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Dietary Pistachio Skin Effects on Antibiotic-Free Lamb: Virulence Traits, Antimicrobial Resistance, and Clonal Relatedness in Commensal *Escherichia coli* Strains

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Abstract

Background/Objectives: In food-producing animal (FPA) environments, healthy animals can act as reservoirs of potentially pathogenic *Escherichia coli*, which can be transmitted through the food chain to humans. This study aimed to evaluate cloacal *E. coli* in healthy Sicilian lambs subjected to an experimental feeding regimen by assessing bacterial levels, antimicrobial resistance, virulence traits, and the clonal relationships, as well as the impact of a pistachio skin as an agro-industrial by-product supplement during a 58-day feeding trial. **Methods**: A total of 295 *E. coli* isolates from the control (CTRL) and treatment (Treated) groups at initial time (T0) and final time (T1) were phenotypically and genotypically characterized using Kirby–Bauer antimicrobial testing, multiplex PCR for virulence genes, and PFGE for clonal analysis. **Results**: The feeding regimen did not significantly influence the prevalence, abundance, or virulence of the *E. coli* isolates. Shiga toxin-producing *E. coli* (STEC) were the most common pathotype, mainly carrying the *stx1* gene, while the Enteroinvasive (EIEC) type was detected only sporadically. Enteropathogenic *E. coli* (EPEC) predominated at T0, while enteroaggregative *E. coli* (EAEC) at T1, and enterotoxigenic *E. coli* (ETEC), initially prevalent in Treated samples, disappeared by T1. Antimicrobial resistance profiles varied among isolates, with the highest resistance observed in the CTRL group. However, both groups exhibited high resistance to streptomycin, and 9% of CTRL isolates were multidrug resistant. A notable reduction in overall resistance rates, especially in the Treated group, was observed, indicating a dietary effect on the *E. coli* resistome. PFGE genotyping showed high genetic diversity, with resistance traits more frequently detected than virulence factors. **Conclusions**: This study highlights that healthy lambs serve as reservoirs for potentially human-pathogenic *E. coli* and suggests that dietary regimes could effectively reduce antibiotic resistance.

Keywords: healthy lamb; agro-industrial by-product; diet regime; fecal samples; resistome; PFGE

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1. Introduction

Escherichia coli is one of the most common bacteria found in the intestine of mammals, including humans and other warm-blooded animals [1], and maintains a symbiotic relation-

ship with its host [2]. Although it is generally considered harmless and even beneficial as a commensal organism in the digestive system, some strains can be highly pathogenic and medically significant [3,4]. The pathogenicity of *E. coli* is often associated with the presence of virulence factors that categorize strains into various pathotypes responsible for intestinal or extraintestinal diseases (DEC, designated diarrheagenic), or carrying genes linked to antibiotic resistance [1,5,6]. Additionally, its highly adaptable genome, which involves gene gain and loss through genetic modifications, confers a strong capacity to evolve and coexist with other enteric pathogens in the same ecological niche. This allows it to colonize and persist in animal and human hosts, leading to the emergence of pathogenic strains from commensal ones [7,8]. As a model microorganism for antimicrobial resistance (AMR) in both livestock and humans, it is extensively studied to understand population dynamics in these contexts [6,9–11]. The close association of *E. coli* with Food-Producing Animals (FPAs), such as sheep, cattle, pigs, and poultry, which are important natural carriers of potentially dangerous strains, has significant implications for human infections. The bacterium can be transmitted during food handling or by consuming contaminated products [12–14]. The presence of *E. coli* in animal feces allows it to enter the food chain through fecal contamination of meat and milk during slaughter. Direct or indirect contact between animals and humans, as well as human-to-human interactions, also plays a key role in spreading infections [12,15]. Therefore, detecting virulence factors from various sources helps develop strategies for preventing and controlling the safety of animal products [1]. Among the various approaches to improve animal health and welfare, preventing disease, combating antibiotic resistance in FPAs, viewed through a circular economy perspective, has led to increased interest in adopting alternative feeding strategies [16–18]. Recently, the suitability of agro-industrial by-products, such as citrus fruit pulp [19], molasses [20], pistachios [21], and olive cake [22], has been thoroughly evaluated for inclusion in livestock diets to meet nutritional needs [23], modulate gut microbiota [24,25], and enhance microbiological, chemical, and nutritional profiles of animal-derived foods, while reducing costs [26–31]. This approach, aligned with the broader One Health initiative, calls for a comprehensive and collaborative effort across multiple sectors, emphasizing the interconnectedness of human, animal, and environmental health to promote public health, prevent infectious diseases, and support resilient, sustainable health systems [32,33]. This study aimed to assess the prevalence, phenotypic resistance profiles, presence of virulence genes, and clonal relationships of *E. coli* strains isolated from fecal samples of healthy, antibiotic-free Sicilian lambs previously involved in an in vivo feeding trial conducted by Musati and co-workers [34]. In that trial, the effects of supplementing lamb diets with 120 g/kg DM of pistachio skin on growth performance and meat quality parameters of twenty-four Valle del Belice × Comisana male lambs fed ad libitum for 60 days were evaluated. Accordingly, this study, based on the hypothesis that pistachio skin supplementation may influence gut microbiota modulation, emphasized the need to monitor the risks associated with asymptomatic carriage of harmful bacteria in healthy livestock environments.

2. Results

2.1. Isolation and Identification of E. coli

Overall, very slight differences in abundance and distribution of *E. coli* strains among the CTRL and Treated groups were revealed at each sampling time. Moreover, in all 48 tested fecal samples, *E. coli* was always detected. Specifically, both phenotypic and genotypic screening revealed that 66 and 73 isolates from CTRL fecal samples, and 83 and 73 isolates from Treated fecal samples, at T0 and T1 time, respectively, were attributable to the *E. coli* species.

2.2. Detection of Virulence Factors

Among the isolates from both CTRL and Treated samples, 65% (90/139) and 64% (100/156), respectively, carried at least one gene encoding a virulence factor. The precise distribution of virulence factors by groups and experimental periods is shown in Figure 1. In detail, the *stx* gene was present in 78% (109/139) of isolates from CTRL samples, classified as STEC pathotypes, and in 54% (85/156) of isolates from Treated samples. Temporal analysis showed no significant variation in the prevalence of virulence factors over time ($p > 0.05$, Fisher's exact test). Specifically, from the *stx*-positive isolates at T0 in CTRL samples, 32% (21/66) were *stx1*, and 9% (6/66) were *stx2*. At T1, the percentage of *stx1*-positive strains increased to 73% (53/73), whereas 40% (29/73) were *stx2*-positive.

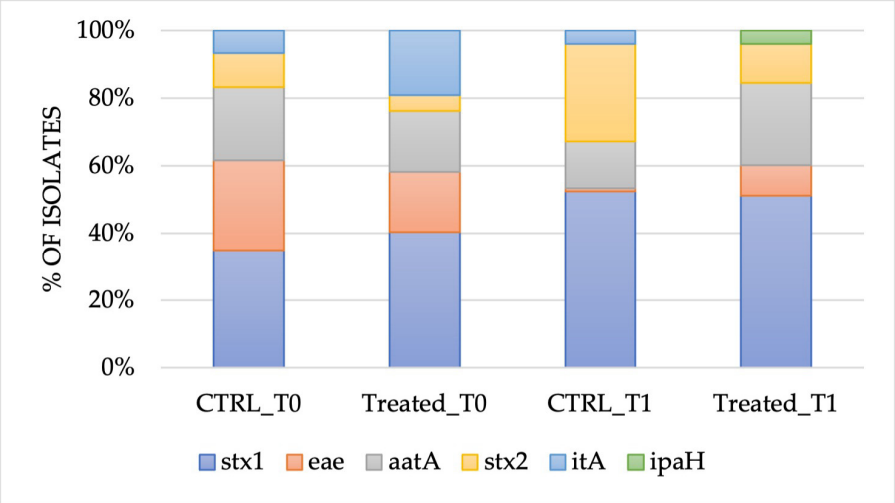

Figure 1. Virulence factor distribution among *E. coli* isolates in both CTRL and Treated groups at T0 and T1 sampling periods.

Regarding *E. coli* strains from Treated samples, at T0, 41% (34/83) were *stx1* positive, and 5% (4/83) were *stx*2 positive. At T1, 53% (39/73) were *stx*1 positive, and 11% (8/73) were *stx*2 positive. Among them, 32 strains (23%) from CTRL samples (5 at T0 and 27 at T1) and 12 strains (8%) from Treated samples (4 at T0 and 8 at T1) simultaneously carried genes *stx*1 and *stx*2. At T0, the *eae* gene was the second most detected virulence gene in *E. coli* strains (EPEC pathotype) from CTRL samples (24%; 16/66), while the *it*A gene (ETEC strains) was found in the Treated group but was absent in Treated isolates at T1. However, by T1, the *aat*A gene became one of the most common in *E. coli* isolates from both sample types, categorizing the strains into EAEC pathotypes. Notably, the *ipa*H gene, which was not detected in all *E. coli* strains, appeared in 3 strains (4%, identified as EIEC) from Treated samples at T1. None of the isolates presented the virulence gene *st*A.

2.3. Antibiotic Resistance

The AMR of the tested isolates was assessed against 9 antibiotics, following the CLSI guidelines [35] and the EUCAST guidelines [36]. The isolates were classified as sensitive, intermediate, or resistant. Overall, 25% (75/295) of isolates were resistant, 62% (183/295) were intermediate, and 13% (37/295) were sensitive, exhibiting a significant temporal variation ($p < 0.05$). The highest percentage of intermediate (73%; 114/156) and sensitive (16%; 25/156) isolates was observed in *E. coli* strains from Treated samples. The most resistant strains in the CTRL group reached 42% (58/139) (Figure 2). Significant differences ($p < 0.05$) in resistance prevalence were also observed among CTRL and Treated isolates.

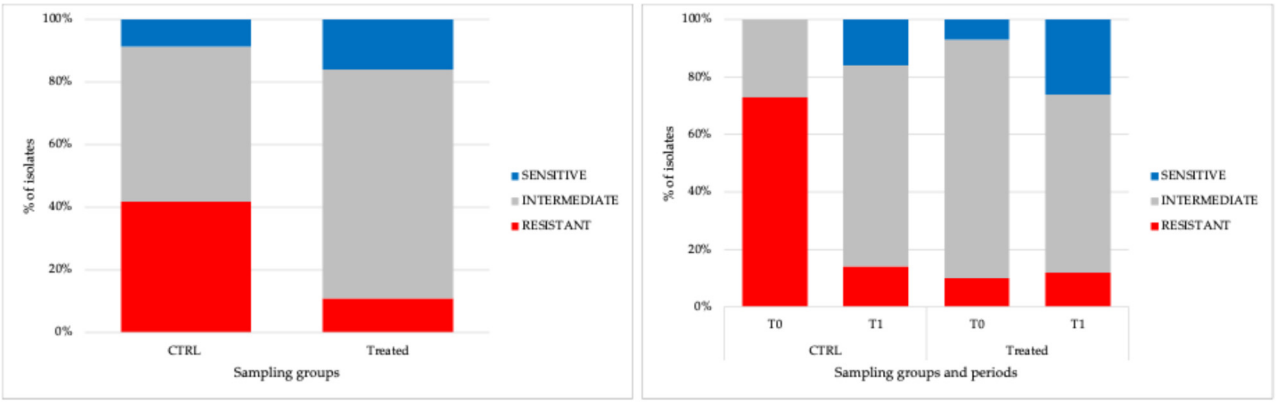

Figure 2. Categorization and distribution of antibiotic resistance in *E. coli* isolates.

Indeed, the most significant contribution to the resistome pattern came from *E. coli* isolated from CTRL samples. At T0, resistance to S was the most common for strains from the CTRL group, followed by TE, with 70% (46/66) and 15% (10/66) of resistant strains, respectively. By T1, the prevalence of resistance to the antibiotics above decreased and reached 8% (6/73 strains), while both AMP and NA resistance slightly increased to 12% (9/73 strains) and 11% (8/73 strains), respectively (Table 1).

Table 1. Antimicrobial-resistant profile of *E. coli* isolates from the CTRL group.

Antimicrobial Molecules	N. Strains Isolated from CTRL at T0	Resistant n. (%)	Intermediate n. (%)	Sensitive n. (%)	N. Strains Isolated from CTRL at T1	Resistant n. (%)	Intermediate n. (%)	Sensitive n. (%)
CIP 5	66	0	16 (24.2)	50 (75.8)	73	0	58 (6.8)	68 (93)
LEV 5	66	1 (1.5)	14 (21.2)	51 (77.3)	73	0	0	73 (100)
CN 10	66	0	14 (21.2)	52 (78.8)	73	1 (1.37)	9 (12.3)	63 (86)
S 10	66	46 (69.7)	20 (30.3)	0	73	6 (8.22)	50 (68.5)	17 (23)
SXT 25	66	7 (10.6)	0	59 (89.4)	73	6 (8.22)	0	67 (92)
AMP 10	66	7 (10.6)	0	59 (89.4)	73	9 (12.33)	0	64 (88)
C 30	66	0	0	66 (100)	73	1 (1.37)	0	72 (99)
NA 30	66	7 (10.6)	0	59 (89.4)	73	8 (10.9)	0	65 (89)
TE 30	66	10 (15.2)	0	56 (84.8)	73	6 (8.22)	1 (1.4)	66 (90)
COL	66	0	0	66 (100)	73	0	0	73 (100)

Regarding *E. coli* from the Treated group, the only resistance registered was against S and AMP at time T0, the first increasing to 11% (8/73) and the second disappearing at time T1. Remarkably, COL resistance was exclusively detected in 4 strains (2 at T0 and 2 at T1) from Treated samples, representing approximately 2.5%, and was not observed in any other strains or at any other time during the study period (Table 2). Furthermore, based on the EDTA-Agar Spot principle, all 4 *E. coli* were *mcr*-mediated COL-resistant strains. Of the *E. coli* strains from CTRL samples, 11% (7/66) at T0 and 8% (6/73) at T1 were multidrug-resistant bacteria, demonstrating concurrent resistance toward five classes of antibiotics (S-SXT-AMP-NA-TE). On the contrary, no multidrug resistance was observed in any *E. coli* isolates from Treated samples, at any sampling period, where 16% (25/156) displayed sensitivities to all tested antibiotics. Finally, the randomly selected *E. coli* isolates were confirmed to be ESBL-negative strains.

Table 2. Antimicrobial-resistant profile of *E. coli* isolated from the Treated group.

Antibiotic Molecules	N. Strains Isolated from Treated at T0	Resistant n. (%)	Intermediate n. (%)	Sensitive n. (%)	N. Strains Isolated from Treated at T1	Resistant n. (%)	Intermediate n. (%)	Sensitive n. (%)
CIP 5	83	0	14 (16.9)	69 (83)	73	0	13 (17.8)	60 (82)
LEV 5	83	0	14 (16.9)	69 (83)	73	0	16 (21.9)	57 (78)
CN 10	83	0	15 (18.1)	68 (82)	73	0	16 (21.9)	57 (78)
S 10	83	6 (7.2)	71 (85.5)	6 (7.2)	73	8 (11)	45 (61.6)	20 (27)
SXT 25	83	0	0	83 (100)	73	0	0	73 (100)
AMP 10	83	1 (1.2)	2 (2.4)	80 (96)	73	0	0	73 (100)
C 30	83	0	1 (1.2)	82 (99)	73	0	0	73 (100)
NA 30	83	0	0	83 (100)	73	0	0	73 (100)
TE 30	83	0	0	83 (100)	73	0	0	73 (100)
COL	83	2 (2.4)	0	81 (98)	73	2 (2.7)	0	71 (97)

To further evaluate the AMR patterns and to summarize multidrug resistance levels across clusters, the MAR Index and R-Score were calculated. Comprehensively, the MAR index of the isolates ranged from 0 to 0.50 for *E. coli* isolated from CTRL samples and from 0 to 0.2 for *E. coli* from Treated samples. A MAR index of 0.5, exhibited by 4% (13) of isolates, was indicative of a high resistance rate in that specific isolate. Furthermore, the R-score showed comparable values between the isolates from the CTRL group at T1 and the isolates from the Treated group at both sampling times. At the same time, the highest R-Score was observed in *E. coli* isolated at T0. In addition, to test the effects of both the feeding regimes and the sampling period on the level of resistance of *E. coli*, the comparison of the strain resistance score and the MAR index indicated the highest value in isolates from the CTRL group at T0, with a value almost equal to 2.0, indicating a group with a high risk of potential contamination by MDR strains. Surprisingly, for the remaining groups (CTRL at T1 and Treated at T0 and T1), quite similar and lower values were observed. Although they individually showed lower levels of resistance than other strains, the greater number of strains with intermediate resistance, belonging to the aforementioned groups, collectively contributed to the MAR index reaching a certain value.

2.4. PFGE Analysis

Of the 295 *E. coli* isolates obtained in this study, 273 were successfully analyzed by *Xba*I-PFGE. The isolates were divided into three datasets (Table 3). The first dataset included 139 isolates collected from 22 animals at the beginning of the experiment (referred to as T0). The remaining two datasets included 69 isolates from 11 control animals (CTRL_T1) and 65 isolates from 13 treated animals (Treated_T1), both collected at the end of the experiment (T1). PFGE genotyping revealed a high level of genetic diversity, identifying 71 distinct PFGE types among the 273 *E. coli* isolates. These comprised 46 subclusters and 25 singletons, i.e., isolates that did not cluster with any others. Specifically, dataset T0 included 16 subclusters and 5 singletons; dataset CTRL_T1 included 17 subclusters and 9 singletons; and dataset Treated_T1 included 13 subclusters and 11 singletons (Figure 3).

Table 3. Overview of genotyping analysis of *E. coli* isolates.

Parameters	T0	CTRL_T1	Treated_T1
animals	22	11	13
isolates	139	69	65
subcluster	16	17	13
singletons	5	9	11
TOTAL pulsotypes	21	26	24

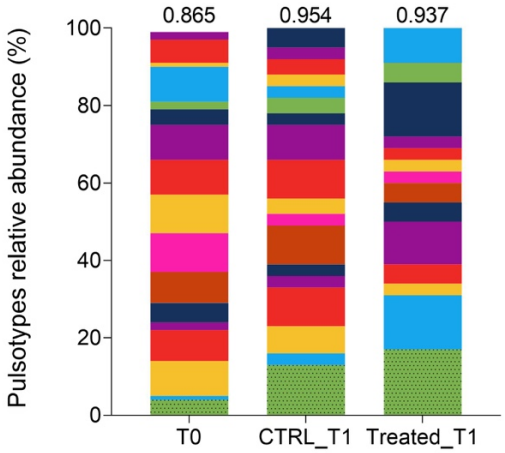

Figure 3. Distribution patterns of pulsotypes. *E. coli* isolates were divided into three datasets: T0 (139 isolates collected from 22 animals at the beginning of the experiment), CTRL_T1 (69 isolates from the 11-control cohort at the end of the experiment), and Treated_T1 (65 isolates from 13 treated animals at the end of the experiment), respectively. Relative abundance (%) was calculated based on the number of isolates belonging to each subcluster or classified as singletons, relative to the total number of isolates in each dataset. Green dotted columns represent singletons (i.e., isolates that did not cluster with any others). Numbers above the columns represent Simpson's Index of Diversity (SID).

In the T0 dataset, 96.4% of isolates were grouped into subclusters, with a Simpson's Index of Diversity (SID) value of 0.865. Several clusters comprise isolates from different fecal samples, indicating the clonal dissemination of the same PFGE types across multiple animals. In other cases, such as clusters S10 and S11, isolates originated mainly from single fecal samples, indicating that these likely represent the same strain. The most frequent pulsotypes were S2 (n = 13), S7 (n = 15), and S8 (n = 13), grouping isolates from five, eight, and six different animals, respectively (Supplementary Figures S1–S3).

In the CTRL_T1 dataset, 17 subclusters encompassed 88.4% of the isolates, with a SID value of 0.954. In the Treated_T1 dataset, 84.4% of isolates were grouped into subclusters, with a SID value of 0.937 (Figure 3). A slight increase in genetic diversity was observed over time, with no apparent effect of the treatment. The distribution of pulsotypes in the CTRL_T1 and Treated_T1 datasets resembled that observed in the T0 dataset (Supplementary Figures S2 and S3). Some subclusters grouped isolates from different animals, suggesting clonal dissemination, while others contained isolates primarily from a single animal. In CTRL_T1, the most frequent pulsotypes were S3 (n = 7) and S9 (n = 7), each grouping isolates from two different animals (Supplementary Figure S2). In Treated_T1, the most frequent pulsotypes, namely S1 (n = 9) and S11 (n = 9), grouped isolates from two and three distinct sources, respectively (Supplementary Figure S3).

3. Discussion

This study investigated the prevalence, AMR, virulence traits, and clonal diversity of *E. coli* isolated from fecal samples of healthy, antibiotic-free Sicilian lambs subjected to a pistachio skin supplementation feeding regimen. Results showed that the feeding strategy did not significantly influence either the prevalence or the amount of *E. coli* in the lamb feces, nor the prevalence and occurrence of virulence traits among the isolates. Although an increase in both STEC and EAEC *E. coli* was observed after 58 days of feeding, these differences were not statistically significant across sampling times. These findings support earlier studies indicating that the host animal actively impacts intestinal colonization and Shiga toxin production through its genetics and immune response, rather than

merely serving as a passive carrier, thereby affecting the prevalence of diarrheagenic *E. coli* (DEC) pathotypes [37,38]. These host-driven processes, combined with exposure to contaminated and improperly cooked or processed meat and dairy products, can contribute to the emergence and spread of strains carrying virulence genes linked to multiple DEC pathotypes, ultimately increasing their pathogenic potential [9,12,39–41]. In this study, the presence of both *stx1* and *stx2* genes, along with the *eae* gene, encoding intimin, which, as widely reported, is associated with increased virulence and more severe clinical outcomes in humans compared to strains carrying *stx1* alone [42–46]. In addition, detection of the EAEC strains as the second most common DEC pathotype in the Treated group confirmed previous studies, where the EAEC-associated *aat*A gene in isolates from farm animals were linked to human diarrhea outbreaks [47,48]. Notably, the disappearance of ETEC (Enterotoxigenic, *it*A gene-mediated) strains and the concurrent emergence of EIEC (Enteroinvasive, *ipa*H gene-mediated) in the Treated group after 58 days of feeding may reflect environmental or management factors during sampling, since humans are their primary reservoirs [49]. Regarding the resistome, many isolates showed intermediate susceptibility, confirming previous observations that attributed the environmental persistence of Antibiotic Resistance Genes (ARGs) to past antimicrobial use, which may exert long-term selective pressure on bacterial populations even without recent treatments [32,50,51]. Of particular concern, the lower resistance levels observed in the Treated group with respect to the control indicate that feeding strategies, based on pistachio skin supplementation, significantly limited the development and persistence of resistance over time. Overall, among the antibiotic resistance, as expected, isolates exhibited resistance against S and TE, reflecting their widespread historical use in livestock for therapeutic, prophylactic, or growth-promoting purposes, often at sub-therapeutic levels, which helps spread resistant bacteria [51–53]. In addition, COL resistance, mediated by *mcr* genes, and detected in *E. coli* strains from Treated samples, emphasizes the ongoing selective pressure from prophylactic use of polymyxins in livestock [6,33,52,53]. The transfer of *mcr*-1-mediated colistin resistance from animals to humans has been reported in several countries, raising serious public health concerns [54,55]. Despite increasing reports of ESBL-producing and MDR *E. coli* in FPAs globally, this study identified no ESBL producers, although MDR isolates were detected [56–58]. This finding supports previous research suggesting that antimicrobial resistance and virulence traits can evolve independently in certain *E. coli* lineages [59]. Most isolates displayed an intermediate resistance profile, possibly reflecting the lack of recent antibiotic exposure and the influence of the feeding strategies. As Ma et al. [60] highlighted, reduced susceptibility, often without acquired resistance genes, may result from spontaneous mutations in environments without antibiotics or from dietary factors. These could influence virulence gene expression, alter microbial communities, and shape the fecal resistome, promoting intermediate resistance. In this context, alternative feeding strategies, such as using herbal extracts or food by-products, have been proposed to modify the gut microbiota and reduce both AMR and pathogen carriage in livestock [61]. Along with resistome and virulence characterization, this study provides the first PFGE-based genetic analysis of DEC *E. coli* isolates from Sicilian lambs, enabling assessments of clonal diversity and dominant genotypes in the area. The high genetic heterogeneity suggested the presence of multiple unrelated lineages rather than the dominance of a single clone [62]. Nonetheless, the detection of genetically related strains across animals, feeding groups, and sampling times implies widespread dissemination and possible cross-contamination within environments and animal populations [63]. Inter- and intra-cluster variability likely reflects different exposures to resistance determinants from direct (antibiotic treatments) and indirect (environmental contamination) sources. The variability within clusters suggests

possible sub-structuring, which warrants further study into the genetic and environmental factors shaping these populations.

4. Materials and Methods

4.1. Sample Collection

A total of 48 fecal samples were collected from individual rectal swabs of healthy, antibiotic-free lambs, divided into two groups: control (CTRL) (n = 24) and experimental (Treated) diets (n = 24). Samples from each animal were taken at the start (T0) and the end (T1) of a 58-day feeding trial at the pilot farm of the University of Catania (37°24′35.3″ N 15°03′34.9″ E), aseptically collected in sterile polypropylene containers, kept on wet ice, and transported to the microbiology laboratory.

4.2. Isolation and Identification of E. coli Strains from Fecal Samples

Ten grams of each fecal sample were weighed in a commercial stomacher bag, 90 mL of buffered peptone water was added, and homogenized in a stomacher (Interscience) for 2 min at 230 rpm. Thereafter, the mixture was incubated at 42 °C overnight before isolating *E. coli*. From each overnight non-selective enrichment, a 10 µL loopful was streaked onto MacConkey agar and incubated at 37 °C overnight. After incubation, plates were inspected to identify colonies with typical *E. coli* morphology (i.e., violet to pink, convex, circular, and dry colonies with a surrounding pink zone), and 12 positive colonies from plate of fecal sample of each animal, at T0 and T1 sampling time, were randomly selected, purified on chromogenic Rapid 2 *E. coli* medium (Biorad Laboratories, Inc., Segrate, Milan, Italy), and initially characterized using conventional methods, including Gram staining and cell morphology. Afterward, almost 300 well-isolated presumptive *E. coli* strains were confirmed by genotypic analysis through PCR. The DNA template for PCR was obtained by dissolving an *E. coli* colony, cultivated on Tryptic Soy agar, in 20 µL of DNAase-free water [2], and analyzed using specific primers (*mdh*): F 5′-GGTATGGATCGTTCCGACCT-3′ and R 5′-GGCAGAATGGTAACACCAGAGT-3′ [64]. The PCR mixture consisted of 12.5 µL of DreamTaq 2X master mix (Thermo Fisher Scientific, Rodano, Italy), 0.5 µL of each primer, 2.0 µL of DNA template, and sterile water, to a final volume of 25 µL. PCR conditions included an initial denaturation at 95 °C for 1 min, followed by 30 cycles of denaturation at 95 °C for 45 s, annealing at 53 °C for 45 s, extension at 72 °C for 45 s, and a final cycle at 72 °C for 7 min. PCR products were verified on 1.5% agarose gels at 100 V for approximately 45 min. All confirmed *E. coli* strains were stored at −80 °C in Tryptic Soy Broth with 20% glycerol stocks until further analysis. All media were purchased from Biolife Italiana S.r.l. (Segrate, Milan, Italy).

4.3. Detection of Virulence Factors by Multiplex PCR

Template DNA from each strain for the PCR reaction, produced as above, was assayed for the presence of genes specific to the pathotype, defining the five most relevant DEC *E. coli* strains: STEC (*stx1*, *stx2*), EPEC (*eae*), ETEC (*lt*A, *st*A), EIEC (*ipa*H), and EAEC (*aat*A). A multiplex PCR was performed according to the protocol previously described [2]. In detail, DreamTaq master mix 2X, 0.5 µL of each primer for all samples was prepared and dispersed into PCR tubes, and 2 µL of DNA template was added to each tube to a final volume of 25 µL. The primer sequences and the predicted size of the amplified products for the different pathogenic gene coding regions are shown in Table S1. PCR products were separated and visualized by gel electrophoresis in 1.5% agarose in 0.5X TBE buffer (25 mM Tris-borate, 0.5 mM EDTA) at 90 V. A 100 bp DNA Ladder (Sigma Aldrich, Milan, Italy) was included in each agarose run, and amplicon sizes from each DEC sample were compared to those in the control strains.

4.4. Antibiotic Susceptibility Test

Susceptibility of *E. coli* isolates to a panel of 9 antimicrobial agents, selected to reflect both veterinary clinical practice and surveillance of critical resistances, was assessed using the disk diffusion (Kirby–Bauer) assay as previously described [1]. The following antimicrobial, disks were used: ampicillin (AMP, 10 mg), ciprofloxacin (CIP, 5 mg), gentamicin (CN, 10 mg), nalidixic acid (NA, 30 mg), streptomycin (S, 10 mg), chloramphenicol (C, 30 mg), trimethoprim-sulfamethoxazole (SXT, 23.75 mg), tetracycline (TE, 30 mg), and levofloxacin (LEV, 5 mg). Moreover, ESBL production was randomly confirmed by the double-disk synergy test (DDST), composed of cefpodoxime-clavulanic acid (CDO1, 10-1 g) and cefpodoxime (CPD10, 10 µg). The susceptibility of strains was determined according to the inhibition zone diameter interpretative standards recommended by the Clinical and Laboratory Standards Institute (CLSI) in 2018 [35]. All antibiotics were purchased from Oxoid Thermo Scientific™ (Basingstoke, UK). In addition, the susceptibility of *E. coli* to colistin was determined via EDTA-Agar Spot, a chelator-based test able to differentiate colistin-resistant and *mcr*-positive/negative isolates [65]. *E. coli* ATCC 25922 was used as a reference. Multidrug resistance was defined as isolates resistant to three or more classes of antimicrobial agents. The multiple antibiotic resistance (MAR) index of the *E. coli* isolates was calculated as previously described [66], following the formula *a/b*, where *a* represents the number of antibiotics to which the isolate resulted resistant, and *b* represents the number of antibiotics to which the isolate was exposed [67]. For a given *E. coli* isolate, the R-score represents the number of antibiotics against which the isolate exhibited intermediate or complete resistance. Resistance scores of 0.5 and 1 were attributed to isolates exhibiting intermediate or complete resistance, respectively, against a given antibiotic [68].

4.5. Pulsed-Field Gel Electrophoresis (PFGE) Analysis

All *E. coli* isolates were analyzed and classified into clusters by PFGE using a modified PulseNet protocol. Briefly, the *E. coli* isolates were grown on Brain Heart Infusion agar overnight at 37 °C and then resuspended in TE buffer to an OD_{600} nm value of 0.8 to 1. Subsequently, 200 µL of bacterial dilutions were embedded in 1% (*v*/*v*) low-melting-point agarose and immersed in 1 mL of lysis buffer (2 M Tris-HCl, pH 7.6; 5 M NaCl; 0.5 M EDTAH 7.6; 5% Brij58; 10% deoxycholate (Thermo Fisher Scientific, Waltham, MA, USA); 20% sarcosyl (Sigma Aldrich, St. Louis, MO, USA); 10 mg/mL lysozyme (Thermo Fisher Scientific), then incubated at 37 °C for 12 h with slow agitation. This was followed by treatment with ESP solution (0.5 M EDTA, pH 9; 20% sarcosyl; 20 mg/mL proteinase K (Thermo Fisher Scientific) at 50 °C for 12 h, again with slow agitation. The agarose plugs were washed three times with ultrapure water (Thermo Fisher Scientific) at 50 °C for 10 min each and then washed three times in 1X TE buffer at 50 °C for 10 min each. The genomic DNA embedded in agarose plugs was digested with the XbaI restriction enzyme (Thermo Fisher Scientific). The restriction fragments were separated in 1.0% Seakem Gold agarose (Lonza, Rockland, ME, USA) using pulse times of 6.76–35.38 s for 22 h at 14 °C with a CHEF-MAPPER System (Bio-Rad, Hercules, CA, USA). The Lambda Ladder (Biorad) served as a molecular weight marker. BioNumerics v8.1 (Applied Maths, BioMérieux, Sint-Martens-Latem, Belgium) was used to analyze the PFGE patterns. Similarity was assessed using the Pearson correlation coefficient, and clustering was performed using the unweighted pair group method with arithmetic mean (UPGMA). Optimization and curve-smoothing parameters were established based on BioNumerics scripts and are provided in Supplementary Table S1. Distinct biotypes (also referred to as pulsotypes in the context of PFGE) were assigned based on a similarity cutoff of less than 96%. This cutoff was established by comparing PFGE fingerprints of the two randomly selected isolates obtained

from different runs. The Simpson's index of diversity (SID) was calculated according to Hunter and Gaston [68].

4.6. Statistical Analyses

Statistical analyses were performed using GraphPad Prism (Version 10.1.1). Fisher's exact test assessed the prevalence of AR and VF, as well as changes over time and the effect of diet on the resistome. Differences in AR and VF presence among clusters were examined with the Kruskal–Wallis test followed by Dunn's post hoc correction, while inter-cluster differences were tested using the Wilcoxon matched-pairs test. Additionally, relationships between AR and VF counts were analyzed with Spearman's rank correlation. Statistical significance was defined as $p < 0.05$.

5. Conclusions

This study offers, for the first time, insights into the prevalence of virulence genes and antibiotic resistance among commensal *E. coli* isolates recovered from fecal samples of healthy lambs raised on an antibiotic-free farm in Sicily. High resistance, especially to streptomycin, was observed, whereas the reduced resistance in the Treated group suggests that dietary interventions may influence the commensal *E. coli* resistome in lambs. High genetic diversity among the isolates was observed, indicating intra- and inter-cluster variation. These results emphasize the need for early monitoring of sentinel organisms like *E. coli* along the food chain within a One Health framework, and suggest that further studies on feeding-related gut microbiota modulation could help develop strategies to improve food safety and safeguard public health.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics15020160/s1. Figure S1. Genotyping of *E. coli* isolates collected at T0. The pulsotype dendrogram was built using the Pearson correlation coefficient and UPGMA. Collapsed clades represent subclusters with more than 96% similarity. Subclusters are indicated from S1 to S16. When visible, gray bars represent error flags. Numbers on the branches represent branch quality and similarity percentage, respectively. Figure S2. Genotyping of *E. coli* isolates collected in control samples at T1 (CTRL_T1). The pulsotype dendrogram was built using the Pearson correlation coefficient and UPGMA. Collapsed clades represent subclusters with more than 96% similarity. When visible, gray bars represent error flags. Subclusters are indicated from S1 to S17. Numbers on the branches represent branch quality and similarity percentage, respectively. Figure S3. Genotyping of *E. coli* isolates collected in treatments at T1 (Treated_T1). The pulsotype dendrogram was built using the Pearson correlation coefficient and UPGMA. Collapsed clades represent subclusters with more than 96% similarity. When visible, gray bars represent error flags. Subclusters are indicated from S1 to S13. Numbers on the branches represent branch quality and similarity percentage, respectively. Table S1. Setting parameters for the Pearson correlation analysis of PFGE profiles.

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Institutional Review Board Statement: The study was conducted in accordance with European Union (Council Directive 2010/63/EU) legislation for the protection of animals used for experimental and other scientific purposes. The animal study protocol was reviewed and approved by the Research Ethics Committees of the University of Catania (protocol number: 153763).

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding author.

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Conflicts of Interest: Nunziatina Russo, Alessandra Pino, Cinzia Caggia, and Cinzia L. Randazzo are founders of ProBioEtna Srl, a spin-off of the University of Catania. All the authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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