimethoprim	*dhfr1*	CGGTCGTAACACGTTCAAGT	CTGGGGATTTCAGGAAAGTA	220	
	dhfr5	CTGCAAAAGCGAAAAACGG	AGCAATAGTTAATGTTTGAGCTAAAG	432	
	dhfr12	AAATTCCGGGTGAGCAGAAG	CCCGTTGACGGAATGGTTAG	429	[16]
	dhfr13	GCAGTCGCCCTAAAACAAAG	GATACGTGTGACAGCGTTGA	294	
sulfisoxazole	*sul1*	TCACCGAGGACTCCTTCTTC	CAGTCCGCCTCAGCAATATC	331	[16]
	sul2	CCTGTTTCGTCCGACACAGA	GAAGCGCAGCCGCAATTCAT	435	
azithromycin	*mph*(A)	GTGAGGAGGAGCTTCGCGAG	TGCCGCAGGACTCGGAGGTC	403	[16]
ciprofloxacin	*gyrA*	CGACCTTGCGAGAGAAAT	GTTCCATCAGCCCTTCAA	626	[16]
nalidixic acid	*qnrA*	GGGTATGGATATTATTGATAAAG	CTAATCCGGCAGCACTATTA	660	[17]
chloramphenicol	*cmlA*	TAC TCG GAT CCA TGC TGG CC	TCC TCG AAG AGC GCC ATT GG	578	[18]
gentamicin	aac(3)-IV	AGTTGACCCAGGGCTGTCGC	GTGTGCTGCTGGTCCACA GC	627	[19]
colistin	*mcr-1*	AGTCCGTTTGTTCTTGTGGC	AGATCCTTGGTCTCGGCTTG	320	[20]
tigecyclin	*tet (X3)*	GTGGATGCTTTGCTATTGTCTGA	TCTGTTGATTCGTCCTGCGTAT	125	[21]

2.5.1. DNA Extraction

Genomic bacterial DNA was isolated from a fresh culture of a pure bacterial isolate on nonselective TSA agar (Oxoid, Hampshire, UK). A loop full (10 µL) of colonies was suspended in 990 µL of DNase- and Rnase free water. The bacterial suspension was then incubated for 15 min at 100 °C without shaking in a thermoblock (MRC, Holon, Israel). The resulting thermolysate was then centrifuged (Hettich, Tuttlingen, Germany) for five minutes at 18,000× *g*, and the supernatant was used for further research.

2.5.2. PCR Protocol

The PCR protocol used included initial denaturation at 95 °C for 15 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 61 °C for 1 min, and elongation at 72 °C for 1 min, with a final extension at 72 °C for 10 min [16]. All PCR experiments included both negative and positive controls. Amplified PCR products were analyzed with electrophoresis using a 2% agarose gel. Additionally, the gel was stained with ethidium bromide and visualized with the Gel Doc XR+ molecular imager (BioRad, Hercules, CA, USA).

3. Results

3.1. Identification of Presumptive ESBL/AmpC and Carbapenemase Producing Commensal E. coli

In all 159 examined fecal samples, the presence of commensal *E. coli* was determined, which was confirmed using the VITEK 2 Compact System.

The results of inoculation of selective MacConkey agar with Cefotaxime showed the presence of 39 (24.52%) presumptive ESBL/AmpC producing strains of *E. coli*, which belonged to 11 (32.3%) farms. As for the presence of carbapenemases-producing strains, tested on a bi-plate selective chromogenic medium, none of the 34 farms were isolated. The resistance percentage on farms where presumptive ESBL/AmpC producing strains of *E. coli* were confirmed ranged from 20% to 100% (Figure 1). On more than half of the farms, the percentage of presumed ESBL/AmpC producing *E. coli* strains varied between 45% and 60%.

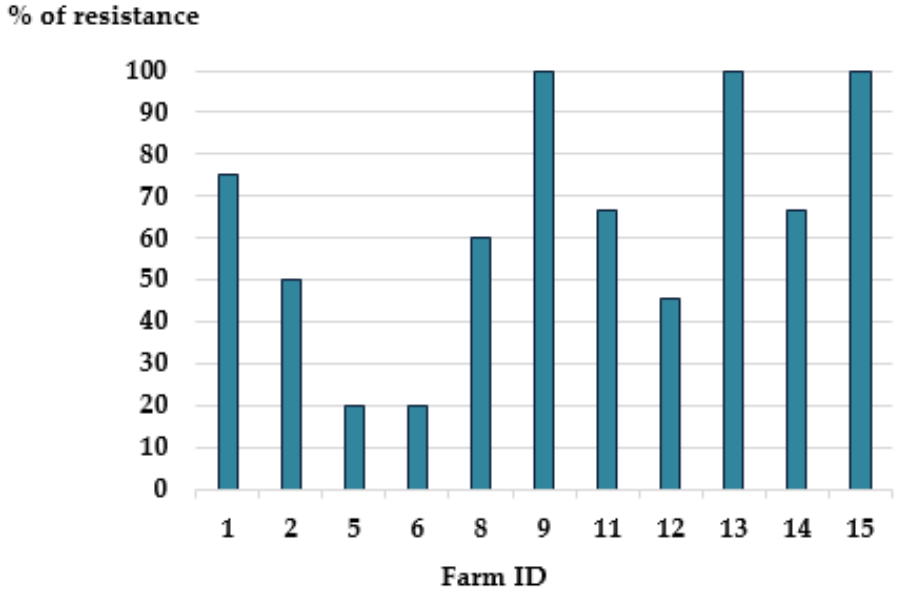

Figure 1. Percentage of resistant commensal *E. coli* isolates per farm recovered from fecal samples obtained from dairy cows.

All strains that showed cephalosporin resistance on the McConkey+Cefotaxime plate, were subjected to phenotypic resistance determination using the broth microdilution method. After 18 h of incubation on the first EUVSEC1 panel, all 39 isolates (100%) showed

resistance to ampicillin and 38 isolates to cefotaxime and ceftazidime. The lowest resistance (18%) was observed to gentamicin in 7 isolates. However, all the isolates were found to be susceptible to colistin (COL), meropenem (MERO), and tigecycline (TGC). Figure 2 shows the overall resistance of all isolates to each antimicrobial agent.

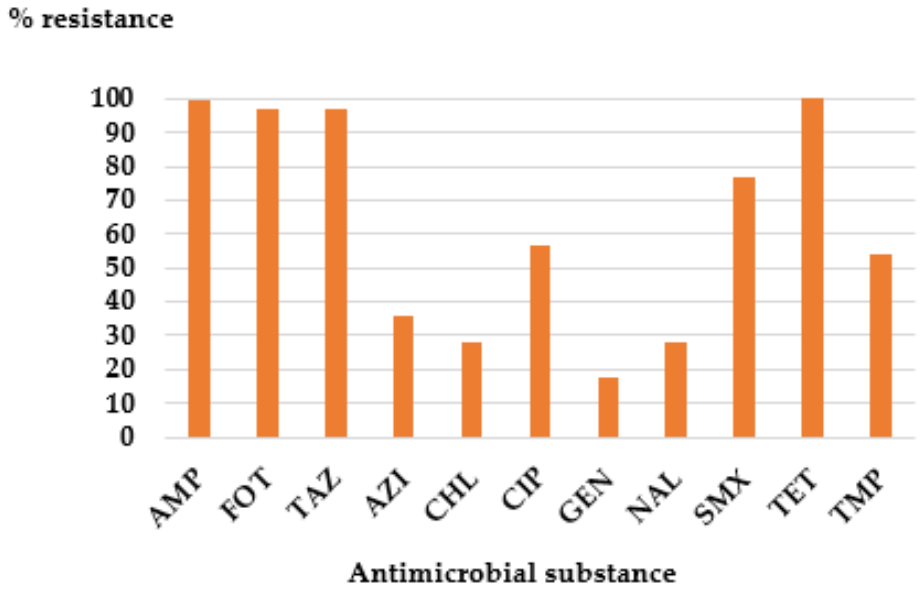

Figure 2. The overall resistance of all commensal *E. coli* isolates to antimicrobial substances from the EUVSEC1 panel.

Concerning the MIC values, the isolates showed resistance to several concentrations above the ECOFF value for 8 antimicrobial substances. For example, for ciprofloxacin (CIP), 22 (56,39%) isolates showed resistance to 5 different concentrations that are above the ECOFF value: 1 (2.56%) at 0.125 mg/L, 7 (17.94%) at 0.25 mg/L, 5 (12.82%) at 0.5 mg/L, 1 (2.56%) at 1 mg/L and 8 (20.51%) isolates ≥8 mg/L, which is the maximal MIC value. The resistance to ceftazidime (TAZ) included 4 different concentrations, 14 (35.89%) isolates showed resistance to the highest MIC value, which is ≥8 mg/L, 6 (15.38%) at the lowest 1 mg/L and 8 (20.51%) and 10 (25.64%) isolates at 2 mg /L and 4 mg/L, respectively. The MIC distribution for all antimicrobial substances for all isolates tested is given in Table 2.

3.2. Categorization of the Isolates

The isolates of presumptive ESBL and/or AmpC producing commensal *E. coli* that showed MIC values for FOT and/or TAZ > 1 mg/L on EUVSEC1 panel, were further tested on the EUVSEC2 panel. Based on the MIC values of the second panel, the strain's phenotype was categorized as follows: 37 (94.87%) were ESBL phenotypes, 1 (2.56%) was an ESBL/AmpC phenotype, and 1 (2.56%) belonged to the "other phenotypic" group.

According to EFSA, multidrug resistance (MDR) refers to the ability of bacteria isolates to resist the effects of antibiotics belonging to three or more classes of antimicrobial agents. The study found that a significant percentage of these bacteria were resistant to multiple antibiotics belonging to three or more classes of antimicrobial agents. The resistance patterns were categorized into two groups, with one group showing resistance to drugs from one or two generations, and the other group showing resistance to drugs from three to seven generations. Among the 29 (74.35%) MDR isolates distributed on 9 (81.82%) farms, 37.9% showed resistance to four classes of antimicrobial agents, while 27.5% showed resistance to seven classes of antimicrobial agents (Table 3). Only two farms (18.18%) had no MDR isolates.

Table 2. MIC distribution of 14 antimicrobial substances from the EUVSEC1 commercial plate for all commensal *E. coli* isolates. The number of resistant isolates is given in percent as well for each antimicrobial.

	Antimicrobial Concentration in mg/L																
AMS	0.015	0.03	0.064	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024
AMP													39 (100)				
AZI								5 (12.8)	5 (12)	8 (20.5)	7 (18)	2 (5.1)	12 (30.7)				
FOT					1 (2.5)				38 (97.4)								
CHL										27 (69.2)	1 (2.5)		1 (2.5)	10 (25.6)			
CIP	13 (33.3)	4 (10.2)		1 (2.56)	7 (17.9)	5 (12.8)	1 (2.56)			8 (20.5)							
COL							39 (100)										
GEN						3 (7.7)	18 (46.1)	11 (28.2)			1 (2.56)	6 (15.3)					
MERO		39 (100)															
NAL									21 (53.8)	6 (15.38)	1 (2.5)	1 (2.5)		10 (25.6)			
SMX											3 (7.7)	4 (10.2)	2 (5.1)	1 (2.5)			29 (4.3)
TAZ						1 (2.56)	6 (15.3)	8 (20.5)	10 (25.6)	14 (35.8)							
TET								10 (25.6)	1 (2.5)		1 (2.5)	2 (5.1)	25 (64.1)				
TGC					26 (66.6)	13 (33.3)											
TMP					13 (33.3)	4 (10.2)		1 (2.5)				21 (53.8)					

EUVSEC1: Ampicillin (AMP) 1–64 mg/L, ECOFF > 8 mg/L; Azithromycin (AZI) 2–64 mg/L, ECOFF > 16 mg/L, Cefotaxime (FOT) 0.25–4 mg/L, ECOFF > 0.25 mg/L; Chloramphenicol (CHL) 8–128 mg/L, ECOFF > 16 mg/L, Ciprofloxacin (CIP) 0.015–8 mg/L, ECOFF > 0.064 mg/L; Colistin (COL) 1–16 mg/L, ECOFF > 2 mg/L; Gentamicin GEN) 0.5–32 mg/L ECOFF > 2 mg/L; Meropenem (MERO) 0.03–16 mg/L ECOFF > 0.125 mg/L; Nalidixic acid (NAL) 4–128 mg/L ECOFF > 16 mg/L; Sulphametoxasole (SMX) 8–1024 mg/L, ECOFF > 64 mg/L; Ceftazidime (TAZ) 0.5–8 mg/L, ECOFF > 0.5 mg/L; Tetracycline (TET) 2–64 mg/L, ECOFF > 8 mg/L; Tigecycline (TGC) 0.25–8 mg/L, ECOFF > 1 mg/L; Trimethoprim (TMP) 0.25–32 mg/L, ECOFF > 2 mg/L. For each antimicrobial agent, white fields represent the range of dilutions tested. Vertical lines represent epidemiological cutoff (ECOFF) values established by the European Committee for Antimicrobial Susceptibility Testing (EUCAST).

Table 3. Multidrug-resistant vs. resistant commensal *E. coli* isolates recovered from fecal samples obtained from dairy cows.

No.	Isolates	Total Number of Isolates	% of Resistance for Each Group of Isolates	No. of Generations	Resistant vs. MDR
1.	4	10	40	I	25.64
2.	6		60	II	
3.	2	29	6.9	III	74.35
4.	11		37.9	IV	
5.	7		24.1	V	
6.	1		3.4	VI	
7.	8		27.5	VII	

Concerning the distribution of MDR isolates per farm, it was noticed that more than half of the farms 6 (66.66 %) had isolates that showed 100% resistance to more than three classes of antimicrobial agents (Figure 3).

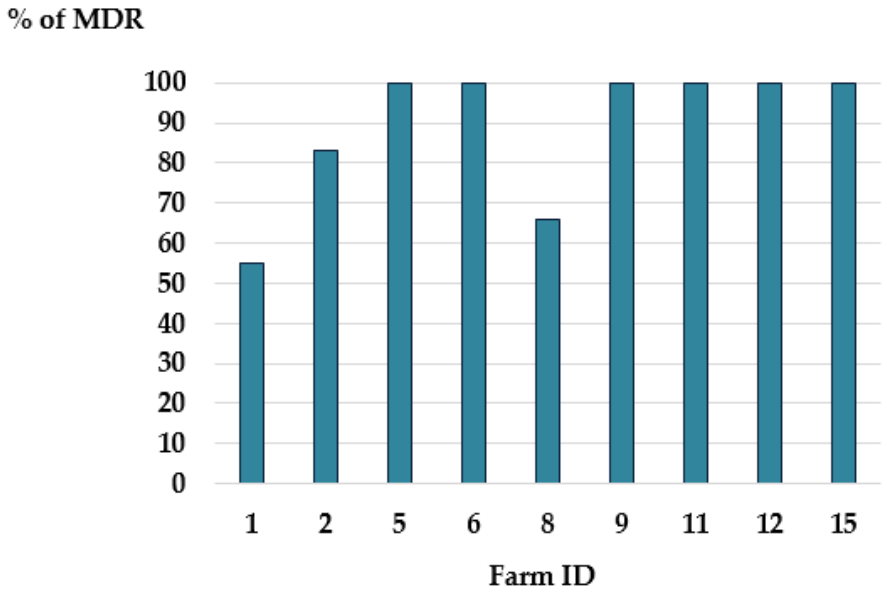

Figure 3. Percentage of MDR commensal *E. coli* isolates recovered from fecal samples obtained from dairy cows, per farm.

3.3. Phenotypic Resistance Profiles of the Isolates

The analysis of the EUVSEC1 plate, revealed the existence of 18 different phenotypic resistance profiles, where isolates showed resistance to three to a maximum of ten antimicrobial substances (Figure 4). The number of isolates showing a phenotypic resistance profile to each of the 18 different profiles varied from one to six, where nine (50 %) profiles were represented with only one isolate per profile. Six (15.38 %) isolates carried the only phenotypic resistance profile with ten antimicrobial substances (AMP, FOT, TAZ, AZI, CHL, CIP, NAL, SMX, TET, and TMP).

AMP-FOT-TAZ is the most prevalent antimicrobial agent combination, present in almost all isolates (97.4%), followed by AMP-SMX, which appears in 30 (77%) of the isolates.

3.4. Detection of Antimicrobial Resistant Genes with Conventional PCR

Across all isolates, out of the 19 ARGs tested, 18 (94.7%) were found to be present, carrying resistance to tetracyclines, sulphonamides, fluoroquinolones, β-lactams aminoglycosides, macrolides, folate synthesis inhibitors, and phenicols (Table 4). None of the isolates was found to carry *bla*OXA2 gene.

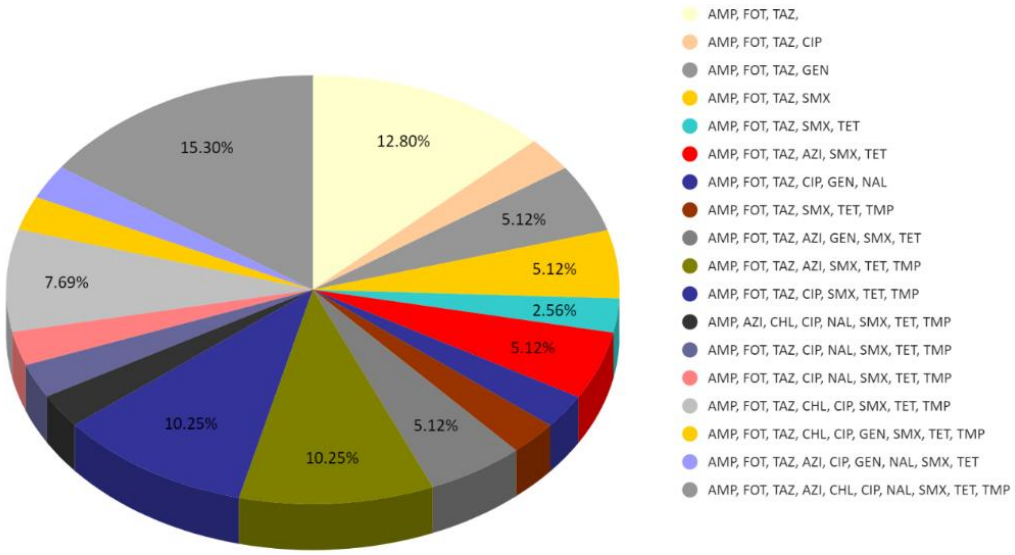

Figure 4. Phenotypic resistance profiles of commensal *E. coli* isolates recovered from fecal samples obtained from dairy cows.

Table 4. Prevalence of resistant genes.

Resistant Genes	Antimicrobial Substance	Class	Number (%) of Isolates
sul1 *sul2*	sulfisoxazole	sulphonamides	8 (20.51%) 21 (53.84%)
tetA *tetB* *tetC*	tetracycline	tetracyclines	28 (71.79%) 20 (51.28%) 1 (2.56%)
dhfr1 *dhfr5* *dhfr12* *dhfr13*	trimethoprim	folic acid blocators	9 (23.07%) 4 (10.25%) 5 (12.82%) 3 (7.07%)
gyrA	ciprofloxacin	fluoroquinolones	27 (69.23%)
qnrA	nalidixic acid	quinolones	15 (38.46%)
cmlA	chloramphenicol	phenicoles	14 (35.89%)
aac (3)- IV	gentamicin	aminoglycosides	7 (17.94%)
mphA	azithromycin	macrolides	12 (30.76%)

PCR results showed the presence of at least one resistance gene in each isolate. The incidence of the examined genes varied between isolates. The most prevalent resistant gene was *tetA* (28/39) 71.79%, followed by *gyrA* (27/39) 69.23%; *sul2* (21/39) 53.84%; *tetB* (20/39) 51.28%, which confer resistance to tetracyclines, fluoroquinolones, and sulphonamides. Sulfamethoxazole-resistant genes *sul1* and *sul*2 were identified among 22 isolates, with *sul1* present in 8 (20.51%) isolates and *sul2* present in 21 (53.84%) isolates. Seven (31.81%) isolates harbored both *sul1* and *sul2*. Tetracycline-resistant genes *tetA*, *tetB*, and *tetC* were identified among 32 (82.05%) isolates, where 16 (50%) of the isolates carried both *tetA* and *tetB* and one isolate (3.12%) had both *tetA* and *tetC*. Trimethoprim-resistant genes *dhfr1* and *dhfr5* were identified in 3 (7.69%) tetracycline-resistant isolates, with *dhfr1* present in two (5.12%) of the isolates and *dhfr5* in one (2.56%) isolate.

Depending on the number of resistant genes detected per isolate, seven different groups emerged, carrying between 3 and 9 genes. Ten (25.64%) of the isolates carried

6 genes, while the maximal number of 9 genes was present in only three (7.69%) of the isolates. The different ARG combination profiles are given in (Table 5).

Table 5. AMG combination profiles of commensal *E. coli* isolates from dairy cows' feces.

Number of Genes	Genotype Profile	Number of Antimicrobial Classes	Farm ID
3	*gyrA, CTX-M, TEM*	2	1
	sul2, tetA, gyrA	3	2
	gyrA, CTX-M, TEM	2	1
	sul2, aac(3)-IV, CTX-M	3	10
4	*gyrA, aac(3)-IV, CTX-M, TEM*	3	10
	tetA, gyrA, CTX-M, TEM	3	1
	tetA, gyrA, cmlA, TEM	4	1
	sul2, tetA, gyrA, TEM	4	2
	tetA, tetB, dhfr12, CTX-M	3	11
5	*tetB, qnrA, cmlA, CTX-M, TEM*	4	5
	sul1, sul2, gyrA, CTX-M, TEM	3	2
	sul2, tetA, tetB, gyrA, TEM	4	1
	sul2, tetA, tetB, gyrA, CTX-M	4	6
	sul2, tetA, gyrA, CTX-M, TEM	4	1
6	*tetA, tetB, dhfr12, qnrA, cmlA, CTX-M*	5	7
	sul2, dhfr1, dhfr12, gyrA, mphA, CTX-M	5	5
	su1l, sul2, dhfr1, dhfr12, CTX-M, TEM	3	1
	tetA, tetB, dhfr12, cmlA, qnrA, TEM	5	7
	tetA, tetB, gyrA, qnrA, aac(3)-IV, TEM	5	1
	teteA, tetB, gyrA, qnrA, cmlA, CTX-M	5	5
	tetA, gyrA, qnrA, cmlA, CTX-M, TEM	5	5
	tetA, dhfr1, dhfr13, qnrA, mphA, TEM	5	6
	tetA, dhfr1, gyrA, mphA, CTX-M, TEM	5	9
	sul2, tetB, mphA, CTX-M, TEM, OXA1	4	4
7	*sul1, sul2, tetB, gyrA, mphA, CTX-M, TEM*	5	8
	sul1, sul2, tetA, dhfr5, gyrA, CTX-M, TEM	5	2
	tetA, dhfr1, dhfr13, gyrA, qnrA, cmla, CTX-M	6	7
	sul2, tetA, tetB, dhfr1, gyrA, mphA, TEM	6	8
	sul2, tetA, tetB, gyrA, mphA, CTX-M, TEM	5	6
	tetA, tetB, dhfr5, qnrA, cmlA, mphA, CTX-M	6	1
	sul2, tetA, tetB, gyrA, aac(3)-IV, mphA, CTX-M	6	6
8	*sul1, sul2, tetA, tetB, qnrA, cmlA, CTX-M, TEM*	5	7
	sul1, tetA, tetB, dhfr5, cmlA, mphA, CTX-M, TEM	6	5
	sul2, tetA, tetB, qnrA, cmlA, mphA, CTX-M, OXA1	6	7
	sul1, sul2, tetA, tetB, dhfr1, gyrA, CTX-M, TEM	5	5
	sul2, tetA, dhfr1, gyrA, aac(3)-IV, mphA, CTX-M, TEM	6	6
9	*sul2, tetA, tetC, dhfr1, gyrA, cmlA, aac (3)-IV, CTX-M, TEM*	7	2
	tetA, tetB, dhfr5, gyrA, cmlA, qnrA, aac(3)-IV, TEM, SHV	6	2
	sul1, sul2, tetB, dhfr13, gyrA, qnrA, cmlA, CTX-M, TEM	7	3

4. Discussion

Besides the differences in sampling strategies and isolation methods among studies, which complicate comparisons, reported data on the prevalence rates of ESBL/AmpC and carbapenemase-producing *E. coli* in food-producing animals not only vary by country and animal species but also depend on the hosts and antimicrobial substances used [22]. Our results regarding the phenotypic categorization of the isolates show a percentage that is consistent and within ranges reported by different European countries. In our study, out of 24.52% of presumptive ESBL/AmpC isolates, 94.87% were categorized as ESBL, while 2.56% had the AmpC phenotype. In Europe, prevalence in veal calves under 1 year of age

ranged from 7.1% in Denmark to 89 % in Italy (in the European Union, average 44.5%) in 2017 [23].

This study examined 39 *E. coli* isolates, and out of the 22 analyzed genes, a total of 19 genes were detected and identified as responsible for conferring resistance to the tested antimicrobials. The results from this study for the prevalence of β-lactam coding genes have previously been described [24]. Tetracycline resistance is very common in resistant strains isolated from dairy cattle and farm environments, as is the prevalence of genes conferring resistance to tetracycline [25]. The most commonly identified resistance genes in this study were *tetA* (71.79%) and *gyrA* (69.23%), which confer resistance to tetracycline and fluoroquinolones, respectively. This finding is not surprising considering the extensive use of tetracycline as a widely prescribed antibiotic in R. North Macedonia. In a previous study by Navajas-Benito et al. [26], a higher number of tetracycline resistance genes (73.33%) was also reported in *E. coli* associated with dairy farms. Similar results to ours (76%) for the *gyrA* gene prevalence were found in *E. coli* isolated from calf diarrhea [27]. National drug resistance statistics from Japan in 2018 indicate an average tetracycline resistance rate of 26.5% in *E. coli* from healthy cattle on livestock farms [28]. However, various studies have reported high rates of tetracycline resistance ranging from 33.3% to 93% in tetracycline-resistant *E. coli* isolated from dairy cows in farms across Asia, the UK, and the USA [25,29,30]. Furthermore, a study conducted on small-scale dairy cattle revealed a tetracycline resistance rate of 50.4% among commensal *E. coli* isolates [31]. Discrepancies between these studies may be attributed to differences in sampling methods, the presence of infectious diseases, varying pathogenicity, different treatment protocols, geographical locations, laboratory techniques, interpretative criteria, or the inherent resistance characteristics of the isolates, which can be influenced by the widespread use of tetracycline. The high rates of tetracycline resistance are primarily attributed to its continued extensive use in human medicine and animal husbandry due to its affordability, oral administration, and minimal side effects [32].

The resistance exhibited by bacteria against third generation cephalosporins and other β-lactam antibiotics is a significant concern for both animal and human health. Since their initial discovery in 1983 [33], extended-spectrum β-lactamases (ESBLs) have garnered considerable attention from the scientific and medical communities. Our findings demonstrate a remarkably high prevalence of resistance against third generation cephalosporins and other β-lactam antibiotics across all farms. All isolates showed resistance to ampicillin, with a range of 97.43% to 100% exhibiting resistance to third- and fourth generation cephalosporins such as cefotaxime, ceftazidime, and cefepime, respectively. Similarly, a study conducted in Tanzania reported a high rate of resistance among commensal *E. coli*, with resistance rates of 96.7% for ampicillin and 95% for cefotaxime [31]. Data from other authors regarding this particular resistance are also absent from the study of healthy cattle, pigs, and chickens—0.6%, 1.2%, and 3.3%, respectively [22]. Unlike the research data mentioned above, our isolates confer a very high rate of resistance toward third- and fourth generation cephalosporins such as cefotaxime, ceftazidime, and cefepime—97.43%, 97.43%, and 100%, respectively. This situation indicates that dairy cattle farms in this territory should be considered potential reservoirs for the emergence and dissemination of cephalosporin resistance. The fact that cephalosporins are known as the last line of antimicrobials fighting against severely infectious diseases in human medicine, should not be neglected.

Gentamicin, an older member of the aminoglycoside class, exhibited interesting findings in our study. We observed that 18% of the resistant isolates did not demonstrate high levels of resistance. Similar results for gentamicin (23.7%) were obtained in *E. coli* isolates from healthy cattle and sheep in Northern Spain [22]. Concerning the prevalence of the *aac(3)-IV* gene conferring resistance to gentamicin, in our study we observed the presence of the gene in 18% of the isolates. These findings differ from those in *E. coli* isolates from dairy farm manure in the USA, where the prevalence of the *aac(3)-IV* gene was 84% [34].

The prevalence of nalidixic acid-resistant *E. coli* strains was found to be 28%, indicating a moderate level of resistance. Similar results were observed in small-scale dairy cattle farms in Tanzania (33.1%) [31] and Tunisia (28.7%) [35], while data from a dairy farm in South Korea showed a low rate of resistance in commensal *E. coli* (8.2%) [36]. Our results for the phenotypic resistance to nalidixic acid (28.2%) fall in the quinolone resistance range in animals from low- and middle-income countries (20% to 60%) [37]. Contrary to the quinolone resistance, in our study we observed high resistance to ciprofloxacin (56.41%). Regarding the prevalence of the *qnrA* and *gyrA* genes, conferring resistance to fluoro(quinolones) in our study, it was 38.46% and 69.23%, respectively. These numbers differ from the ones for the phenotypic resistance towards both antimicrobials, which is probably attributed to mutations occurring in the quinolone resistance determining regions (QRDR) or due to the presence of active efflux or outer membrane permeability. Additionally, plasmid-mediated quinolone resistance (PMQR) was observed as well, as indicated by the presence of *qnrA,* and *gyrA* genes in the resistant isolates. Thus, the acquisition of quinolone resistance in these isolates was facilitated by plasmid-mediated mechanisms [38].

In both our study and the cross-sectional survey conducted by Tello et al. [22], it was found that the resistance rate to chloramphenicol was 28%. The survey by Tello et al. specifically focused on ESBL-/AmpC-producing *E. coli* isolated from dairy cattle herds.

The EUVSEC1 plates used allowed the performance of an independent antimicrobial susceptibility test for trimethoprim and sulfamethoxazole, revealing a resistance rate of 54% and 77%, respectively. In different studies, depending on their protocol design, territory, and antimicrobial targets, different results were obtained, but a similar rate for trimethoprim-sulfamethoxazole—a 42.1% rate of resistance—was found [31]. Molecular detection of the *sul1* and *sul2* genes showed a higher prevalence of *sul2* (53.84%) over the *sul1* gene (20.51%), where 6 (15.38%) carried both genes.

Due to their inherent low permeability and multidrug efflux systems, *E. coli* isolates are frequently intrinsically resistant to macrolides, though azithromycin has some activity against some Gram-negative bacteria. In our case, we detected moderate resistance toward macrolides, particularly to azithromycin (36%), compared to other studies.

Regarding the presence of carbapenem-resistant *E.coli,* in our study, not a single isolate was recovered from the carbapenem-containing medium. All isolates were susceptible to meropenem, ertapenem, and imipenem. Numerous studies have selectively screened samples for the presence of carbapenem-resistant *Enterobacteriaceae* (CRE), and a prevalence of <1% was found among livestock and companion animals in Europe, 2–26% in Africa, and 1–15% in Asia. Wildlife (gulls) in Australia and Europe carried CRE at a prevalence of 16–19% [39].

5. Conclusions

This study aimed to determine the prevalence of the phenotypic and genotypic antimicrobial resistance (AMR) profiles of commensal *E. coli* isolated from dairy cows' feces. The findings revealed that despite the official limited use of antimicrobials, there is an unexpectedly high prevalence of resistance to different antimicrobials. The most surprising fact was the high incidence of ESBL producing *E. coli* and the high prevalence of resistant genes towards tetracyclines, fluoroquinolones, and sulfonamides. The researchers hypothesized that factors other than antimicrobial treatment, such as feed, environment, farm type, and management practices, may play a role in the development and spread of AMR in *E. coli* in beef feedlot cattle.

Regular monitoring would enable timely identification of both emerging and existing forms of resistance and AMR genes in bacteria originating from food-producing animals, including those on dairy cow farms.

Author Contributions: Conceptualization, M.K., D.J., M.R.M. and K.B.; methodology, M.R.M., M.P. and B.S.-D.; investigation, M.K., MRM, B.S.-D., M.P. and Z.H.-M.; resources, Z.H.-M. and M.P.; project administration, D.J. and K.B.; writing—original draft preparation, M.K.; writing—review and editing, K.B., D.J. and B.S.-D.; supervision, D.J. and K.B.; funding acquisition, D.J. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Faculty of Veterinary Medicine—Skopje, Code FVM-IPR-01 (Decision no. 0202/2090/3, from 3 December 2019).

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: This research was part of the project financed by the Faculty of Veterinary Medicine—Skopje, Code FVM-IPR-01 (Decision no. 0202/2090/3, from 3 December 2019) with the title "Antimicrobial resistance of commensal *Escherichia coli* in Republic of Macedonia". The authors would like to thank the colleagues from the Laboratory of Food and Feed Microbiology and the Laboratory for Molecular Food Analyses and GMO at the Food Institute at the Faculty of Veterinary Medicine in Skopje for their cooperation and contribution to this study.

Conflicts of Interest: The authors declare that they have no potential conflict of interest with respect to the authorship and/or publication of this article.

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