zoonotic diseases

MDPI

Article

Dominance of Diarrheagenic *E. coli* Virulent Types in Integrated Crop–Livestock Farms and Their Antibiotic Resistance Patterns

Arpita Aditya [1,†], Dita Julianingsih [1,†], Zajeba Tabashsum [2], Zabdiel Alvarado-Martinez [2], Chuan-Wei Tung [1], Matthew Wall [1] and Debabrata Biswas [1,2,*]

[1] Department of Animal and Avian Sciences, University of Maryland, College Park, MD 20742, USA; aaditya1@umd.edu (A.A.); djuliani@umd.edu (D.J.); vmtung@umd.edu (C.-W.T.); mattjwall@gmail.com (M.W.)
[2] Biological Sciences Program, University of Maryland, College Park, MD 20742, USA; ztabashs@umd.edu (Z.T.); zalvara1@umd.edu (Z.A.-M.)
* Correspondence: dbiswas@umd.edu; Tel.: +1-301-405-3791; Fax: +1-301-405-7980
† These authors have contributed equally to this work and share first authorship.

Simple Summary: This study delves into the microbial ecology of integrated crop–livestock farms (ICLFs) and its impact on food safety, specifically addressing the prevalence and antibiotic resistance of diarrheagenic *E. coli*. Analyzing 2973 samples over two years in Maryland and Washington DC, the study reveals a 4.30% incidence of diarrheagenic *E. coli*, with livestock bedding materials showing the highest prevalence at 8.51%. Notably, 92.30% of virulent type *E. coli* displayed resistance to common antibiotics. The findings underscore the potential risks associated with integrated farming practices, emphasizing the need for vigilant on-farm and market-level precautions to mitigate the threat of antibiotic-resistant virulent type *E. coli* in locally integrated farming contexts.

Abstract: Microbial ecology on integrated crop–livestock farms (ICLFs) can impact food safety through pathogen transfer between animals and crops. Recent reports of pathogen-contaminated products sold in local organic retail, roadside, and farmers markets highlight the need for assessment of the ecological patterns of bacterial pathogens. This study investigated the prevalence and antibiotic resistance of the virulent type of diarrheagenic *E. coli* in ICLFs. Over two years, 2973 samples from ICLFs and markets in Maryland and Washington DC were analyzed. Diarrheagenic *E. coli* was found in 4.30% (128/2973) of collected samples, with a higher isolation rate in environmental (4.42%, 59/1332) and produce (4.20%, 69/1641) samples. Overall, livestock bedding materials had the highest prevalence (8.51%, 4/47). Post-harvest produce exhibited a lower contamination rate of 1.32% (10/756), whereas pre-harvest produce had a higher incidence with 6.67% contamination (59/885), indicating the presence of *E. coli*. Alarmingly, 92.30% (72/78) of pathogenic *E. coli* isolates were resistant to common antibiotics. The findings highlight potential risks associated with integrated farming practices and emphasize the importance of safe harvesting and post-harvesting measures, particularly in the context of the growing popularity of local integrated farming. Implementing precautions at on-farm and market levels is crucial to mitigate the risk of antibiotic-resistant *E. coli*-related enteric illnesses, safeguarding both consumers and the integrity of integrated farming systems.

Keywords: mixed farm; prevalence; *E. coli*; antibiotic resistance; Maryland and Washington D.C. metropolitan area; integrated crop–livestock farms (ICLF)

Citation: Aditya, A.; Julianingsih, D.; Tabashsum, Z.; Alvarado-Martinez, Z.; Tung, C.-W.; Wall, M.; Biswas, D. Dominance of Diarrheagenic *E. coli* Virulent Types in Integrated Crop–Livestock Farms and Their Antibiotic Resistance Patterns. *Zoonotic Dis.* **2024**, *4*, 11–21. https://doi.org/10.3390/zoonoticdis4010003

Academic Editor: Stephen K. Wikel

Received: 26 November 2023
Revised: 9 January 2024
Accepted: 10 January 2024
Published: 12 January 2024

1. Introduction

According to the Centers for Disease Control and Prevention (CDC), more than 10,225 foodborne outbreaks occurred in the US between 2009 and 2021 which caused at least 176,502 illnesses, 11,953 hospitalizations, and 316 deaths [1]. Out of these numbers of outbreaks, plant products including fruits, vegetables, spices, and grains are responsible for

more than 51% of foodborne illnesses in the US [2]. Further, fresh fruits and vegetables including spinach, carrots, lettuce, tomatoes, cucumber, melons, apples, and strawberries are higher risk foods as these are commonly consumed raw. Shiga toxin-producing *Escherichia coli* (STEC), non-typhoidal *Salmonella enterica*, and *Listeria monocytogenes* are the most common bacterial pathogens associated with reported produce-related outbreaks [1,3]. Farm animals, such as cattle, goats, pigs, and poultry, serve as major reservoirs for STEC [4,5]. In addition to STEC, other diarrheagenic *E. coli* virulent types include enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and enterotoxigenic *E. coli* (ETEC), further contributing to the complexity of potential foodborne illnesses associated with contaminated produce. These pathogens colonize in these farm animal intestines as normal microbiota and contaminate the animal food products during inappropriate processing. In addition to contaminating animal food products, they can contaminate soil, water, and grasses/plants and survive for a long time in soil, water, and plants through fecal runoff [6,7]. Given the numbers of foodborne illnesses with the pathogen STEC, the economic impacts are more than USD 1.6 billion [8]. Our study goes further into the dynamics of foodborne outbreaks by focusing on integrated crop–livestock farms (ICLFs) in response to the request for creative research. We want to discover new issues and potential sources of contamination by investigating the complicated link between animal and vegetable production in these farms.

When animals and vegetables are produced in the same farm, the facility is known as an integrated crop–livestock farm (ICLF). Food products, specifically fresh produce cultivated in ICLFs, are more vulnerable to becoming contaminated with zoonotic pathogens [9–16]. ICLF practices are gaining popularity across various states in the US, particularly in the Northeast. The numbers of ICLFs are on the rise, aligning with ecological and conservation principles [13–16]. These farms employ the recycling of animal manure as fertilizer and utilize plant residuals as animal feed. However, the effectiveness of these practices depends on proper management, as improper recycling, such as inadequate composting, can elevate the risk of introducing pathogenic microbes into crop production environments and sustaining pathogen reservoirs in livestock [13–16]. The survival potential of pathogens during the recycling of animal manure in ICLFs is notably high when not following recommended composting guidelines or lacking trained workers. Compliance with suggested guidelines at ICLFs is not universal, and some farms, which may even be open to visitors, are susceptible to intrusion by wild animals, birds, rodents, and insects [13–16]. Notably, the contamination of produce samples from organic integrated farms in Europe tends to be higher than those from organic produce farms without livestock [9]. In ICLFs, the shared use of tools and the introduction of new chicks, calves, and other agricultural animals can contribute to the transfer of pathogen loads between different animals [9,10]. The source of contamination of fresh produce with enteric pathogens is often traceable to environmental reservoirs associated with farm operations and wild animals [9].

Further, food products grown in ICLFs are commonly sold in either farmers markets, roadside stands, or local retail markets [7,15]. Currently, more than 8000 farmers markets are listed in the National Farmers Market Directory (NFMD) and this number is rising as the United States Department of Agriculture (USDA) is promoting farmers markets across the country [5,17]. Though the chances of large or widespread outbreaks with contaminated produce sold in farmers markets are very low, this sector may contribute to sporadic cases and localized outbreaks [7,15].

The proximity of animal and produce operations in the same farm may increase the potential for cross-contamination of pathogens between animal reservoirs (poultry, pig, sheep, goat, cattle, and other livestock) and fresh produce [18,19]. A fundamental focus during transitioning to organic involves building soil health and microbial diversity [20,21]. For ICLFs, that involves developing sustainable manure management and use practices, particularly for manure-based soil amendments used to fertilize soils cropped to fresh produce [18,19]. Animal manure and compost not only are fertilizers, but they may improve soil health by increasing soil organic matter, and accompanying properties

that could play a role in how long zoonotic pathogens will survive and transfer to fresh produce crops [22]. This study distinguishes itself by focusing on the specific setting of ICLFs, offering insights into the complex relationships between farming methods and the prevalence of diarrheagenic *E. coli* virulent types. We hope to provide novel perspectives that can inform targeted strategies for enhancing food safety in this increasingly popular agricultural paradigm through a comprehensive analysis.

In this study, we aim to investigate the presence of diarrheagenic *E. coli* virulent types in ICLF environments including soil, compost, grasses, animal feed, waters, animal feces, on-farm vegetables, as well as post-harvested vegetables from local farmers markets and organic grocery stores over a period, and determine their antibiotic resistance.

2. Materials and Methods

2.1. Sample Collection

A wide variety of environmental (n = 1332) and pre-harvest produce (n = 885) samples (Table 1) were collected from three ICLFs (practicing organic farming), located in the Maryland and Washington D.C. metropolitan area. We also collected post-harvest (n = 756) produce samples from two established chain organic grocery stores and one local farmers market. All samples were collected during the summer months (May to September) between the years 2019 and 2021. However, due to the COVID-19 pandemic, field visits for sample collection were hindered in 2020. Therefore, no sample collection took place during that year. All the ICLFs and grocery stores chosen for this study were visited twice each year within a week interval to create biological replicates. Multiple samples (between 5 and 15) from the same category were considered as technical replicates. All samples were aseptically collected, transported, and processed to the laboratory for analysis following the method previously published by our research group [6].

Table 1. Samples collected from various sources at the ICLFs.

Sample Category	Description	Total Sample No.
Livestock drinking water	Water collected from the drinking tubs of various farm animals, such as cow, pig, turkey, and chicken	221
Feces	Fresh fecal excreta of the farm animals	266
Feed	Dry feed such as hay, and salts collected from barns	196
Soil	Soil collected from various locations of farms: grazing land, produce garden, etc.	359
Bedding	Bedding material of farm animals including hay, grass, etc.	47
Grass	Grass collected from the grazing land of animals and the produce garden	178
Compost	Collected from different depths of the compost heap	65
Produce [a] (pre-harvest)	Aseptically collected from the garden	885
Produce (post-harvest)	Collected from organic grocery stores and a local farmers market	756
Total		2973

[a] A wide variety of produce samples were collected from all farms including garden vegetables, fruits, herbs, spices, etc.

2.2. Presumptive Isolation of Diarrheagenic E. coli

The collected samples underwent processing and enrichment, with the selective isolation of presumptive diarrheagenic *E. coli* based on cultural characteristics, following the protocol established by our group [6,7]. In summary, 1 g of each solid environmental sample (Table 1) was combined with 25 mL of 1× PBS (pH 7.4). Meanwhile, liquid samples, such as livestock drinking water, were directly used without additional 1× PBS. Pre- and post-harvest produce samples were aseptically separated into individual bags and immersed in 1× PBS, following the same procedure for leafy vegetables, herbs, and

grass. The resulting suspensions or washed liquids were inoculated into Luria–Bertani (LB) broth (Becton, Dickinson and Co., Sparks, MD, USA), supplemented with 5% sheep blood (Ward's Science, Rochester, NY, USA), at a final ratio of 1:9 (*v*/*v*) of sample per volume of media. Following an overnight aerobic incubation at 37 °C, the enriched broth cultures were streaked on sorbitol-MacConkey (SMAC) agar (Becton, Dickinson and Co., Sparks, MD, USA) to selectively isolate diarrheagenic *E. coli*. Presumptive colonies of diarrheagenic *E. coli* were obtained through two subsequent rounds of subculturing and preserved in glycerol stock for further primer-specific (Table 2) polymerase chain reaction (PCR)-based analysis.

Table 2. Primers used in this study to identify the virulent types of diarrheagenic *E. coli*.

Genes	Primer Names	Sequences (5′-3′)	Product Sizes (bp)	References
uid [a]	*uid*-1	ATGGAATTTCGCCGATTTTGC	187	[23]
	uid-2	ATTGTTTGCCTCCCTGCTGC		
stx [c]	*stx*-VT1	GAGCGAAATAATTTATATGTG	518	[24]
	stx-VT2	TGATGATGGCAATTCAGTAT		
est [e]	est-AL1	TTAATAGCACCCGGTACAAGCAGG	147	[24]
	est-AL2	CCTGACTCTTCAAAAGAGAAAATTAC		
elt [e]	*elt*-LT1	TCTCTATGTGCATACGGAGC	322	[24]
	elt-LT2	CCATACTGATTGCCGCAAT		
ipa [d]	*ipa*-H1	GTTCCTTGACCGCCTTTCCGATACCGTC	619	[24]
	ipa-H2	GCCGGTCAGCCACCCTCTGAGAGTAC		
agg [f]	*agg*-R1	GTATACACAAAAGAAGGAAGC	254	[24]
	agg-R2	ACAGAATCGTCAGCATCAGC		
bfp [b]	*bfp*-1	GGAAGTCAAATTCATGGGGGTAT	300	[24]
	bfp-2	GGAATCAGACGCAGACTGGTAGT		
eae [b,c]	*eae*-SK1	CCCGAATTCGGCACAAGCATAAGC	881	[24]
	eae-SK2	CCCGGATCCGTCTCGCCAGTATTCG		

Identification of *E. coli*: [a] EPEC; [b] STEC; [c] EIEC; [d] ETEC; [e] EAEC; [f] EAEC.

2.3. Confirmation of E. coli and Identifying Their Virulent Types

The presumptive isolates of diarrheagenic *E. coli* underwent further confirmation and identification of specific virulence genes using designated primers (Table 2), following a previously published protocol [6]. In brief, glycerol stocks were revitalized by streaking on LB agar and aerobic incubation at 37 °C. A colony from the presumptive isolation was selected and fully suspended in 50 µL PBS. DNA extraction employed the thermal lysis of the suspended cells at 95 °C for 15 min [25]. The heat-treated bacterial suspension was centrifuged to collect DNA in the supernatant, serving as the template for the PCR reaction. This study investigated the distribution of five major virotypes including STEC, EPEC, ETEC, EHEC, and EAEC which are commonly detected in clinical cases of human enteric illness. PCR reactions were conducted in a 20 µL final volume, following manufacturer recommendations, consisting of 10 µL of 2× GoTaq® Green Master Mix (Promega, Madison, WI, USA), 0.5 µL of each forward and reverse primer (1 µM working concentration), and 2 µL of template DNA. The PCR reactions were carried out in a thermocycler (BioRad, Hercules, CA, USA) under the following temperature conditions: initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 46.8 °C (annealing temperature) for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 5 min. The visualization of PCR products was achieved through 1.5% (*w*/*v*) agarose gel electrophoresis (Sigma-Aldrich, Burlington, MA, USA) running for 50 min at 80 V/cm. Virotypes were determined

by comparing product sizes with a standard 100 bp DNA marker (Invitrogen, Carlsbad, CA, USA).

2.4. Antibiotic Resistance Pattern of Confirmed Diarrheagenic E. coli Virulent Types

The antibiotic resistance patterns of diarrheagenic *E. coli* virulent types were assessed using the standard agar dilution method, following the guidelines set by the Clinical and Laboratory Standards Institute (CLSI) [26]. A selection of antibiotics commonly employed for therapeutic purposes was chosen for this investigation (Table 3) [6]. The preparation of agar plates containing antibiotics and the execution of the antibiogram followed a previously published study [6,7]. In summary, Muller–Hinton (MH) agar plates (Becton Dickinson and Co, Franklin Lakes, NJ, USA) were individually supplemented with three different concentrations of each antibiotic by combining the antibiotic at specific concentrations with molten MH agar, as detailed before [7]. Confirmed diarrheagenic *E. coli* virulent types *i* were cultured on MH agar and incubated overnight at 37 °C. A colony from each isolate was selected and suspended in 0.85% saline solution to create an inoculum for the antibiogram. Before inoculation, the optical density of each positive sample was adjusted to fall between 0.08 and 0.1 (equivalent to 0.5 McFarland standard) and then diluted tenfold to achieve approximately ~10^7 CFU/mL. Subsequently, 2 µL of the diluted suspension was inoculated on the antibiotic MH agar plates. After inoculating all samples, the antibiotic plates underwent an overnight incubation at 37 °C. The antibiotic resistance of diarrheagenic *E. coli* isolates was interpreted based on CLSI breakpoints (Table 3).

Table 3. Antibiotics, antibiotic groups, and resistance breakpoints used in antimicrobial susceptibility tests for confirmed diarrheagenic *E. coli* virulent types [a].

Antimicrobial Class	Antimicrobial Agent	Breakpoints (µg/mL)		
		Susceptible	Intermediate	Resistant
Aminoglycosides	Gentamicin	≥4	≥8	≥16
	Streptomycin	≥16	≥24	≥32
β-Lactam	Amoxicillin	≥8	≥16	≥32
Cephems	Ceftriaxone	≥1	≥2	≥4
Folate pathway inhibitors	Trimethoprim/sulfamethoxazole	≥2 and ≥38	≥3 and ≥57	≥4 and ≥76
Macrolides	Azithromycin	≥16	≥24	≥32
Penicillin	Ampicillin	≥8	≥16	≥32
Phenicol	Chloramphenicol	≥8	≥16	≥32
Quinolones	Ciprofloxacin	≥0.06	≥0.12	≥1
Tetracyclines	Tetracycline	≥4	≥8	≥16

[a] The antimicrobial susceptibility test was performed using the agar dilution method according to guidelines established by the CLSI. *E. coli* ATCC 25922 was used as the quality control organism.

2.5. Statistical Analysis

Prevalence comparisons of diarrheagenic *E. coli* and its virotypes across different sample categories, along with analyses of antibiotic resistance, were conducted in MS Excel using Fisher's exact tests and the Cochran–Mantel–Haenszel (CMH) test.

3. Results

3.1. Prevalence of Diarrheagenic E. coli in Various Categories of Samples Collected at Pre- and Post-Harvest Levels

In this study, the overall prevalence of diarrheagenic *E. coli* was 4.30% (128/2973) ($p < 0.05$) (Figure 1). While comparing the prevalence of this pathogen between two major sample categories, a prevalence of diarrheagenic *E. coli* of 4.42% (59/1332) and 4.20% (69/1641) was observed in environmental and produce samples, respectively. Among the

sub-categories, all types of environmental samples (except compost) harbored diarrheagenic *E. coli* with a variation based on the source. Among the various categories of samples collected from ICLF environment, the livestock bedding materials harbored the highest amount of diarrheagenic *E. coli* at 8.51% (4/47) followed by drinking water at 6.78% (15/221). Feed and feces samples had a similar prevalence of 5.61% (11/196) and 5.26% (14/266), respectively. Among the produce, samples collected at the pre-harvest level exhibited a significantly ($p < 0.05$) higher prevalence of diarrheagenic *E. coli* (6.67%, 59/885) compared to the prevalence of this pathogen in produce samples collected at the post-harvest (1.32%, 10/756) level. The overall prevalence of diarrheagenic *E. coli* virulent types and their distribution in various sources are presented in Figure 1.

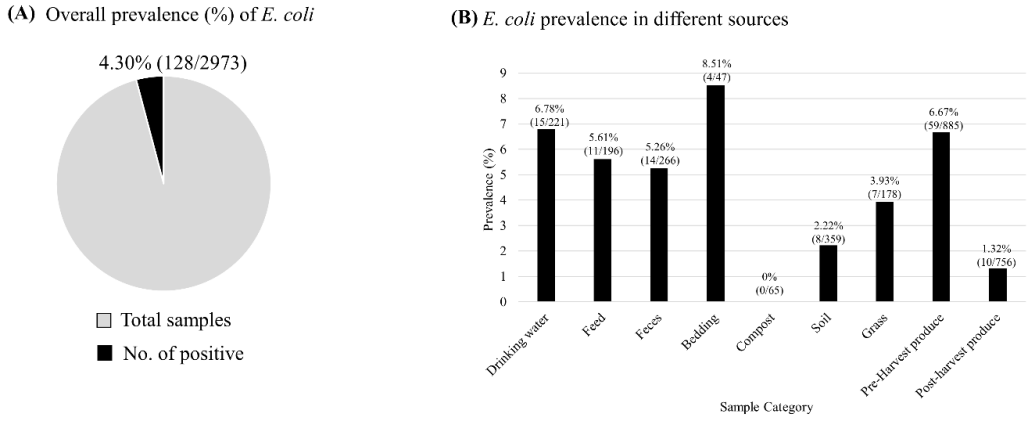

Figure 1. Prevalence of diarrheagenic *E. coli* in various samples collected from ICLFs. (**A**) Overall percentage of samples positive with diarrheagenic *E. coli*. (**B**) Percentages of samples positive with diarrheagenic *E. coli* in environmental samples (drinking water, feed, feces, compost, bedding, grass, and soil) (59/1332) ($p < 0.05$) and pre-harvest and post-harvest produce (69/1641) ($p < 0.05$).

3.2. Virulent-Type-Specific Distribution of Isolated E. coli

A total of 78 isolates (out of 128) were identified under these five major diarrheagenic *E. coli* virulent types, either EPEC, STEC, EIEC, EAEC, or ETEC. Environmental samples as well as pre-harvest produce samples harbored all these *E. coli* isolates. On the other hand, none of the post-harvest produce samples contained any of these virulent types. The highest virulent type distribution of isolated *E. coli* in the major pre-harvest and environmental sample categories concerned EIEC and STEC. Among the environmental samples, both of these types were equally distributed (16.66%, 13/78), whereas the predominant type in the pre-harvest produce was EIEC at 29.48% (23/78), followed by STEC at 8.97% (7/78). The distribution of other major *E. coli* virulent types was seen to follow a similar pattern in the environmental and pre-harvest categories (Table 4).

Table 4. Distribution of isolated *E. coli* and its virulent types in various samples categories.

Sample Category	EPEC	STEC	EIEC	EAEC	ETEC
Environmental sample	8.97% (7/78)	16.66% (13/78)	16.66% (13/78)	2.56% (2/78)	3.84% (3/78)
Pre-harvest	5.12% (4/78)	8.97% (7/78)	29.48% (23/78)	5.12% (4/78)	2.56% (2/78)
Post-harvest	0.0	0.0	0.0	0.0	0.0

Comparing the distribution of the major virotypes of isolated *E. coli* across the environmental samples, livestock drinking water had the highest distribution of *E. coli* virulent types at 19.23% (15/78) ($p < 0.05$), followed by feed at 11.53% (9/78) (Table 5), whereas feces (6.41%, 5/78), soil (5.12%, 4/78), and grass (5.12%, 4/78) showed a similar distribution rate. Pre-harvest produce had all major diarrheagenic *E. coli* virotypes present in 4.51% (40/885) of the samples evaluated (Table 5).

Table 5. Distribution of major virulent types of isolated *E. coli* across various sources.

Sources	EPEC	STEC	EIEC	EAEC	ETEC	Distribution (%)
Water	3	3	8	1	0	19.23
Feed	2	5	2	0	0	11.53
Feces	0	1	1	0	3	6.41
Bedding	0	1	0	0	0	1.28
Soil	1	0	2	1	0	5.12
Grass	1	3	0	0	0	5.12
Pre-harvest produce	4	7	23	4	2	51.28
Post-harvest produce	0	0	0	0	0	0

3.3. Resistance Pattern of Isolated E. coli Virulent Types against Major Antibiotics

Overall, 92.30% (72/78) of the isolated *E. coli* virulent type were resistant to at least one antibiotic, while only 6.41% (5/78) were sensitive to all tested antibiotics (Table 3). The highest percentage of antibiotic sensitivity was documented against gentamicin (46.15%; 36/78) and chloramphenicol (46.15%; 36/78), respectively. Also, gentamicin was the most potent antibiotic found in this study because only 1.28% (1/78) of isolated virulent type *E. coli* was resistant against it, while streptomycin was another strong antibiotic like gentamicin and chloramphenicol, because 8.97% (7/78) of virulent type *E. coli* showed resistance against streptomycin (Figure 2). Moreover, chloramphenicol has a 10.25% (8/78) resistance which is similar to streptomycin (Figure 2). For other tested antibiotics, 21.79% (17/78), 23.07% (18/78), and 25.64% (20/78) of virulent type *E. coli* presented resistance against ciprofloxacin, trimethoprim/sulfamethoxazole, and ceftriaxone, respectively. However, an alarming percentage of *E. coli* virulent types could grow below the resistant breakpoint concentrations of ciprofloxacin (50%; 39/78), trimethoprim/sulfamethoxazole (55.12%; 43/78), and ceftriaxone (39.74%; 31/78), which has been indicated as "intermediate" (Figure 2). A higher percentage of resistant *E. coli* virulent type was documented against amoxicillin (84.61%; 66/78), ampicillin (58.97%, 46/78), and tetracycline (47.43%, 37/78). The detailed antibiotic resistance patterns of the isolated *E. coli* is illustrated in Figure 2.

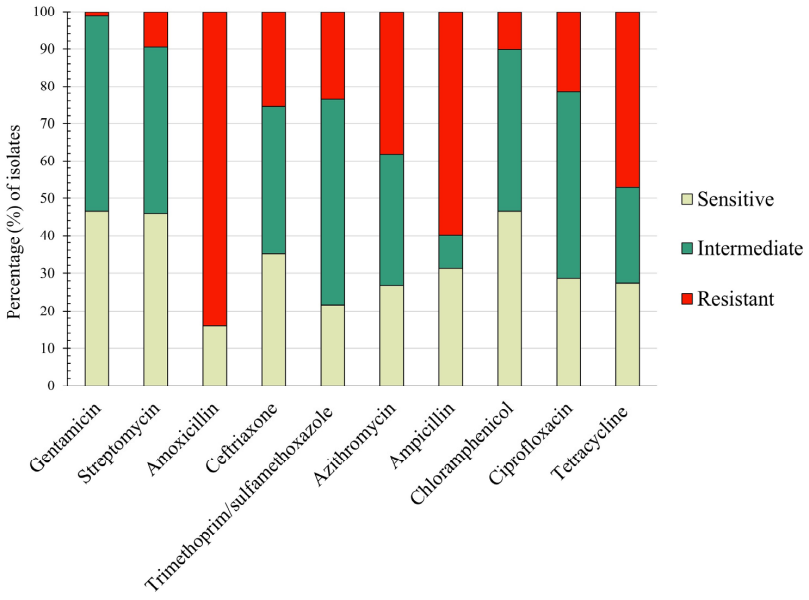

Figure 2. Antibiotic resistance patterns of diarrheagenic *E. coli* isolated from samples collected from ICLFs.

4. Discussion

According to CDC, diarrheagenic *E. coli*, specifically STEC, is one of the major public health concerns in the US which cause multiple foodborne outbreaks each year [1]. Many of these outbreaks occur from the consumption of leafy greens or raw produce/salads which are now part of a popular healthy diet [27]. Factors responsible for diarrheagenic *E. coli* contamination and spreading through produce include improper handling, particularly post-harvest processing and handling, improper storage, and transportation. In this study, we measured the overall prevalence of diarrheagenic *E. coli* in ICLF environments and products, specifically pre-harvest produce/leafy greens. It was observed that the prevalence of these pathogens was very similar in both environmental (4.50%) and pre-harvest produce (4.30%) samples, which recommended urgent attention. This outcome is compatible with a previously published study where it was found that 8.93% of environmental samples collected from conventional dairy farm environments were also contaminated with pathogenic *E. coli* [6]. Among the various environmental samples collected from the ICLFs, we observed the highest prevalence of *E. coli* in bedding material, followed by feces and feed materials (Figure 1). Several research teams also observed a similar prevalence of diarrheagenic *E. coli* in environmental samples and confirmed its transmission to produce samples [28,29]. Pathogens found in livestock fecal materials (manure) have the potential to be transferred to produce, entering the food chain [6]. The utilization of farm animal manure is widespread to enhance soil quality by providing essential nutrients and minerals, including potassium, nitrogen, and phosphorus, crucial for promoting plant growth [30]. When applying fresh manure or incomplete compost as fertilizer, it is imperative to incorporate it into or inject it beneath the soil to mitigate pathogen exposure, particularly in small farms or backyard gardens [31]. Employing proper on-farm composting of livestock manure proves to be an effective method for pathogen eradication during the process of fertilizing the soil with manure nutrients [31,32]. This study corroborates such effectiveness, as no diarrheagenic *E. coli* was detected in the compost category (Figure 1). The detection of virulent *E. coli* strains, such as EPEC, STEC, EIEC, EAEC, and ETEC, in environmental and pre-harvest samples is crucial for guaranteeing food safety and averting possible outbreaks. Implementing comprehensive surveillance and testing procedures in these environments enables the early detection of pathogenic strains, offering vital insights into the contamination risks connected with agricultural and environmental sources. We can improve preventive measures and contribute to the overall safety of the food supply chain by addressing the occurrence of virulent *E. coli* in pre-harvest conditions.

Considering the increasing popularity of agrotourism/U-pick and/or the increasing number of roadside/on-farm/farmers markets, it is now time to take appropriate measures or precautions while coming in direct contact with animals such as in petting zoos and farm animal fairs [33]. Further, the farm animals' and specifically the ruminants' fecal material constantly adds billions of coliforms to the soil and grass which can cross-contaminate the whole farm ecology when the animals are rotated across the farm [14]. Alternatively, farm animals can also be contaminated with fecal materials which can be transmitted to people from the subsequent touching of the face, mouth, wounded skin, or even clothing [6]. Additionally, diminutive wildlife such as birds, rodents, and insects may serve as vectors for pathogens, as these microorganisms can be transmitted through direct contact with their body parts. Subsequently, these vectors have the potential to spread pathogens to various areas of the farm, including fresh produce [34].

The spread of enteric pathogens including *E. coli* often occurs through direct contact with humans and animals or surface water and the environment, which are already contaminated due to direct exposure to animals and humans. In this study, the higher prevalence rate of diarrheagenic *E. coli* isolates in environmental and produce samples also indicate that the environment is the source of transmission of most pathogens. In this study, we detected that 51.28% (40/78) of diarrheagenic *E. coli* virulent types were distributed in pre-harvest produce samples, whereas no diarrheagenic *E. coli* virulent type was detected in the post-harvest produce samples. One of the reasons could be that the mass awareness of personal

hygiene such as hand washing, maintaining physical distance, and an overall decrease in outdoor activities played a significant role. Besides that, the strict quality control measures taken by the Food and Drug Administration (FDA) to minimize pathogen contamination in leafy greens are also effective [27]. This finding implies that improving worker hygiene and applying efficient farm management techniques, particularly in terms of avoiding pre-harvest contamination, could be additional measures for producing safer food products at ICLFs or other small- to medium-sized farms. Farm workers who pay close attention to personal hygiene and follow adequate sanitation methods can considerably lower the risk of introducing diarrheagenic *E. coli* into the environment, contributing to enhanced farm hygiene. Crop rotation, proper irrigation practices, and the timely removal of animal waste can limit crop exposure to potential sources of contamination, while monitoring and enforcing biosecurity measures within the farm environment play a critical role in minimizing the presence and spread of pathogenic strains, fostering a safer and healthier agricultural setting.

Globally, antibiotic-resistant microbial pathogens are one of the big problems of medical science. Besides being a life-threatening risk, they are also responsible for higher medical expenses, longer hospital stays, and higher mortality [35]. In this study, about 92.30% (72/78) of *E. coli* virulent types were resistant to at least one medically important antibiotic, while only 6.41% (5/78) were sensitive even though these farms were antibiotic-/chemical-free. Antibiotic resistance was observed in both the environmental and pre-harvest sample category. This finding agreed with our previous study in which we observed that removing antibiotics or chemicals for a short period of time could not reverse the antibiotic resistance patterns of enteric pathogens [14]. Comparing the resistance patterns of diarrheagenic *E. coli* against different antibiotics, we found that gentamicin, streptomycin, and chloramphenicol were comparatively effective antibiotics; however, a substantial percentage of diarrheagenic *E. coli* virulent types survived just under the resistant breakpoint. This is problematic because it indicates that antibiotics which are effective in treating some *E. coil* infections might not be effective in the future [36]. Our findings are also consistent with already published research. For example, in Nigeria, 88% and 78% of *E. coli* isolates from dairy-origin food products are sensitive to gentamicin and ciprofloxacin, respectively [35]. The antibiotic resistance patterns also vary based on geographic location and the economic status of the location. For example, one study reported a 96% tetracycline sensitivity of *E. coli* in Nigeria whereas another study conducted in the US reported a 65% tetracycline resistance [37]. In Asia, countries within the WHO South East Asia region reported the highest risk of emergence and spread of antibiotic-resistant pathogens compared to all other WHO regions [23]. This finding reiterates the necessity of restraining antibiotic application in livestock growth promotion worldwide.

5. Conclusions

The ubiquitous presence of diarrheagenic *E. coli* virulent types in environmental samples and pre-harvest produce of ICLFs necessitated the reinforcement of precautionary steps to prevent pathogen transmission. Our findings recommend practicing proper composting, and the application of sustainable natural disinfectants, such as plant phenolics, to reduce the pathogen load of the grazing land grass before rotating the livestock (Peng et al. [14]). Moreover, farm workers and visitors should be educated about the risk of pathogen transmission between the ICLF ecology and humans.

Author Contributions: Methodology, A.A., D.J., Z.T., Z.A.-M., M.W. and C.-W.T.; writing—original draft, A.A.; writing—review and editing, A.A., D.J., Z.T., Z.A.-M. and D.B.; supervision, project administration, and funding acquisition, D.B. All authors have read and agreed to the published version of the manuscript.

Funding: USDA-National Institute of Food and Agriculture (grant number 20185110628809).

Institutional Review Board Statement: Ethical approval was not necessary for this study, as the samples were gathered through standard procedures that did not pose any harm to animals. The

samples were exclusively sourced from environmental samples and chicken meat acquired from markets, with no use of live animals in the process.

Informed Consent Statement: Not applicable.

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

Acknowledgments: We are grateful for the support, tremendous interest, and cordial cooperation of the participating farm owners and the workers located in Maryland and Washington, D.C., USA. Finally, we would like to thank all undergraduate students from our lab for their enthusiastic technical support.

Conflicts of Interest: There are no conflicts to declare.

References

1. CDC (Centers for Disease Control and Prevention). National Outbreak Reporting System (NORS) Dashboard. 2023. Available online: https://wwwn.cdc.gov/norsdashboard/ (accessed on 1 October 2023).
2. CDC (Centers for Disease Control and Prevention). Reports of Selected *E. coli* Outbreak Investigations. 2022. Available online: https://www.cdc.gov/ecoli/outbreaks.html (accessed on 1 October 2023).
3. Tack, D.M. Preliminary Incidence and Trends of Infections with Pathogens Transmitted Commonly through Food—Foodborne Diseases Active Surveillance Network, 10 U.S. Sites, 2016–2019. *MMWR Morb. Mortal. Wkly. Rep.* **2020**, *69*, 509. [CrossRef] [PubMed]
4. Ferens, W.A.; Hovde, C.J. *Escherichia coli* O157:H7: Animal Reservoir and Sources of Human Infection. *Foodborne Pathog. Dis.* **2011**, *8*, 465–487. [CrossRef]
5. Jackson, C.R.; Davis, J.A.; Barrett, J.B. Prevalence and Characterization of Methicillin-Resistant *Staphylococcus aureus* Isolates from Retail Meat and Humans in Georgia. *J. Clin. Microbiol.* **2013**, *51*, 1199–1207. [CrossRef] [PubMed]
6. Aditya, A.; Tabashsum, Z.; Alvarado Martinez, Z.; Wei Tung, C.; Suh, G.; Nguyen, P.; Biswas, D. Diarrheagenic *Escherichia coli* and Their Antibiotic Resistance Patterns in Dairy Farms and Their Microbial Ecosystems. *J. Food Prot.* **2023**, *86*, 100051. [CrossRef]
7. Peng, M.; Salaheen, S.; Almario, J.A.; Tesfaye, B.; Buchanan, R.; Biswas, D. Prevalence and Antibiotic Resistance Pattern of *Salmonella* Serovars in Integrated Crop-Livestock Farms and Their Products Sold in Local Markets. *Environ. Microbiol.* **2016**, *18*, 1654–1665. [CrossRef]
8. Painter, J.A.; Hoekstra, R.M.; Ayers, T.; Tauxe, R.V.; Braden, C.R.; Angulo, F.J.; Griffin, P.M. Attribution of Foodborne Illnesses, Hospitalizations, and Deaths to Food Commodities by Using Outbreak Data, United States, 1998–2008. *Emerg. Infect. Dis.* **2013**, *19*, 407–415. [CrossRef] [PubMed]
9. Berger, C.N.; Sodha, S.V.; Shaw, R.K.; Griffin, P.M.; Pink, D.; Hand, P.; Frankel, G. Fresh Fruit and Vegetables as Vehicles for the Transmission of Human Pathogens. *Environ. Microbiol.* **2010**, *12*, 2385–2397. [CrossRef] [PubMed]
10. Bolton, D.J.; O'Neill, C.J.; Fanning, S. A Preliminary Study of *Salmonella*, Verocytotoxigenic *Escherichia coli* O157 and *Campylobacter* on Four Mixed Farms. *Zoonoses Public Health* **2012**, *59*, 217–228. [CrossRef]
11. Johnston, L.M.; Jaykus, L.-A.; Moll, D.; Martinez, M.C.; Anciso, J.; Mora, B.; Moe, C.L. A Field Study of the Microbiological Quality of Fresh Produce. *J. Food Prot.* **2005**, *68*, 1840–1847. [CrossRef] [PubMed]
12. Kozak, G.K.; MacDonald, D.; Landry, L.; Farber, J.M. Foodborne Outbreaks in Canada Linked to Produce: 2001 through 2009. *J. Food Prot.* **2013**, *76*, 173–183. [CrossRef]
13. Mukherjee, A.; Speh, D.; Jones, A.T.; Buesing, K.M.; Diez-Gonzalez, F. Longitudinal Microbiological Survey of Fresh Produce Grown by Farmers in the Upper Midwest. *J. Food Prot.* **2006**, *69*, 1928–1936. [CrossRef]
14. Peng, M.; Salaheen, S.; Buchanan, R.L. Alterations of *Salmonella* Enterica Serovar Typhimurium Antibiotic Resistance under Environmental Pressure. *Appl. Environ. Microbiol.* **2018**, *84*, e01173-18. [CrossRef]
15. Salaheen, S.; Chowdhury, N.; Hanning, I.; Biswas, D. Zoonotic Bacterial Pathogens and Mixed Crop-Livestock Farming. *Poult. Sci.* **2015**, *94*, 1398–1410. [CrossRef]
16. Teramoto, H.; Salaheen, S.; Biswas, D. Contamination of Post-Harvest Poultry Products with Multidrug Resistant *Staphylococcus aureus* in Maryland-Washington DC Metro Area. *Food Control* **2016**, *65*, 132–135. [CrossRef]
17. NFMD. National Farmers Market Directory. 2023. Available online: https://nfmd.org (accessed on 1 October 2023).
18. Hoffmann, I. Climate Change and the Characterization, Breeding and Conservation of Animal Genetic Resources. *Anim. Genet.* **2010**, *41* (Suppl. S1), 32–46. [CrossRef]
19. Strawn, L.K.; Fortes, E.D.; Bihn, E.A.; Nightingale, K.K.; Gröhn, Y.T.; Worobo, R.W.; Wiedmann, M.; Bergholz, P.W. Landscape and Meteorological Factors Affecting Prevalence of Three Food-Borne Pathogens in Fruit and Vegetable Farms. *Appl. Environ. Microbiol.* **2013**, *79*, 588–600. [CrossRef]
20. Barton, J.; Henderson, K. Ohio Ecological Food and Farm Association. 2008. Available online: http://certification.oeffa.org/certfiles/transition/Organic%20Transition%20Guide.pdf (accessed on 1 October 2023).
21. Bullock, J.M.; Pywell, R.F.; Burke, M.J.W.; Walker, K.J. Restoration of Biodiversity Enhances Agricultural Production. *Ecol. Lett.* **2001**, *4*, 185–189. [CrossRef]

22. Nielsen, M.N.; Winding, A. Microorganisms as Indicators of Soil Health. 2002. Available online: https://www2.dmu.dk/1_viden/2_publikationer/3_fagrapporter/rapporter/fr388.pdf (accessed on 1 October 2023).
23. Maheux, A.F.; Picard, F.J.; Boissinot, M.; Bissonnette, L.; Paradis, S.; Bergeron, M.G. Analytical Comparison of Nine PCR Primer Sets Designed to Detect the Presence of *Escherichia coli/Shigella* in Water Samples. *Water Res.* **2009**, *43*, 3019–3028. [CrossRef] [PubMed]
24. Botkin, D.J.; Galli, L.; Sankarapani, V.; Soler, M.; Rivas, M.; Torres, A.G. Development of a Multiplex PCR Assay for Detection of Shiga Toxin-Producing *Escherichia coli*, Enterohemorrhagic *E. coli*, and Enteropathogenic *E. coli* Strains. *Front. Cell. Infect. Microbiol.* **2012**, *2*, 8. [CrossRef]
25. Ahmed, O.B.; Dablool, A.S. Quality Improvement of the DNA Extracted by Boiling Method in Gram Negative Bacteria. *Int. J. Bioassays* **2017**, *6*, 5347. [CrossRef]
26. CLSI (Clinical and Laboratory Standards Institute). EM100 Connect—CLSI M100 ED33:2023. 2023. Available online: http://em100.edaptivedocs.net/GetDoc.aspx?doc=CLSI%20M100%20ED33:2023andscope=user (accessed on 1 October 2023).
27. FDA (Food and Drug Administration). Leafy Greens STEC Action Plan. 2022. Available online: https://www.fda.gov/food/foodborne-pathogens/leafy-greens-stec-action-plan (accessed on 1 October 2023).
28. Atidégla, S.C.; Huat, J.; Agbossou, E.K.; Saint-Macary, H.; Glèlè Kakai, R. Vegetable Contamination by the Fecal Bacteria of Poultry Manure: Case Study of Gardening Sites in Southern Benin. *Int. J. Food Sci.* **2016**, *2016*, e4767453. [CrossRef]
29. Black, Z.; Balta, I.; Black, L.; Naughton, P.J.; Dooley, J.S.G.; Corcionivoschi, N. The Fate of Foodborne Pathogens in Manure Treated Soil. *Front. Microbiol.* **2021**, *12*, 781357. [CrossRef] [PubMed]
30. MacDonald, J.M.; Ribaudo, M.O.; Livingston, M.J.; Beckman, J.; Huang, W. Manure Use for Fertilizer and for Energy: Report to Congress. 2009. Available online: https://www.ers.usda.gov/webdocs/publications/42731/16739_ap037fm_1_.pdf?v=8487.8 (accessed on 1 October 2023).
31. US EPA (United States Environmental Protection Agency). Animal Feeding Operations—Uses of Manure. National Pollutant Discharge Elimination System (NPDES). 2023. Available online: https://www.epa.gov/npdes/animal-feeding-operations-uses-manure (accessed on 1 October 2023).
32. Augustin, C.; Rahman, S.Q. Composting Animal Manures: A Guide to the Process and Management of Animal Manure Compost. NDSU Extension Service. 2010. Available online: https://www.ag.ndsu.edu/manure/documents/nm1478.pdf (accessed on 1 October 2023).
33. Saulo, A.A. Preventing *E. coli* Infection at Petting Zoos and Farm Animal Fairs. University of Hawaii at Manoa - College of Tropical Agriculture and Human Resources FST-17a. 2013.
34. Lindeberg, Y.L.; Egedal, K.; Hossain, Z.Z.; Phelps, M.; Tulsiani, S.; Farhana, I.; Begum, A.; Jensen, P.K.M. Can *Escherichia coli* Fly? The Role of Flies as Transmitters of *E. coli* to Food in an Urban Slum in Bangladesh. *Trop. Med. Int. Health* **2018**, *23*, 2–9. [CrossRef]
35. WHO (World Health Organization). Antibiotic Resistance. 2020. Available online: https://www.who.int/news-room/fact-sheets/detail/antibiotic-resistance (accessed on 1 October 2023).
36. Osaili, T.M.; Alaboudi, A.R.; Rahahlah, M. Prevalence and Antimicrobial Susceptibility of *Escherichia coli* O157:H7 on Beef Cattle Slaughtered in Amman Abattoir. *Meat Sci.* **2013**, *93*, 463–468. [CrossRef] [PubMed]
37. Gelalcha, B.D.; Ensermu, D.B.; Agga, G.E.; Vancuren, M.; Gillespie, B.E.; D'Souza, D.H.; Okafor, C.C.; Kerro Dego, O. Prevalence of Antimicrobial Resistant and Extended-Spectrum Beta-Lactamase-Producing *Escherichia coli* in Dairy Cattle Farms in East Tennessee. *Foodborne Pathog. Dis.* **2022**, *19*, 408–416. [CrossRef]